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Development of Transgene Containment Technologies for Woody Plants

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University of Connecticut, 2017

Abstract

Transgenic technology provides a powerful tool for woody plant improvement. However, the full utilization of this technology has been stagnant due to gene flow and food safety concerns. This dissertation describes two transgene containment techniques that are particularly useful for woody plants. The first one is a flowerless plant technique that can be used to prevent pollen- and seed-mediated transgene flow. We developed an artificial promoter sequence, *rPTAG2I*, and demonstrated that this sequence is floral-predominant in tobacco with no detectable activity in the vegetative tissues of tobacco and poplar. Transgenic tobacco plants expressing the *Diphtheria toxin A* (*DT-A*) gene driven by the *rPTAG2I* promoter exhibited a flowerless phenotype with enhanced shoot and root biomass. Over two growing seasons in the field, *rPTAG2I::DT-A* transgenic poplar plants displayed similar vegetative growth compared to wild type. The second technique used a root-predominant gene promoter (*SbUGT*) to drive the expression of an auxin biosynthetic gene (*iaaM*) and a cytokinin degradation gene (*CKX*) to improve rootstocks. We demonstrated that expression of both the *iaaM* and *CKX* genes, predominantly in roots, inhibited lateral bud release and enhanced both root initiation and biomass of rootstock. Also, the grafting success rates were improved even though non-transgenic scions were used. We have further shown that the use of the *iaaM+CKX*

transgenic rootstocks led to improved non-transgenic scion growth even when the lateral buds on rootstock remained intact. Because of these characteristics, our technique takes advantages of transgenic technology but also eliminates the concerns of gene flow from flowers and fruits. In summary, the development of both the flowerless and *iaaM+CKX* rootstock techniques may provide useful tools to address gene flow and food safety concerns over transgenic woody plant species.

Development of Transgene Containment Technologies for Woody Plants

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Table of Contents

List of Tables

List of Figures

Chapter 1 Introduction

Transgenic technology provides a powerful tool to improve the performance of woody plant species by conveying herbicide resistance, abiotic stress tolerance, enhanced growth rate, or end-use characteristics (Tzfira et al., 1998; Ye et al., 2011). Any one of these transgenic traits can be of great economic importance. For instance, Sedjo (2005) estimated that herbicide resistance traits could reduce wood production costs by 1 billion USD per year, worldwide. Also, the *Bt* gene has the potential to be used to prevent outbreaks of trunk insect attacks in China, with the potential to reduce wood production costs by 5 billion RMB (Su et al., 2003). A number of tree biotechnology companies, such as ArborGen, are focusing on producing various transgenic trees, such as fast-growing loblolly pine (*Pinus taeda* L.) for the southern US and low-lignin eucalyptus for South America (Hinchee, 2005; Farnum et al., 2007).

Transgenic technologies also provide solutions to the problems associated with traditional woody plant breeding, such as the long juvenile periods. For woody plants, progeny production through sexual crossing, and subsequent selection, is a lengthy and labor-intensive process that can take over a decade to reach fruition (van Nocker and Gardiner, 2014; Xiong et al., 2015). In addition to problems imposed by long juvenile stages, breeding through sexual crossing has additional limitations as some elite traits may be lost in the process due to complex genetic backgrounds (Pinto, 2015). Transgenic approaches help to bypass these issues and can add novel genes which are not available in the germplasm.

Despite the great potential of transgenic technologies, the commercial cultivation of transgenic plants has aroused tremendous biosafety concerns, specifically in regards to environmental safety and food safety (Li et al., 2013). Although many field trials for transgenic woody plants have been conducted, very few of them have led to commercialization in the United States or other developed countries (Kaldorf et al., 2002; Jing et al., 2004; Lu et al., 2017). The undesirable spread of transgenes to native ecosystems, a process known as transgene flow, is one major concern obstructing the deregulation of transgenic woody plants (Strauss et al., 2009). Containment strategies for the prevention of transgene flow are vital for the commercialization of genetically modified (GM) perennial plants (Moon et al., 2010).

Pollen is one of the main vehicles of transgene flow for flowering plants (Chandler and Dunwell, 2008). Controlling the development of viable pollen can be an efficient method to block transgene flow. A great deal of research has centered around efforts to eliminate the possibility of pollen-mediated gene transfer. Creating male-sterile lines using transgenic technology has been explored for many species (De Block et al., 1997; Higginson et al., 2003; Yui et al., 2003). Tapetal ablation has been achieved for tobacco and canola plants by using a tapetum-specific promoter to drive the expression of the cytotoxic gene *barnase* (Mariani et al., 1990). Additionally, antisense RNA of the rice *rts* gene, expressed under the same tapetum-specific promoter, has been used to inhibit tapetum development in creeping bentgrass, leading to the loss of pollen fertility (Luo et al., 2006).

Seeds are another vehicle of transgene flow. Seed-mediated transgene flow may be caused by the natural spread in the field, human error during planting, or during transportation (Heuberger et al., 2010). A recently developed transgene deletion technology, the gene deletor system, has the ability to delete all functional transgenes from both the pollen and seeds of transgenic tobacco plants while maintaining these transgenes in vegetative tissues (Luo et al., 2007). The gene deletor system is an effective tool for eliminating concerns over transgene flow, but does nothing to address other concerns associated with tree pollen production, such as allergenic reactions (Bastl et al., 2014). For most commercial cultivars of woody plants, vegetative propagation is preferred over sexual reproduction (Rupp et al., 2011), therefore, engineering flowerless cultivars through the application of transgenic technologies could serve as an effective tool to address both transgene flow and allergy concerns.

Grafting techniques are commonly used for woody plant propagation. These techniques involve the joining of the upper part (scion) of one plant to the lower part (rootstock) of a second plant (Goldschmidt, 2014; Warschefsky et al., 2016). Elite rootstock can enhance a graft's ability to obtain water and mineral nutrients, as well as improve the performance of scions in various aspects such as adapting to certain soil conditions or resistant to soil-borne pests and diseases (Hartmann and Kester, 1975; Song et al., 2013; Warschefsky et al., 2016). In the case of apple trees, dwarf rootstocks are commonly used to reduce the stem heights of scions, which has been shown to significantly increase fruit production (James and Thurbon, 1981). For tomatoes and melons, using disease-resistant or salt-resistant rootstock can lead to the increased resistance of grafted plants to these stresses (Estañ et al., 2005; King et al.,

2008). For citrus, cold-tolerant rootstock has been used to increase the hardiness of grafted plants in low-temperature environments (Melnyk and Meyerowitz, 2015).

Another common grafting technique is to graft scions from mature plants onto rootstocks of young seedlings (Melnyk and Meyerowitz, 2015). Mature scions readily produce inflorescences, seeds, and fruits, but mature shoot cutting are often difficult to root directly through vegetative propagation (Poethig, 1990). On the other hand, juvenile plants are relatively easy to root, but may require a long time to reach reproductive maturity (Abu-Abied et al., 2014). In extreme cases, the juvenile stage of a woody plant can last up to 40 years (Ahuja, 2013). Grafting mature scions onto juvenile rootstocks results in chimeric plants that readily flower and also have robust root systems.

Many plants with excellent rootstock characteristics are difficult to root from vegetative cuttings (Akbari et al., 2015). For instance, it is difficult to induce rooting for some dwarf apple rootstock varieties (Akbari et al., 2015; Pawlicki and Welander, 1995). Also, the undesirable outgrowth of lateral buds from rootstocks following grafting can reduce grafting success rates and inhibit scion growth (Daley and Hassell, 2014). Transgenic technologies have the potential to improve rootstock characteristics at a much faster rate, compared to traditional breeding, significantly shortening the breeding cycles of rootstock plants (Gambino and Gribaudo, 2012). Transgene insertion has been proven to be efficient in improving a variety of rootstock characteristics for a number of plant taxa, including apples, pears, and olives. For example, the stem height of apple rootstock cultivars can be significantly reduced by the expression of an *Arabidopsis gai* gene (Gambino and Gribaudo,

2012). Also, when grafting non-transgenic scions onto transgenic rootstock, the resulting chimeric plants will produce non-transgenic fruit and seeds, while alleviating both the ecological and food safety concerns associated with wholly transgenic plants.

Flower ablation as well as the combined use of transgenic rootstock with non-transgenic scions, have the potential to improve woody plants while eliminating concerns over transgene flow. These two technologies hold great potentials to accelerate the commercialization of transgenic plants. Both technologies also have benefits outside of the context of transgene flow. For flowerless plants, the risks of pollen allergen-related health hazards can be completely eliminated. Grafted plants with non-transgenic scions, benefit from the superior traits of transgenic plants while producing non-transgenic fruits, thus eliminating concerns over the consumption of genetically modified fruits.

Chapter 2 An *AGAMOUS* intron-driven cytotoxin leads to flowerless tobacco and produces no detrimental effects on vegetative growth of either tobacco or poplar

2.1 Abstract

The flowerless trait is highly desirable for poplar because it can prevent pollen- and seed-mediated transgene flow. We have isolated the second intron of *PTAG2*, an *AGAMOUS* (*AG*) orthologue from *Populus trichocarpa.* By fusing this intron sequence to a minimal *35S* promoter sequence, we created two artificial promoters, *fPTAG2I* (forward orientation of the *PTAG2* intron sequence) and *rPTAG2I* (reverse orientation of the *PTAG2* intron sequence). In tobacco, expression of the β-glucuronidase gene (*uidA*) demonstrates that the *fPTAG2I* promoter is non-floral-specific, while the *rPTAG2I* promoter is active in floral buds but with no detectable vegetative activity. Under greenhouse conditions, transgenic tobacco expressing the *Diphtheria toxin A* (*DT-A*) gene driven by the *rPTAG2I* promoter produced three floral ablation phenotypes: flowerless, neuter (stamen-less and carpel-less), and carpel-less. Further, the vegetative growth of these transgenic lines was similar to the wild type. In field trials during 2014 and 2015, the flowerless transgenic tobacco stably maintained its flowerless phenotype, and also produced more shoot and root biomass when compared to wild-type plants. In poplar, the *rPTAG2I::GUS* gene exhibited no detectable activity in vegetative organs. Under field conditions over two growing seasons (2014 to the end of 2015), vegetative growth of the *rPTAG2I::DT-A* transgenic poplar plants was similar to that of the wild-type plants. Our results demonstrate that the *rPTAG2I* artificial promoter has no detectable activities in vegetative organs of poplar plants, and the *rPTAG2I::DT-A* gene may be useful for producing flowerless poplar plants with normal vegetative growth.

2.2 Introduction

Pollen- and seed- mediated transgene flow is difficult to control (Heuberger et al., 2010). It has been reported that pollen and seeds from poplar (*P. trichocarpa*) can be easily dispersed over long distances via wind, increasing the risk potentials of transgene flow (DiFazio et al., 2012; Kausch et al., 2010; Oliver et al., 2004; Slavov et al., 2009; Zhang et al., 2012). In addition, pollen from poplar can induce allergic reactions in a large segment of the population (Celik et al., 2005). Since vegetative propagation is preferred over sexual reproduction for most commercial cultivars of poplar, engineering flowerless poplar could provide an effective means to address pollen- and seed-mediated transgene flow and allergy problems (DiFazio et al., 2012; Kausch et al., 2010; Oliver et al., 2004; Zhang et al., 2012). Furthermore, with reproductive organs eliminated, more photosynthetic products can be used for vegetative growth, likely leading to improvement of biomass production.

When driving a cytotoxic gene, a flower-specific promoter could direct a flowerless phenotype (Skinner et al., 2003; Wei et al., 2007). However, for all the developed "flower-specific" promoters from woody plant species, when used to control expression of a toxin gene, the resulting transgenic plants displayed reductions in vegetative growth (Brunner et al.2000; Igasaki et al. 2008). For instance, Elorriaga et al. (2014) reported that all transgenic poplar plants, expressing a barnase gene driven by the *TA29* promoter, a tapetum-specific promoter cloned from tobacco, exhibited retarded vegetative growth. Losses of the floral-specificity for these promoter sequences, such as the tobacco pollen-specific *TA29* promoter, in poplar demonstrate that floral-specificity of promoter sequences from one plant species may not be retained in a different species due to evolutionary divergences. Therefore, use of

a floral-specific promoter from poplar may be necessary for engineering flowerless poplar with no negative effects on vegetative growth.

AGAMOUS (*AG*), a gene coding for a MADS protein, is expressed in the third and fourth whorls of *Arabidopsis* flowers and is essential for floral meristem determinacy (Bowman et al., 1991). A 4-kb enhancer sequence located within the second intron of *AG* was found to influence *AG* expression during early stages of flower development of *Arabidopsis* (Deyholos et al., 2000). Chimeric promoters derived from the second intron of *Arabidopsis AG* have been used to drive expression of the cytotoxic gene *Diphtheria toxin A (DT-A)* in *Arabidopsis* plants. Flowerless *Arabidopsis* plants have been achieved with no detrimental impacts on vegetative growth (Liu and Liu, 2008).

However, Wang et al. (2008) reported that using the same chimeric promoter from *Arabidopsis* directed imprecise and inefficient floral specific expression in tobacco plants. These results suggest that *AG* intron-derived chimeric promoters from *Arabidopsis* or other species may not maintain floral specificity and effectiveness if used in poplar or other woody plants. Therefore, it is highly desirable to isolate a floral specific gene promoter from poplar, in order to develop a flower-specific promoter for producing flowerless poplar plants.

2.3 Results

2.3.1 The reverse-oriented second intron of *PTAG2* conferred flower-specific expression

To test the flower specificity of the *PTAG2* second intron, we transformed tobacco and poplar using *A. tumefaciens* strains hosting *fPTAG2I::GUS*, and *rPTAG2I::GUS* genes **(Figure 2.1)**. Both transgenic poplar and tobacco plants were assayed with histochemical staining of GUS activity in leaf, stem, and apical meristem tissues. At vegetative stages, *fPTAG2I::GUS* expression in both poplar and tobacco plants were detected in stem and vegetative apical meristem tissues **(Figure 2.2)**. At reproductive stages, *fPTAG2I::GUS* tobacco plants displayed GUS activity in floral organs and also vegetative tissues **(Figure 2.3)**.

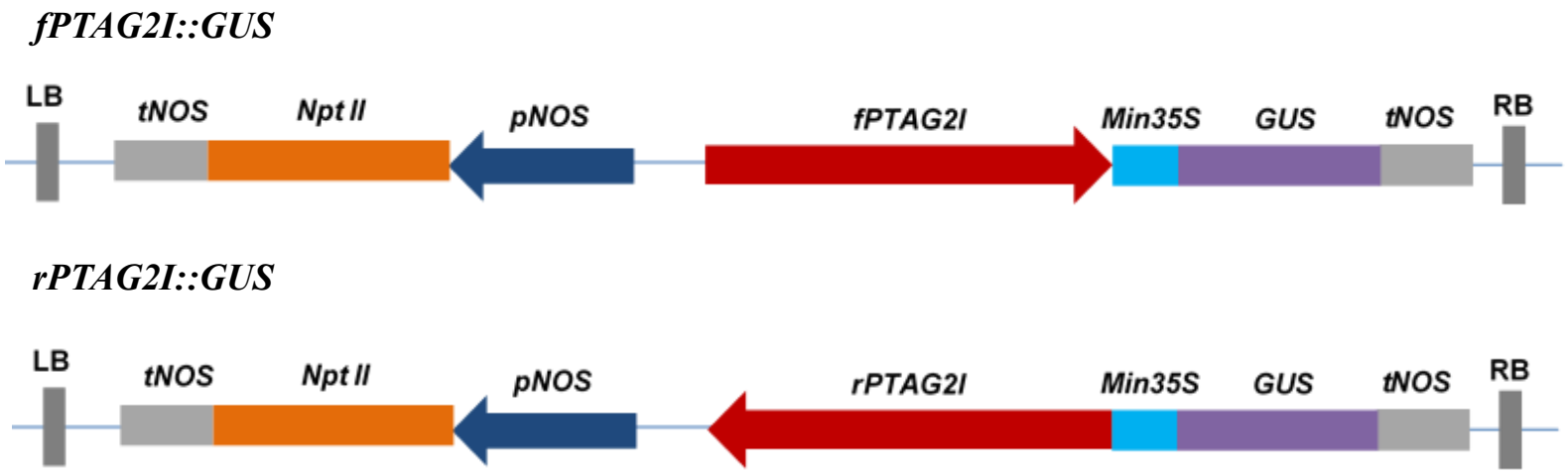


Figure 2.1 T-DNA regions of the *fPTAG2I::GUS* and *rPTAG2I::GUS* constructs. LB: left border sequence of T-DNA. RB: right border sequence of T-DNA. *tNOS*: nopaline synthase terminator. *NptII*: neomycin phosphotransferase gene. *pNOS*: nopaline synthase gene promoter sequence. *GUS*: the coding sequence for the β-glucuronidase gene. *fPTAG2I*: the forward orientation of the second intron of *P. trichocarpa AGAMOUS (AG) 2* gene. *Min35S*: 60-base-pairs of the *35S* gene leader and promoter sequence that has no promoter activity. *rPTAG2I*: the reverse orientation of the second intron sequence of the *P. trichocarpa AGAMOUS* (*AG*) *2* gene.

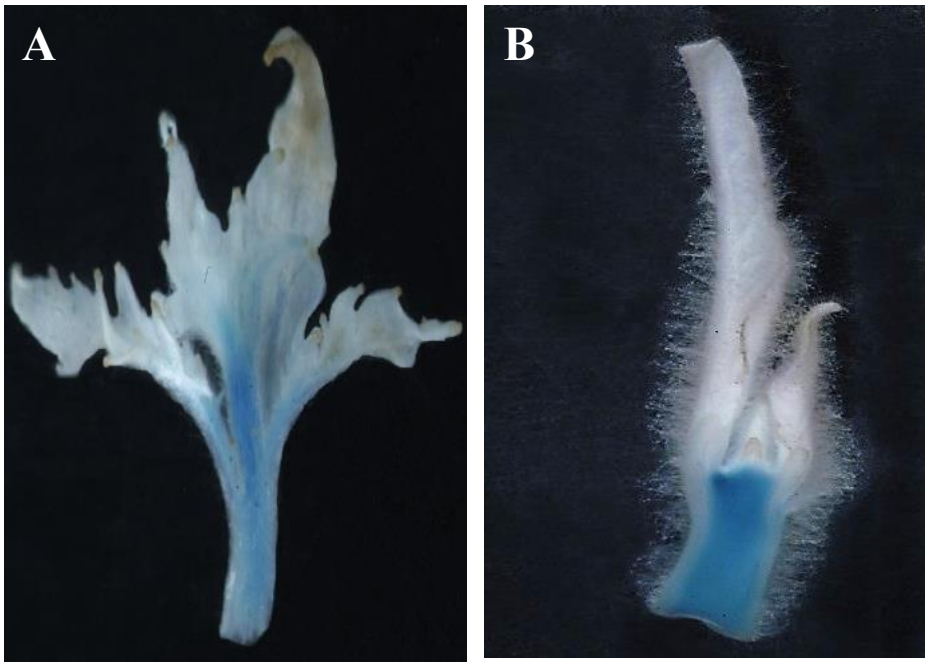


Figure 2.2 Histochemical staining of GUS activity in *fPTAG2I::GUS* poplar and tobacco. (A) Shoot apices from two-month-old transgenic poplar plants harboring *fPTAG2I::GUS*. (B) Longitudinal sections of shoot apices from five-week-old greenhouse-grown transgenic tobacco plants harboring *fPTAG2I::GUS*.

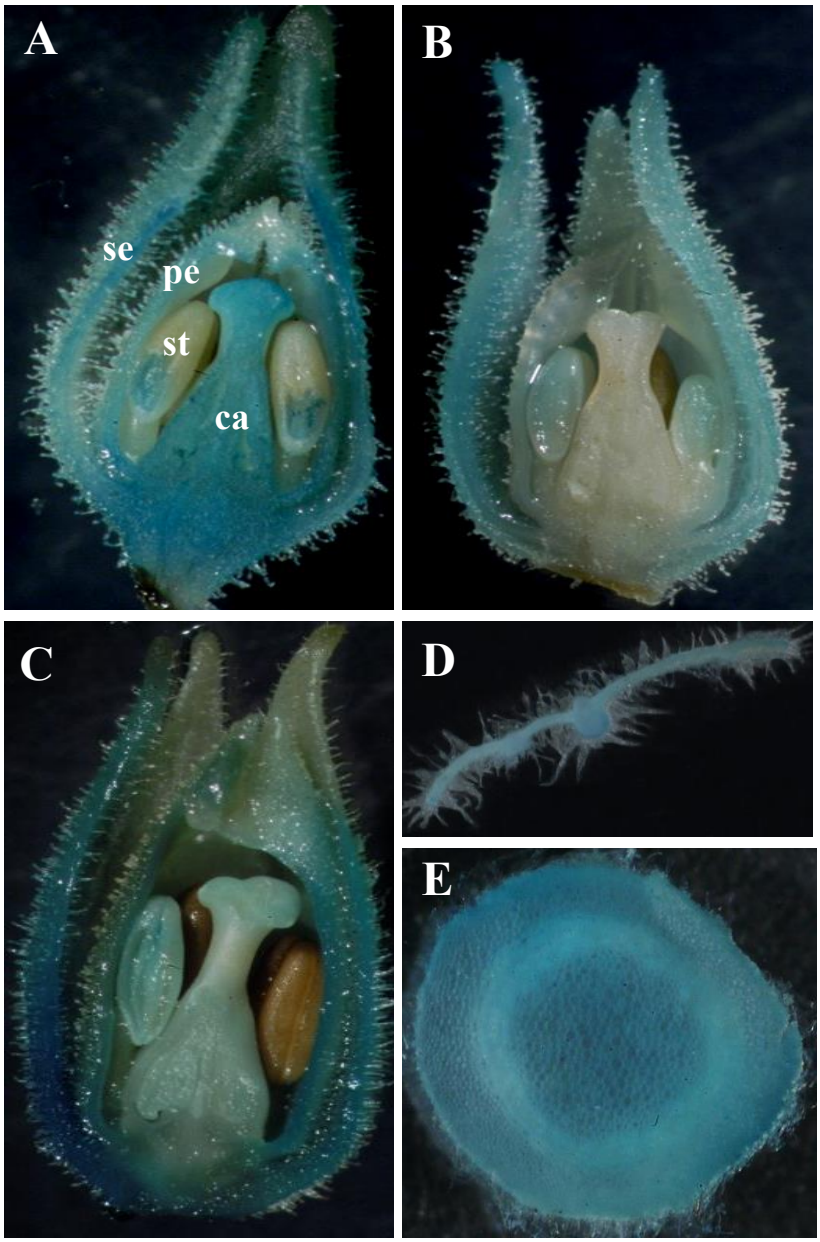


Figure 2.3 Histochemical staining of GUS activity in four-month-old *fPTAG2I::GUS* tobacco. (A-C) Longitudinal sections of floral buds from three different *fPTAG2I::GUS* tobacco lines showed varying levels of *fPTAG2I* promoter activity. (D, E) Cross sections of (D) leaf and (E) stem from four-month-old *fPTAG2I::GUS* tobacco exhibited GUS activity. se: sepal; pe: petal; st: stamen; ca: carpel.

For *rPTAG2I::GUS* transgenic poplar and tobacco lines, none of them had any detectable GUS activity in vegetative tissues before flowering **(Figure 2.4).** At flowering stages, *rPTAG2I::GUS* tobacco lines showed GUS activity in floral organs with three expression patterns: A) expression in carpel **(Figure 2.5A)**; B) expression in stamen and carpel **(Figure 2.5B)**; and C) expression in sepal, petal, stamen, and carpel **(Figure 2.5C)**. None of *rPTAG2I::GUS* tobacco lines showed any detectable GUS activity in vegetative tissues at reproductive stages **(Figure 2.5D, E)**. The histochemical staining for the GUS activity demonstrates that both the forward and reverse orientations of the *PTAG2* second intron are highly active in floral organs, but the forward orientation sequence (*fPTAG2I*) is active in vegetative organs while the reverse orientation sequence (*rPTAG2I*) has no detectable activity in vegetative organs. Based on these observations, we chose the *rPTAG2I* promoter derived from poplar for further experiments.

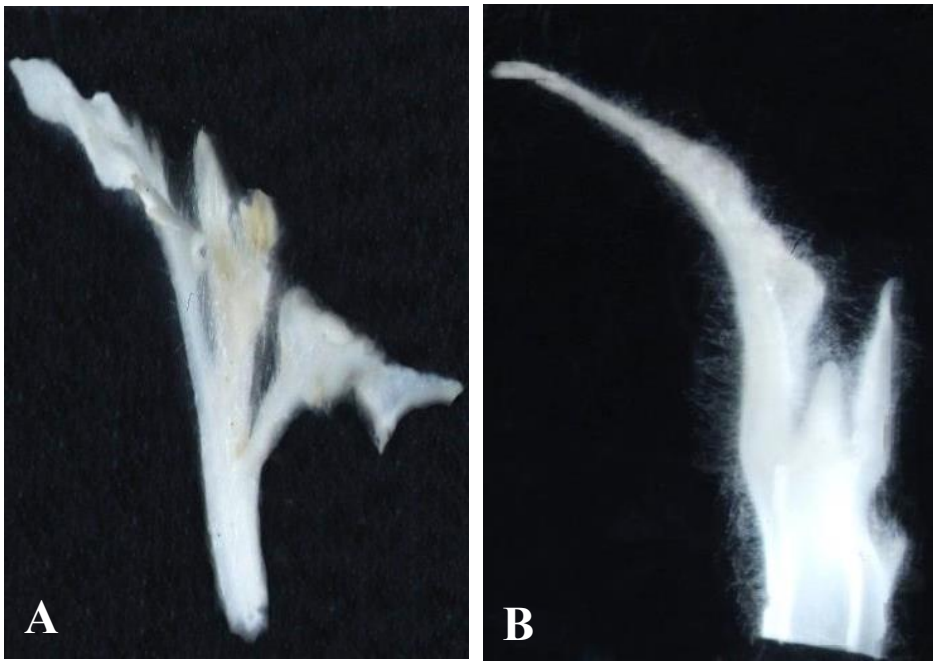


Figure 2.4 Histochemical staining of GUS activity in *rPTAG2I::GUS* poplar and tobacco. (A) Shoot apices from two-month-old transgenic poplar plants harboring *rPTAG2I::GUS*. (B) Longitudinal sections of shoot apices from five-week-old greenhouse-grown transgenic tobacco plants harboring *rPTAG2I::GUS*.

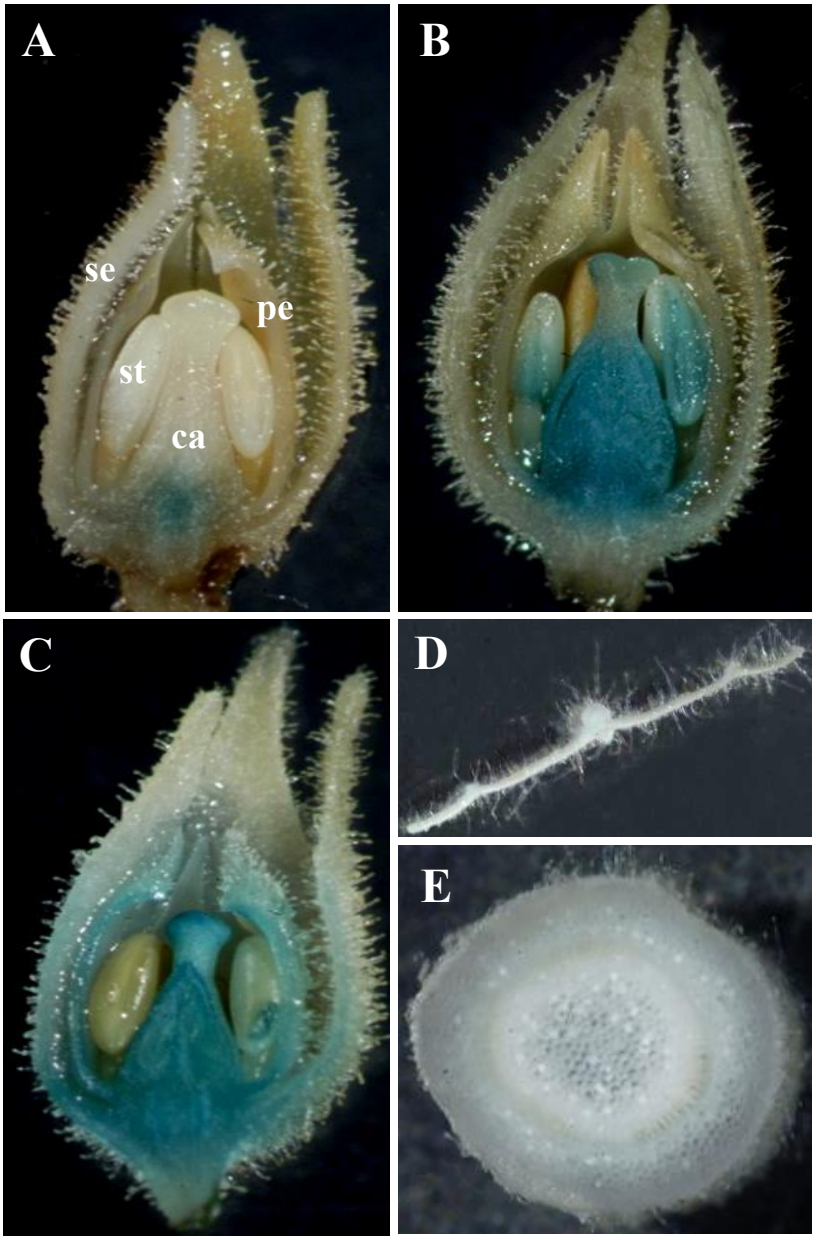
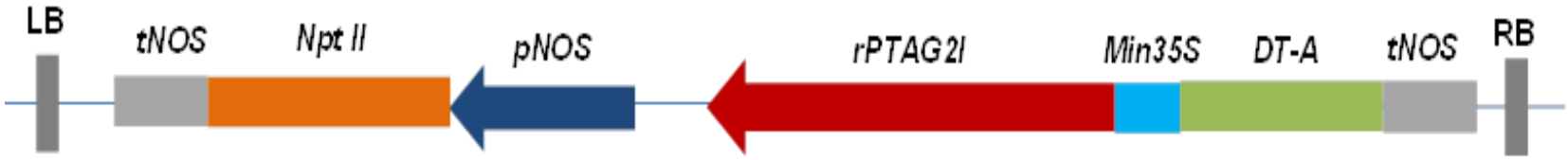


Figure 2.5 Histochemical staining of GUS activity in four-month-old *rPTAG2I::GUS* tobacco. (A-C) Longitudinal sections of floral buds from three different four-month-old *rPTAG2I::GUS* tobacco lines showed varying levels of *rPTAG2I* promoter activity. (D, E) Cross sections of (D) leaf and (E) stem from four-month-old *rPTAG2I::GUS* tobacco exhibited no GUS activity. se: sepal; pe: petal; st: stamen; ca: carpel.

2.3.2 Floral organ ablation directed by the *rPTAG2I* chimeric promoter in greenhouse grown tobacco plants

The *rPTAG2I* chimeric promoter we constructed was used to drive a *Diphtheria toxin A (DT-A)* gene, coding for a ribosome inactivating protein (Palmiter et al., 1987) **(Figure 2.6)**. Among 51 *rPTAG2I::DT-A* tobacco lines produced, 48 lines exhibited floral ablation phenotypes while the other three lines displayed a wild-type floral phenotype. The 48 transgenic lines can be categorized into four groups morphologically. Group I (19 lines), were flowerless, with all floral buds aborted before floral stage 9 **(Figure 2.7)** [see Mandel et al. (1992), for the definitions of tobacco floral development stages]. Group II (5 lines), had some floral buds that lacked stamens and carpels, while the remaining buds were aborted before floral stage 9. Group III (10 lines), were neuter (stamen-less and carpel-less), with all floral buds lacking both stamens and carpels **(Figure 2.8)**. Group IV (14 lines), were carpel-less, all floral buds developing without carpels. For Group IV carpel-less flowers, pollen grain production was reduced to less than 20% of wild-type flowers **(Figure 2.9)**. Our pollen germination experiment showed that transgenic pollen from the carpel-less plants (Group IV) was not viable.



rPTAG2I::DT-A

Figure 2.6 T-DNA region of the *rPTAG2I::DT-A* construct. LB: left border sequence of T-DNA. RB: right border sequence of T-DNA. *tNOS*: nopaline synthase terminator. *NptII*: neomycin phosphotransferase gene. *pNOS*: nopaline synthase gene promoter sequence. *rPTAG2I*: the reverse orientation of the second intron sequence of the *P. trichocarpa AGAMOUS(AG) 2* gene. *Min35S*: 60-base-pairs of the 35S gene leader and promoter sequence that has no promoter activity. *DT-A*: the coding sequence for the Diphtheria toxin A (*DT-A*) gene, which codes for a ribosome inactivating protein.

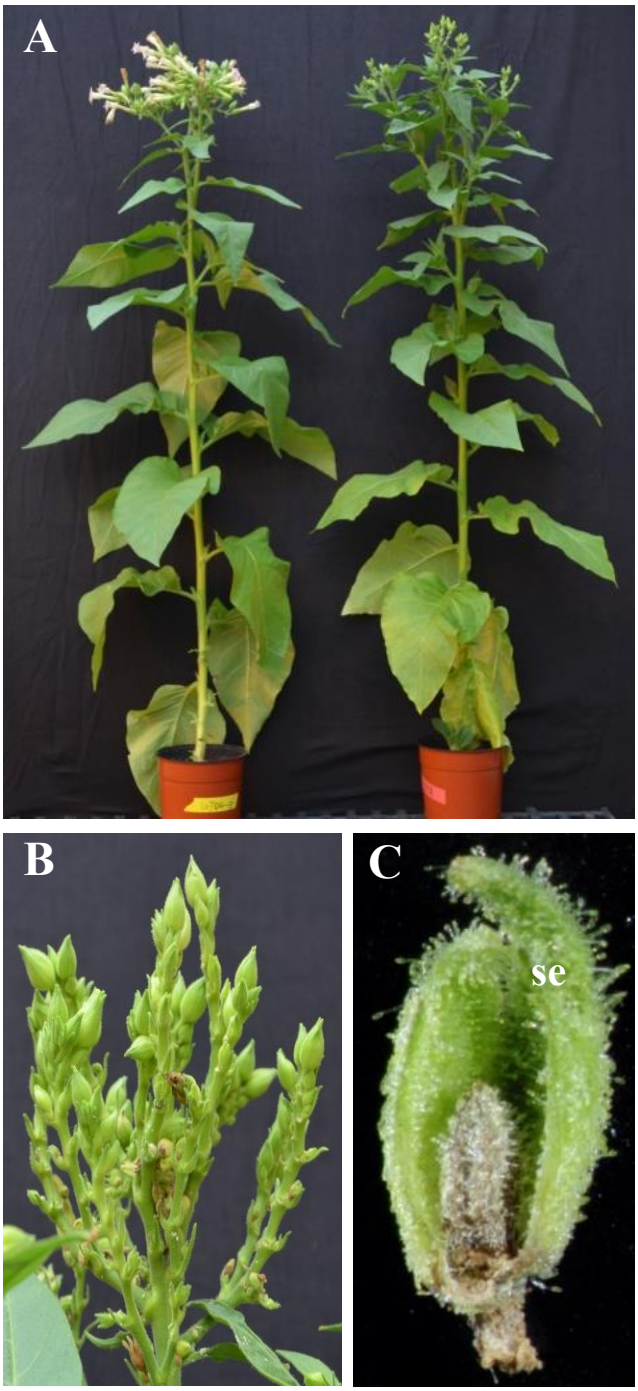


Figure 2.7 Greenhouse-grown flowerless tobacco plants (*rPTAG2I::DT-A*-Line-8). (A) Four-month-old flowerless (right) alongside wild-type plants (left). Flowerless plants displayed normal vegetative growth. (B) A closer look at the floral buds from *rPTAG2I::DT-A*-Line-8. All floral buds were aborted before reaching stage 9 of floral development. (C) A longitudinal section of a floral bud from (B) showing aborted petals, stamens and carpel. se: sepal.

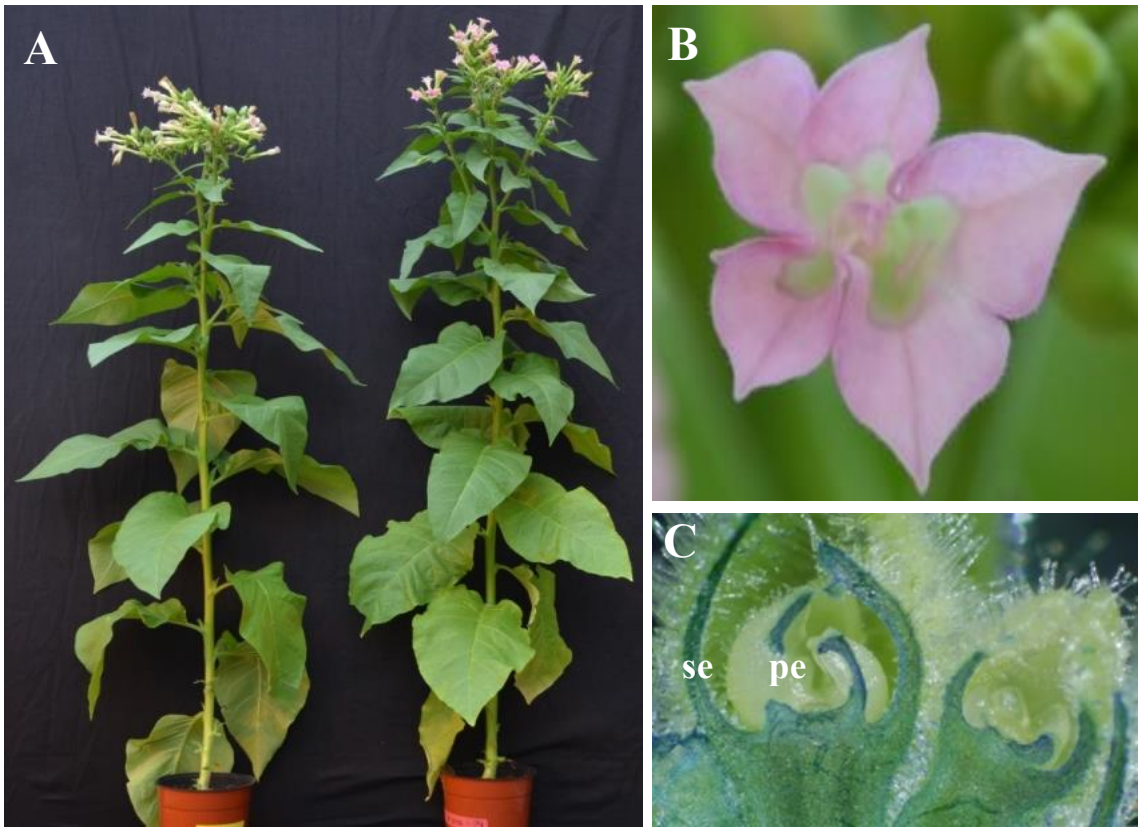


Figure 2.8 Floral organ development of greenhouse-grown neuter tobacco plants (stamen-less and carpel-less, *rPTAG2I::DT-A*-Line-27). (A) Four-month-old neuter (right) alongside wild-type plants (left). Neuter plants displayed normal vegetative growth. (B) A flower with no stamens or carpel. (C) A longitudinal section of floral buds from *rPTAG2I::DT-A*-Line-27 having stamens and carpels aborted. se: sepal; pe: petal.

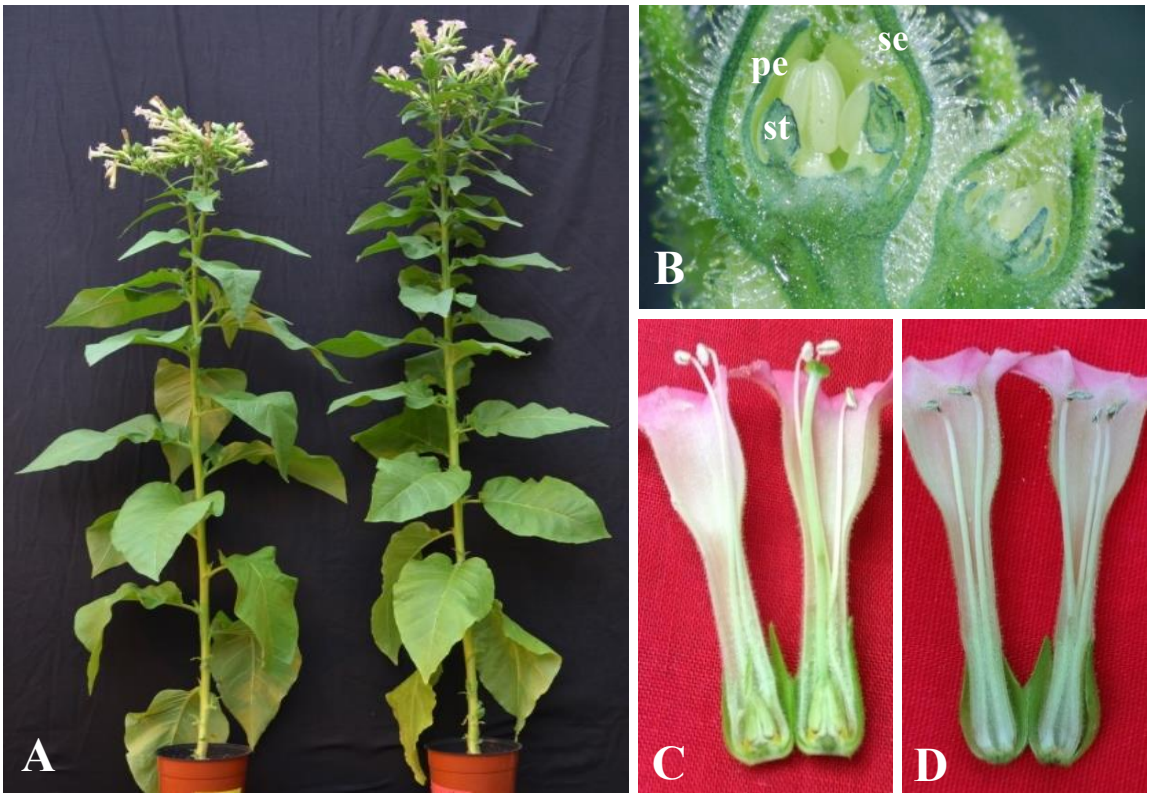


Figure 2.9 Floral organ development of greenhouse-grown carpel-less tobacco plants (*rPTAG2I::DT-A*-Line-35). (A) Four-month-old carpel-less plant (right) alongside a wild-type plant (left). Carpel-less plants displayed normal vegetative growth. (B) A longitudinal section of floral buds from *rPTAG2I::DT-A*-Line-35 with carpels aborted. (C, D) A longitudinal section of a flower from (D) carpel-less plant showing carpel aborted when compared to that from (C) a wild-type plant with a carpel fully developed. se: sepal; pe: petal; st: stamen.

2.3.3 Morphological differences between *rPTAG2I::DT-A* tobacco flowers correlated with differences in *DT-A* expression

We performed a quantitative real-time PCR (qRT-PCR) analysis to confirm the relationship between *DT-A* expression and flower ablation phenotypes observed in *rPTAG2I::DT-A* tobacco plants. Approximately 0.7 mm long floral buds, corresponding to floral stage 6 (Mandel et al., 1992) were used from representative plant lines: Line 8 (flowerless, floral buds dropped before stage 9), Line 27 (neuter, floral buds having no stamens or carpels), and Line 35 (carpel-less, with no carpels). **Figure 2.10** shows that *DT-A* expression was detected in floral buds from all three lines, with a relatively high level in Line 8, a medium level in Line 27, and a low expression level observed in Line 35. A relatively high *DT-A* expression level in the floral buds of Line 8 plants correlates with the flowerless phenotype, the strongest phenotype observed in the *DT-A* transgenic lines. A relatively low expression level observed in Line 35 also correlates with relatively normal floral development except ablation of carpels in these plants. A medium expression level detected in the floral buds of Line 27 plants may explain why these plants had an intermediate phenotype between Lines 8 and 35: ablation of both stamens and carpels. We therefore suggest that the expression level of the *DT-A* gene in floral buds correlate with the severity of the phenotype observed in floral organ ablation.

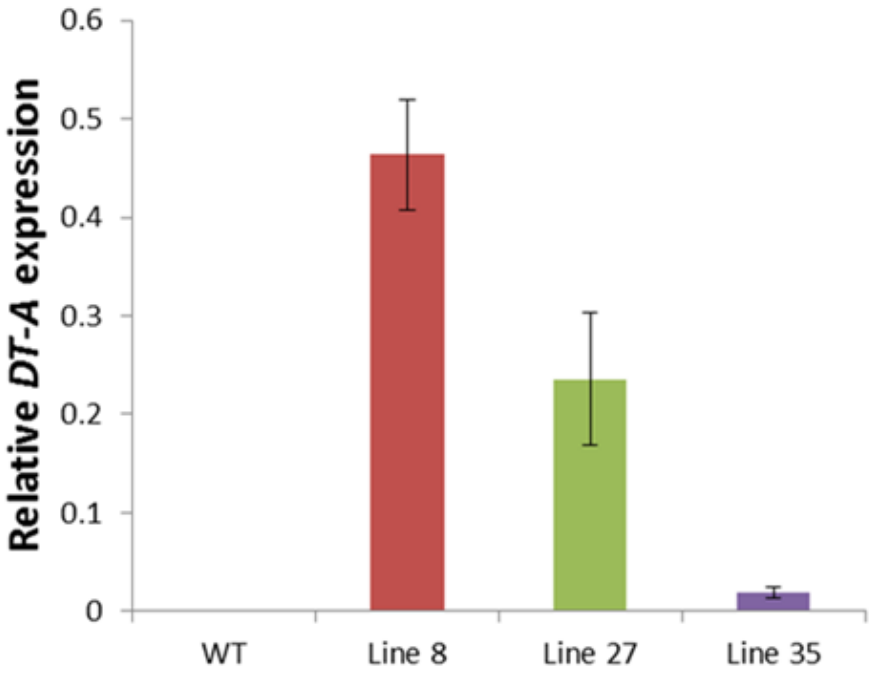


Figure 2.10 Relative expression levels of the *DT-A* gene in the 0.7 mm floral buds (floral stage 6) of three representative *rPTAG2I::DT-A* tobacco plants. WT: wild-type buds; Line 8: flowerless buds from *rPTAG2I::DT-A*-Line 8; Line 27: neuter buds from *rPTAG2I::DT-A*-Line 27; Line 35: carpel-less buds from *rPTAG2I::DT-A*-Line 35. Expression levels of the tobacco elongation factor 1α gene in each biological replicate were used as an internal reference (Schmidt and Delaney, 2010). Data represent means from three independent biological replicates. Bars show standard errors.

2.3.4 Floral ablation phenotypes were stably maintained in vegetatively propagated *rPTAG2I::DT-A* tobacco progeny under greenhouse and field conditions

To examine the stability of floral ablation phenotypes in vegetatively propagated progeny, two representative tobacco lines, Line 8 (flowerless) and Line 27 (neuter, stamen-less and carpel-less), were vegetatively propagated for further evaluation. As shown in **Table 2.1**, all tested plants from vegetatively propagated Line 8 and Line 27 maintained their floral phenotypes respectively under greenhouse conditions. We also planted vegetatively propagated Line 8 and Line 27 plants in the field during the summers of 2014 and 2015. **Table 2.1** shows that in both years, all tested plants from Line 8 maintained the flowerless phenotype and all plants from Line 27 maintained the neuter phenotype. These results demonstrate that flowerless and neuter phenotypes can be maintained in vegetative propagated progeny under both greenhouse and field conditions.

Table 2.1 Characterization of floral morphologies of *rPTAG2I::DT-A* transgenic tobacco plants in greenhouse and field.

Line[a]	No. of plants tested	Normal flower plants	Flowerless plants[b]	Neuter plants[c]	Carpel-less plants[d]
Greenhouse					
Wild type	20	20	0	0	0
Line 8	23	0	23	0	0
Line 27	19	0	0	19	0
Field					
Year 2014					
Wild type	12	12	0	0	0
Line 8	12	0	12	0	0
Line27	12	0	0	12	0
Year 2015					
Wild type	12	12	0	0	0
Line 8	12	0	12	0	0
Line 27	12	0	0	12	0

[a] Line 8 had all floral buds aborted before floral stage 9, was a representative flowerless plant; Line 27 had all floral buds with no stamens or carpels, was a representative neuter plant.

[b] Flowerless plant: all floral buds were aborted before reaching floral stage 9.

[c] Neuter plant: all floral buds had no stamens or carpels.

[d] Carpel-less plant: all flowers had no carpels.

We examined 60 flowers for each wild-type plant, and 100-120 floral buds/ flowers for each transgenic plant.

2.3.5 Field grown flowerless tobacco showed an enhanced biomass production compared to wild-type plants

Both Line 8 (flowerless) and Line 27 (neuter, stamen-less and carpel-less) exhibited similar growth as wild-type plants before flowering **(Table 2.2)**. For the flowerless plants, the floral meristems failed to develop further when reaching reproductive stages **(Figure 2.11A, B)**. After growth of floral meristems ceased, lateral shoots of the flowerless plants were released. Flowerless plants developed significant more lateral shoots than wild-type plants at the end of the growing season **(Figure 2.11C, D)**. As shown in **Table 2.2**, flowerless plants had 72-91% more dry shoot biomass, and 97-139% more dry root biomass than wild-type plants at the end of each growing season, respectively. Similarly, neuter (stamen-less and carpel-less) plants had 91-115% more dry shoot biomass, and 124-130% more dry root biomass than wild-type plants, respectively **(Table 2.2)**. Dry root/shoot biomass ratios of either flowerless plants or neuter plants were not significantly different compared to those of wild-type plants. These results demonstrate that under field conditions in 2014 and 2015, the *rPTAG2I::DT-A* gene did not affect vegetative growth of flowerless tobacco plants before flowering, and enhanced shoot biomass production in the reproductive stages.

Table 2.2 Growth characteristics of field-grown *rPTAG2I::DT-A* tobacco plants in 2014 and 2015.

Line	Height at flowering (cm)[a] (mean ± SE)	Height at harvesting (cm)[b] (mean ± SE)	No. shoots released (mean ± SE)	Dry shoot biomass (g)[c] (mean ± SE)	Dry root biomass (g)[d] (mean ± SE)	Dry root: shoot biomass ratio (mean ± SE)
Year 2014						
Wild type	99.06±2.93	112.18±1.85	6.25±0.75	80.53±8.18	26.53±3.27	0.33±0.011
Line 8 (flowerless)	103.33±4.58	128.69±2.24*	12.0±1.22*	138.73±7.62*	52.17±3.61*	0.38±0.019
Line 27 (neuter)	101.60±1.47	122.77±3.05*	11.25±1.31*	153.60±17.07*	61.13±7.88*	0.40±0.023
Year 2015						
Wild type	86.51±2.87	129.54±6.96	9.50±1.55	142.35±12.59	43.47±3.76	0.31±0.026
Line 8 (flowerless)	90.17±3.20	158.12±1.91*	17.0±1.73*	272.07±7.47*	104.11±3.65*	0.38±0.024
Line 27 (neuter)	84.29±3.42	156.63±4.48*	19.16±1.62*	305.48±9.8*	97.22±11.51*	0.32±0.036

[a] Height at flowering: plant height when just starting flowering

[b] Height at harvesting: plant height when harvesting.

[c] Dry shoot biomass includes all stem and branch biomass above root collar excluding foliage.

[d] Dry root biomass includes all root biomass below root collar.

Data were collected from 12 replicate plants and presented as averages. Asterisks (*) represent a significant difference when compared to wild type in the same year using two-tailed Student's t test with the pooled variance ($p \leq 0.05$) . SE, standard error.

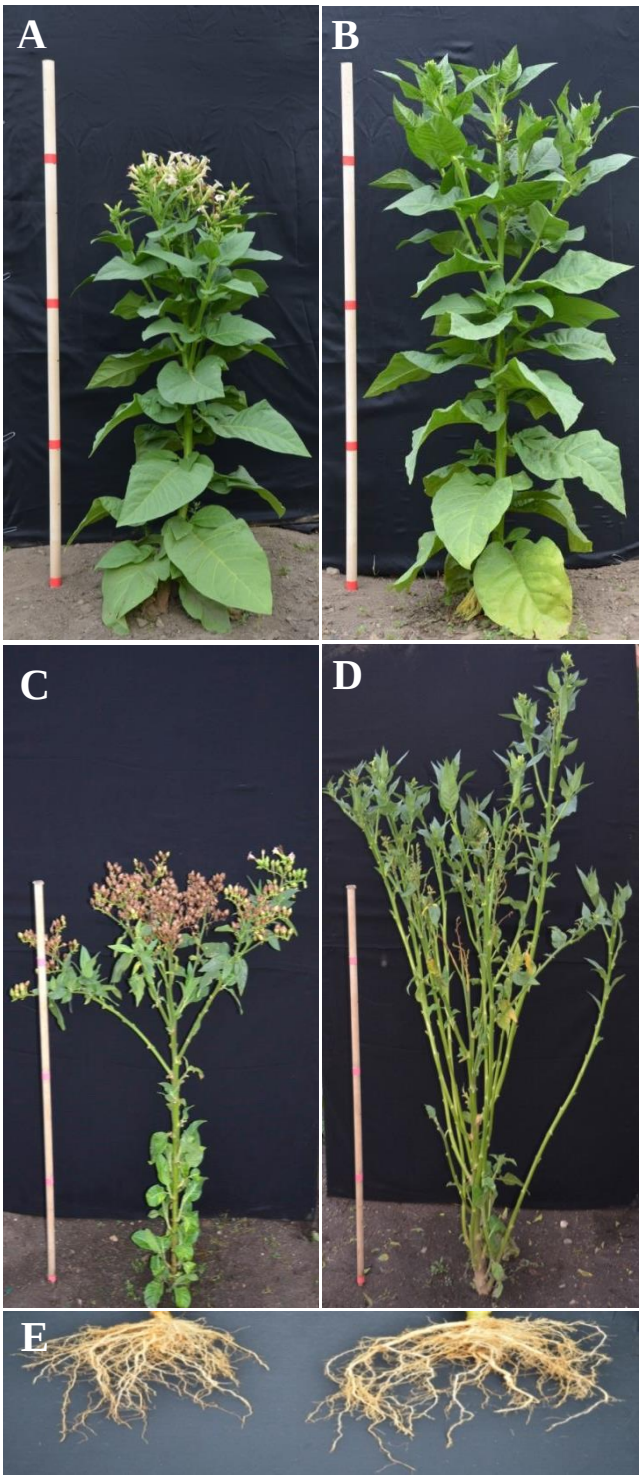


Figure 2.11 Performance of flowerless transgenic tobacco plants under field conditions. (A) Flowers developed from a three-month-old wild-type plant. (B) No flowers observed in a three-month-old flowerless plant (*rPTAG2I::DT-A*-Line 8) because all floral buds were aborted before floral stage 9. (C) Abundant seeds/seed pods produced from a four-month-old wild-type plant. (D) No seeds/seed pods produced but more branch shoots developed from a four-month-old flowerless plant (*rPTAG2I::DT-A*-Line 8). (E) A better root system (in right) observed in a four-month-old flowerless plant (*rPTAG2I::DT-A*-Line 8) when compared to that of a wild-type plant (in left).

2.3.6 *rPTAG2I::DT-A* transgenic and wild-type poplar plants exhibited similar vegetative growth under greenhouse and field conditions

Using the *rPTAG2I::DT-A* gene, we produced a total of 62 independent poplar lines from two transformation events, one in 2014 (27 lines) and the other in 2015 (35 lines) **(Figure 2.12)**. These plants were grown under greenhouse conditions and stem heights and basal stem diameters (2cm above soil) were measured after two months. We estimated the biomass index using (BI: height × diameter^2) according to the method by Wei et al. (2007). Our t-test analysis showed that neither the average height nor biomass index were negatively affected for the transgenic poplar plants from the 2014 transformations (p=0.7049 for average height and p=0.8019 for biomass index, respectively). There were also no negative effects observed for the plants produced from the 2015 transformations (p=0.7773 for the average heights and p=0.8565 for the biomass indices, respectively) **(Figure 2.13)**. On the other hand, we did observe two transgenic poplar lines (Line 36 and Line 45), that displayed significant reductions in height growth (25% and 13%, respectively). We performed a qRT-PCR assay to determine the expression levels of the *DT-A* gene in young shoots of these two poplar lines as well as four representative lines with normal vegetative growth. We observed very low but detectable levels of the *DT-A* gene expression in both Line 36 and Line 45 for which we observed reduction in stem height growth. We also observed no detectable *DT-A* expression in the four transgenic lines with normal vegetative growth **(Table 2.3)**.

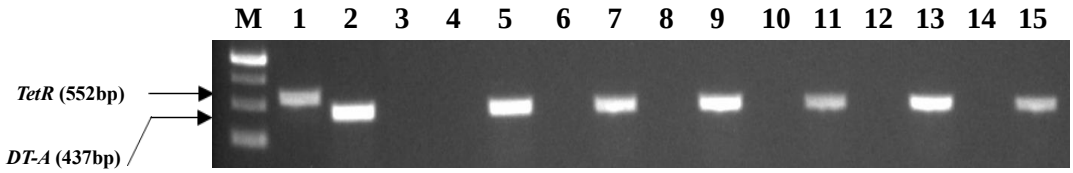


Figure 2.12 PCR confirmation of stable incorporation of the *rPTAG2I::DT-A* gene into the genome of representative poplar plant lines used for field evaluation. PCR reactions were performed as described in Materials and Methods with primer sequences for the *DT-A* gene within the T-DNA region and for the *tetR* gene within the backbone of the Ti-plasmid, using genomic DNA isolated from representative putative transgenic poplar plants as templates. The Lane M: Molecular weight marker. Lane 1 and 2: the *rPTAG2I::DT-A* Ti-plasmid as template with the *tetR* primers (Lane 1) and *DT-A* primers (Lane 2). Lane 3: Wild-type poplar plant DNA as template with the *DT-A* primers. Lanes 4-5, 6-7, 8-9, 10-11, 12-13, and 14-15 are for PCR products using genomic DNA isolated from putative *rPTAG2I::DT-A* transgenic poplar Lines 2, 16, 29, 36, 45 and 57, respectively, with the *tetR* primers for even numbers and the *DT-A* primers for odd numbers. The presence of the *DT-A* gene and the absence of the *tetR* gene in the putative transgenic poplar lines demonstrate these lines should be transgenic. On the other hand, the presence of both the *DT-A* gene and the *tetR* gene indicates that the genomic DNA from that putative transgenic poplar plant is contaminated with the Ti-plasmid DNA and thus the presence of the *DT-A* gene does not necessarily support that the plant is transgenic.

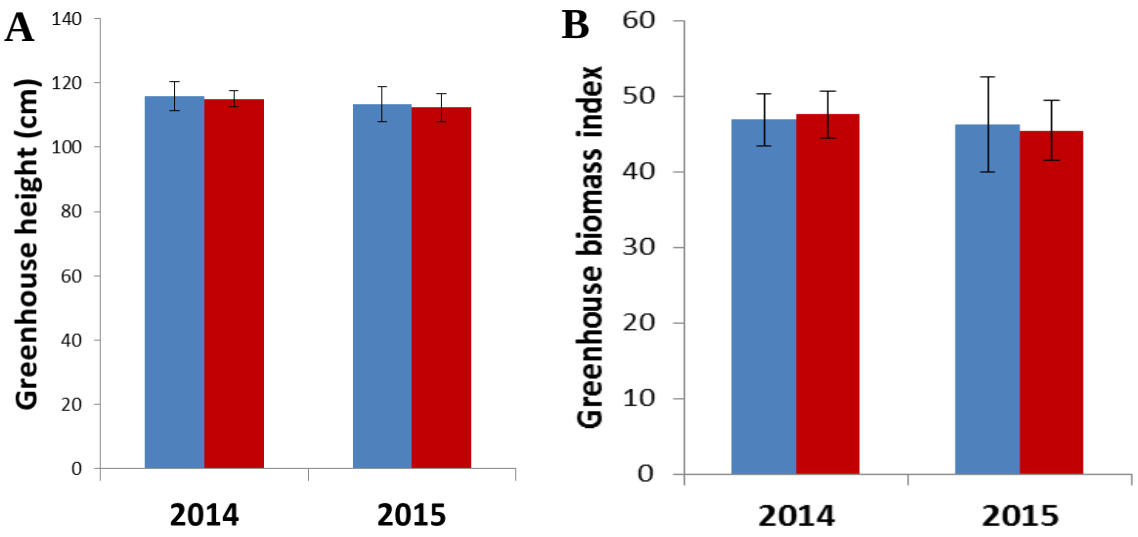


Figure 2.13 Performance of *rPTAG2I::DT-A* transgenic poplar plants under greenhouse conditions. No significant differences in (A) height or (B) biomass index (estimated using height × diameter^2) between WT and transgenic plants were observed after growing for two months in greenhouse for both 2014 and 2015 transformations. Data were analyzed using Student's t-test with the pooled variance at p=0.05. Brackets represent 95 % confidence intervals. Blue bars show data of wild type poplar plants and red bars show data of *rPTAG2I::DT-A* transgenic poplar plants.

Table 2.3 Relative expression levels of the *DT-A* gene in representative *rPTAG2I::DT-A* transgenic poplar lines

Lines	Relative expression of *DT-A* gene[a]
Wild type	Not detectable
Line 2	Not detectable
Line 16	Not detectable
Line 29	Not detectable
Line 36	2.1%
Line 45	Less than 0.1%
Line 57	Not detectable

[a] Relative expression of the *DT-A* gene in each sample was determined and calculated using the expression level of the *DT-A* gene in the same tissue sample versus that of the *UBQ* gene. For instance, the *DT-A* expression level in *rPTAG2I::DT-A* transgenic poplar Line 36 was 2.1% of the *UBQ* gene.

We planted 27 *rPTAG2I::DT-A* poplar plants derived from the 2014 transformation under field conditions. After two growing seasons, we measured the stem height and stem diameter at the base of all 27 *rPTAG2I::DT-A* poplar plants and the wild-type control plants at the end of November 2015. The average height of transgenic plants was similar to that of the wild-type plants ($p=0.7778$) **(Figure 2.14, 2.15A)**. There were no significant differences in the estimated field biomass index between the transgenic and wild-type plants ($p=0.7078$) **(Figure 2.15B)**. These findings show that no significant differences ($p>0.05$) were found between wild type and transgenic poplar for all variables tested. Depending on the variable, growth differences from 4 to 17% could be tolerated before significance would have been declared under the conditions of our experiments.

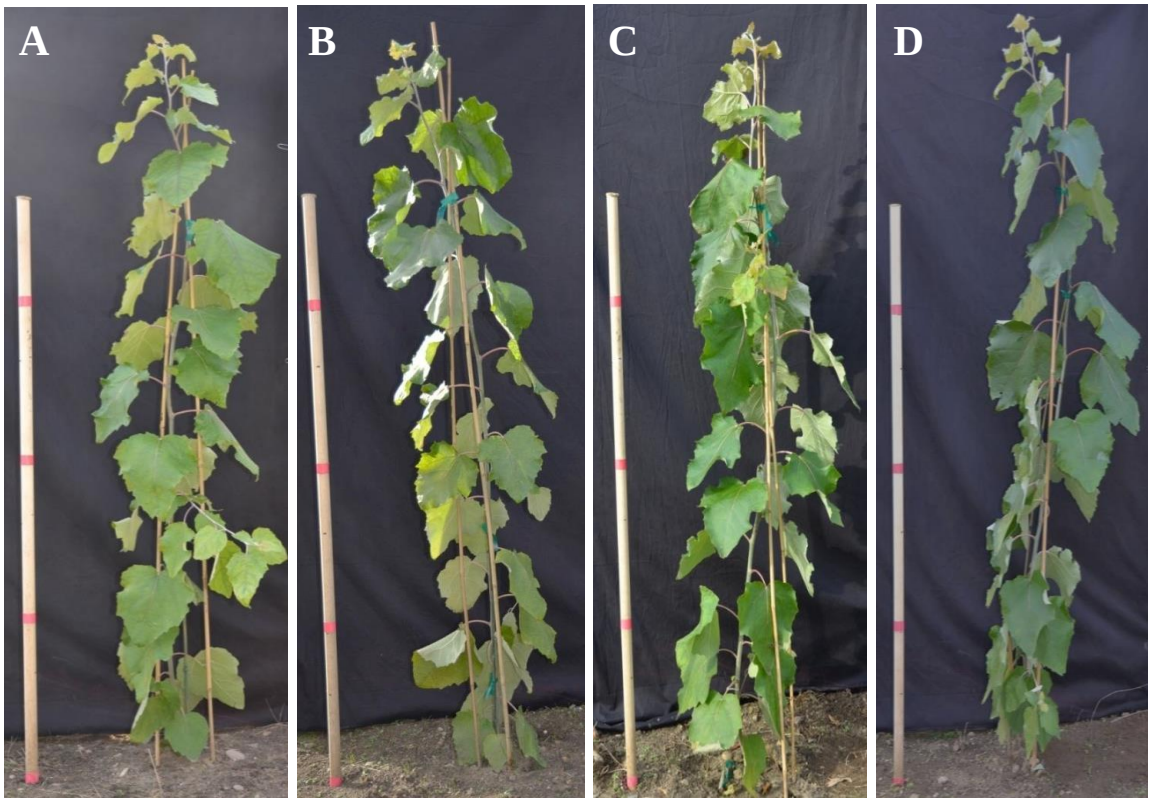


Figure 2.14 Phenotype of *rPTAG2I::DT-A* transgenic poplar plants in field. (A-D) No morphological differences were observed between (A, B) wild-type and (C, D) transgenic poplar plants after one month growth in the field.

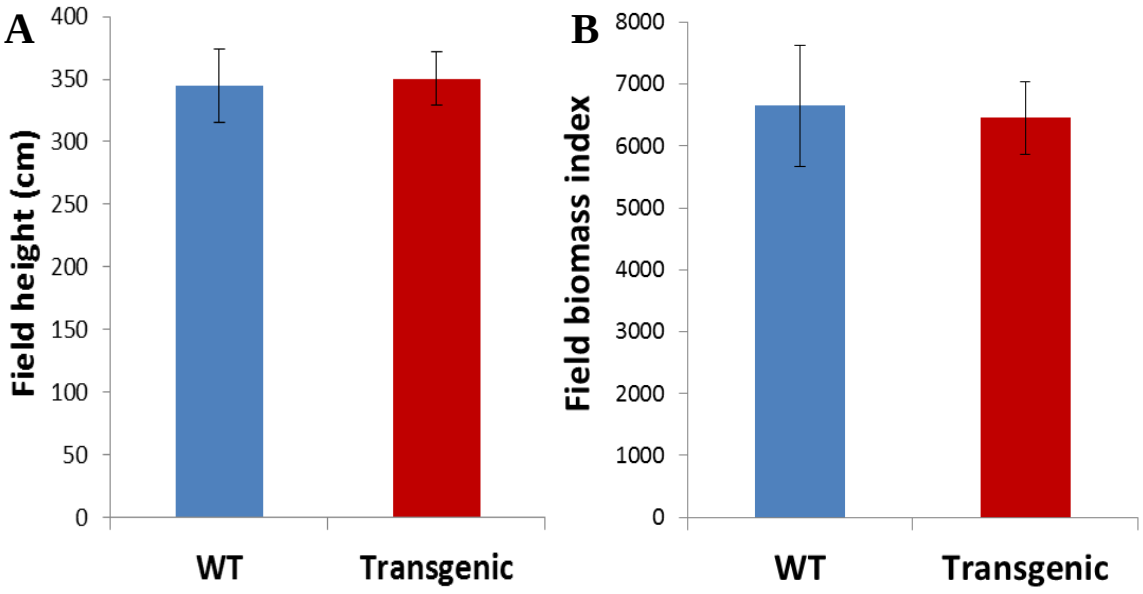


Figure 2.15 Performance of *rPTAG2I::DT-A* transgenic poplar plants under field conditions. No significant differences in (A) height or (B) biomass index (estimated using height × diameter^2) between WT and transgenic plants were observed after two growing seasons in field for 2014 transformation according to Student's t-test with the pooled variance at p=0.05. Brackets represent 95 % confidence intervals. Blue bars show data of wild-type poplar plants and red bars show data of *rPTAG2I::DT-A* transgenic poplar plants.

2.4 Discussion

In this study, we demonstrated that the reverse orientation of the second intron sequence of poplar *PTAG2* gene, when fused with the minimal *35S* promoter (*rPTAG2*), confers floral specific activity as shown with expression of the β-glucuronidase gene (*uidA*) in tobacco. We also showed that the expression of the *rPTAG2I::DT-A* gene in tobacco produced a flowerless phenotype and that the phenotype was maintained under field conditions over two growing seasons in vegetatively propagated progeny plants. We further demonstrated that under field conditions, transgenic tobacco plants expressing the *rPTAG2I::DT-A* gene produced normal vegetative growth before flower induction, and exhibited increases in vegetative growth during flowering and fruiting stages. Similar to tobacco, we found that in poplar the activity of the *rPTAG2I* promoter was not detectable in all *rPTAG2I::GUS* transgenic lines. Further, we observed that the majority of transgenic poplar plants hosting the *rPTAG2I::DT-A* gene grew and developed normally relative to wild-type plants, under both greenhouse and field conditions. The transgenic poplar plants in this study have not reached reproductive maturity and therefore could not yet be evaluated for floral specific expression of the *uidA* or *DT-A* genes. However, we believe that the *rPTAG2I* promoter should be floral dominant in poplar and the *rPTAG2I::DT-A* gene should produce the flowerless phenotype with little side effects on vegetative growth, because of the following reasons: 1) No significant negative effects on the biomass production were observed in flowerless transgenic tobacco under both field and greenhouse conditions; 2) As in *rPTAG2I::GUS* tobacco, no detectable *rPTAG2I::GUS* activity was observed in vegetative organs of poplar plants; 3) No significant negative effects were observed in biomass production for the 27 field-grown *rPTAG2I::DT-A* poplar lines. However, final confirmation

will be obtained after several years when the transgenic poplar plants reach reproductive maturity.

It has been previously reported that the second intron of *Arabidopsis AG (AG)* can direct carpel- and stamen-specific expression of *AG* due to its cis elements (Deyholos and Sieburth, 2000; Sieburth and Meyerowitz, 1997). Bioinformatics analysis of the poplar *PTAG2* second intron has shown that it contains a list of flower-related cis-regulatory elements, such as LFY-binding sites and a repeat sequence of CCAATCA, which have been demonstrated to maintain *AG* expression in *Arabidopsis* flowers (Busch et al., 1999; Hong et al., 2003). It has been reported that *PTAG2* gene exhibited floral expression with weak vegetative expression in poplar (Brunner et al., 2000). Our study has shown that forward orientation of the *PTAG2* second intron (*fPTAG2I*) exhibited activity mainly in floral organs, as well as in some vegetative organs and tissues, consistent with the observations by Brunner et al. (2000). It is, however, not known whether the *PTAG2* second intron sequence plays a role in the expression pattern of the endogenous *PTAG2* gene in poplar.

In contrast to the forward orientation of the *PTAG2* second intron (*fPTAG2I*), reverse orientation (*rPTAG2I*) conferred floral expression with no detectable activity in vegetative organs and tissues. The expression differences between genes driven by either the *fPTAG2I* or *rPTAG2I* promoter reveal that the activity of the poplar *AG* second intron is orientation-dependent. One possible explanation for this difference is that the cis-regulatory elements conferring vegetative tissue expression might be closer to the transcription starting site in the

fPTAG2I sequence, while the same cis elements in the *rPTAG2I* is farther from the transcription starting site.

Highly variable abiotic and biotic conditions in the field could significantly affect transgene expression levels and patterns and therefore the transgenic phenotypes. Remarkable differences of transgene expression or phenotype between field- and greenhouse-grown plants have been reported (Anand et al., 2003; Brandle et al., 1995; Sharp et al., 2002). The flowerless trait has been successfully engineered in a variety of annual plant species (Kobayashi et al., 2006; Wang et al., 2008); however, in all cases these transgenic lines were evaluated only under greenhouse conditions and not in the field. Our study demonstrates that the transgene-mediated flowerless phenotype in tobacco plants could be maintained over two growing seasons under field conditions with no observable adverse effects on vegetative growth. Similarly, we did not observe negative effects on vegetative growth in the majority of the transgenic poplar plants that were engineered with a floral bud toxin gene (the *rPTAG2I::DT-A*). Further, our study demonstrated that engineering a flowerless phenotype in plants could lead to more biomass production. This is most likely due to the reduction in photosynthate partitioning toward generative development, relative to wild type plants, under floral induction conditions. Enhanced biomass production as a result of the flowerless trait could be useful in various energy crops (Kalluri et al., 2014; Poovaiah et al., 2015; Torney et al., 2007).

It has been reported that vegetative growth is very sensitive to toxin gene expression and even low expression levels of a toxin gene in vegetative organs could be detrimental to the

growth of plants (Lännenpää et al., 2005; Lemmetyinen et al., 2004). Also, Wei et al. (2007) reported that all of 59 transgenic poplar lines expressing a *LEAFY* promoter::barnase gene had a substantially reduced growth rate after one or two growing seasons under field conditions, even though the growth of some of the same plant lines was similar to the wild type control plants in the greenhouse. In this study, we found that none of the 27 *rPTAG2I::DT-A* poplar plants tested under field conditions exhibited observable reduction in vegetative growth over two growing seasons, suggesting that the *rPTAG2I* promoter is floral dominant with little activity in vegetative organs. However, we did also observe that two out of the 35 transgenic poplar lines produced in the 2015 transformation had significant reduction in stem height under the greenhouse conditions when compared to the average stem height of the wild type controls. Based on the results of qRT-PCR, we believe that the reduction in stem height of these two transgenic poplar lines are due to the expression of the *DT-A* gene in stem tissues.

We also tested 25 *rPTAG2::GUS* lines in the field, and none of them had any detectable GUS activity in vegetative organs. Based on all data from the tobacco and poplar studies, we concluded that *rPTAG2I::DT-A* could be a useful tool for engineering flowerless poplar plants with normal vegetative growth characteristics. Although the t-test results showed that there were no significant differences in vegetative growth between the wild type control and *rPTAG2I::DT-A* transgenic poplar, the number of the independent transgenic poplar lines used for analysis was relatively small. It is therefore possible that with more transgenic plant lines, our current conclusion of no significant differences in vegetative growth between the wild type and *rPTAG2I::DT-A* transgenic poplar, could change.

While the flowerless phenotype can be used to reduce concerns over transgene flow, neuter (stamen-less and carpel-less) phenotype and carpel-less phenotype could have other applications. Both the neuter and the carpel-less phenotypes would be useful for reducing seed-mediated invasiveness of some exotic ornamental plants, such as purple loosestrife (Brown et al., 2002). Moreover, pollination has been shown to shorten floral duration in a variety of plant species by triggering a number of developmental events, including pigmentation changes, ultimately resulting in petal senescence (Stead et al., 1992; Proctor and Harder, 1995; Martini et al., 2003; Weber and Goodwillie, 2007). Xu and Hanson (2000) reported that incompatible pollination could drastically increase flower longevity in petunia when compared to compatible pollination. Pollination will not occur in the carpel-less transgenic plants, which can be of value for increasing flower longevity of many ornamental plants.

Although toxin genes as shown here could be effective for producing sterile transgenic plants, commercial use of these genes in transgenic plants may cause concerns (Millwood et al., 2015). On the other hand, RNAi or CRISPR/Cas9 techniques that can silence or mutate endogenous genes may offer an alternative tool if abolishing function (s) of an endogenous gene (s) can effectively lead to flowerlessness or sterility. In addition to driving the *DT-A* gene expression, the *rPTAG2I* promoter could also be used to control expression of genes important for flower or fruit development. For example, cytokinins have been shown to regulate flower size in Petunia (Verdonk et al., 2008). However, it has been reported that constitutive up- or downregulation of cytokinin negatively impacted plant growth and

development (Li et al., 1992; Werner et al., 2008). The *rPTAG2I* promoter could be used to specifically drive the expression of a cytokinin biosynthetic or degradation gene in floral buds or floral organs, which could either increase or reduce flower size with no adverse effects of the growth and development of vegetative organs.

2.5 Materials and Methods

Isolation and cloning of the *P. trichocarpa AG* 2 (*PTAG2*) second intron fragments

Total genomic DNA was extracted from leaves of *P. trichocarpa* genotype 'Nisqually-1' grown in a greenhouse using a modified CTAB method (Porebski et al., 1997). Two hundred nanograms of genomic DNA was used as template for amplifying a 4-kb second intron from *PTAG2*, one of two *AG* orthologues in *P. trichocarpa*, using primer pair PTAG2F3154 (5'-GTATATACTTAGTTCCTCGGCT-3') and PTAG2R7035 (5'-CTGCGCATTCATGTCATCATTT-3'). These primers were designed to precisely flank the splice junctions of the *PTAG2* second intron sequence (Brunner et al., 2000; Genbank accession No. AF052571). The amplification condition was as follows: an initial denaturation step at 98℃ for 5 min, followed by 35 cycles of 98℃ for 10 s, 60℃ for 5 s, and 72℃ extension plus a final extension at 72℃ for 10 min. The amplified fragment was cloned into the pGEM-T easy vector and verified by DNA sequencing.

Plasmid construction

The amplified second intron fragment of *PTAG2* was first fused with the 60-bp minimal 35S promoter at the 5′ end in forward and reverse directions to create chimeric promoters of forward orientation (*fPTAG2I*) and reverse orientation (*rPTAG2I*), respectively. The two chimeric promoters were inserted upstream of the *GUS* coding sequence in a pBIN19 vector to create constructs of *fPTAG2I::GUS* and *rPTAG2I::GUS*. Similarly, *rPTAG2I* was inserted upstream of the *DT-A* coding region in a pBIN19 vector to create the construct of *rPTAG2I::DT-A*, as illustrated in Figure 1. The *GUS* gene under the control of the globally

active 35S CaMV promoter in a pBIN19 background (*35S::GUS*) was used as a control vector.

Tobacco and poplar transformation

Plasmid vectors of all built constructs were introduced into *Agrobacterium tumeficiens* strain EHA105 separately and the resulting bacteria were used to transform *Nicotiana tabacum* cv. Xanthi and *Populus tomentosa* Carr.. Tobacco leaf disc transformation was performed as described previously (Zheng et al., 2007). Leave discs of Chinese white poplar (*Populus tomentosa* Carr.), approximately 0.5 × 0.5 cm, were incubated with A. *tumefaciens* EHA105 (OD_{630} = 0.5) for 10 min, and then transferred onto sterile filter paper to remove excess liquid and bacteria. After 2 days of co-cultivation on WPM medium at 28℃ in dark without hormone and antibiotics, infected discs were transferred to callus-inducing medium containing 2 mg l^{-1} BA, 1 mg l^{-1} NAA, 150 mg l^{-1} timentin, and 30 mg l^{-1} kanamycin. After 3 weeks cultivation at 28℃ in a 16 h photoperiod, leaf discs with induced calli were subcultured on shoot-inducing medium containing 1 mg l^{-1} BA, 0.1 mg l^{-1} NAA, 150 mg l^{-1} timentin, and 40 mg l^{-1} kanamycin. Putative transgenic shoots were then transferred to the rooting medium containing 150 mg l^{-1} timentin, and 15 mg l^{-1} kanamycin. The plantlets were transplanted in soil and grown in a greenhouse.

Molecular confirmation of transgenic plants

Genomic DNA was extracted from leaves of putative transgenic plants using a modified CTAB method (Porebski et al., 1997). In order to avoid contamination of Ti-plasmid DNA from Agrobacterium remaining in transgenic plant tissues, the isolated genomic DNA was fractioned on 0.8% (w/v) agarose gel with the related Ti-plasmid DNA loaded on the side as

a reference. Large-sized genomic DNA was recovered from the agarose gel to eliminate all Ti-plasmid DNA from residual Agrobacterium cells, and the purified plant genomic DNA was used as templates (Chen et al, 2006). The primer pair DT-AF (5'-CTTCGTACCACGGGACTAAACTGGTTATGT-3') and DT-AR (5'-AAGTTCTACGCTTAACGCTTTCGCCTGT-3') was used to amplify a 437bp fragment from *DT-A* gene within the T-DNA region of the Ti-plasmid, and the primer pair TET-F (5'-GACGACTGGCGCTCATTTCT-3') and TET-R (5'-GCATGAAAAAGCCCGTAGCG-3') was used to amplify a 552bp fragment containing a partial tetracycline resistance (*tetR*) gene within the backbone sequence of pBIN19 outside the T-DNA region. PCR reaction solution was 20 μl containing 1× PCR buffer (Takara, Japan), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2μl e2TAK DNA polymerase (Takara, Japan), 0.25 μM of each primer and 500 ng DNA. The amplification started with an initial denaturation step at 98℃ for 5 min, followed by 35 cycles of 98℃ for 10 s, 60-65 ℃ for 5 s, and 72℃ extension plus a final extension at 72℃ for 10 min.

Tissue sectioning and histochemical GUS assays

Poplar shoot apices, as well as tobacco hand-sectioned leaf, stem, shoot apices, and floral buds were incubated in X-gluc solution at 37℃ overnight for histochemical GUS staining. Histochemical assays of GUS activity were performed in a solution consisting of 100 mM potassium phosphate buffer, pH 7.0, 10 mM Na_2EDTA, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, 0.1% triton X-100, 1 g/l X-gluc (5-bromo-4-chloro-3-indolyl-β-d-glucuronic acid). Subsequent depigmentation was carried out in ethanol to gradually remove

chlorophyll and other pigments gradually prior to visual inspection and photographically recorded.

Pollen germination assays

Pollen germination assays were performed as described previously (Wang and Jiang, 2011). Basically, pollen was collected from stage 11-12 anthers of both wild type and transgenic tobacco plants, and incubated on glass slides with pollen germination medium [0.01% boric acid, 1mM $CaCl_2$, 1mM $Ca(NO_3)_2 \cdot 4H_2O$, 1mM $MgSO_4 \cdot 7H_2O$, 10% (wt/vol) sucrose, pH 6.5] at 27.5 ℃. After two hours, the pollen germination rates were recorded.

Quantitative real-time PCR analysis of *DT-A* expression in transgenic tobacco plants

Samples for RNA extraction were collected from vegetatively propagated plants per representative flowerless, neuter, carpel-less, and wild type tobacco plant lines. More than 20 0.7-mm floral buds were collected for each replicate. Total plant RNA was extracted using the RNeasy Plant Mini Kit including RNase-Free DNase set (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Richmond, CA) was used to synthesize cDNA, and cDNA products were utilized for quantitative real-time PCR assays using SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories, Richmond, CA) on a CFX96™ Real-Time PCR detection system (Bio-Rad Laboratories, Richmond, CA). The primer pair, DTA-F (5'-GAGTTTATCAAAAGGTTCGGT-3') with DTA-R (5'-TTCGCCTGTTCCCAGTTATT-3') was used for analysis of DT-A transcripts, and the pair, EF1α-F (5'-TGAGATGCACCACGAAGCTC-3') with EF1α-R (5'-

CCAACATTGTCACCAGGAAGTG-3') was used to amplify cDNA of the internal reference gene, elongation factor 1α. Data were analyzed using CFX Manager™ software version 2.0. The *DT-A* gene expression in each sample was normalized using the expression level of the elongation factor 1α gene in the same sample (Schmidt and Delaney, 2010). Three biological replicates were performed with the wild type and each independent transgenic line.

Evaluation of stability of floral ablation phenotypes in vegetative propagated progeny plants in the greenhouse

Representative flowerless, neuter, and wild type plant lines were vegetatively propagated and grown in a greenhouse. After growing for three months, the tobacco plants started flowering. To determine the floral phenotype of each plant, 100-120 floral buds/flowers were examined for each transgenic plant.

Field evaluations of flowerless transgenic tobacco plants

Representative flowerless, neuter, and wild type tobacco plants were vegetatively propagated and grown in a greenhouse. One month old plants with heights of approximately 20 cm were planted in the field located in Storrs, Connecticut, USA in July, 2014 and June, 2015. Field test plots employed a randomized design with 12 replicates. Heights of both transgenic and wild type tobacco plants were recorded at initial flowering and final harvest at the end of the growing season (October in 2014 and 2015). At harvest, all tobacco plants were carefully dug out and cut just above the root collars. After removing leaves of all plants, shoot materials (above root collars) and root materials (below root collars) were oven-dried at 70°C for 10 days and then weighed. Shoot biomass, root biomass and ratio of

root:shoot biomass were determined for each replicate. Data were reported as means of all 12 replicates. Means between field-grown transgenic and wild type tobacco plants were compared using the two-tailed Student's t-test with the pooled variance (Steel et al., 1997).

Vegetative growth evaluation of poplar plants under greenhouse and field conditions

Plants of the 62 *rPTAG2I::DT-A* transgenic poplar lines and 20 wild type plants were acclimatized in pots at 25 °C under a 14 h photoperiod with a humidity of 80% and then transferred to a greenhouse maintained under ambient light cycles (27 lines and 10 wild type plants in the year of 2014, 35 lines and 10 wild type plants in 2015). Once acclimated, poplar lines were arranged in a randomized block design. After two months of growth in greenhouse, height and basal diameter (2 cm above soil) were measured using a ruler and vernier caliper, respectively. Greenhouse biomass index was estimated using (height × diameter^2) (Wei et al., 2007). After greenhouse evaluation, the 27 *rPTAG2I::DT-A* transgenic poplar plant lines derived from the 2014 transformation and 10 wild type were planted in the field located in Storrs, Connecticut in August, 2014 at a spacing of 5×5 feet (1.5 × 1.5 m). Transgenic plants and wild type controls were randomly planted. Plants were watered as needed until fully established. Height and basal diameter (both in cm) were taken on all poplar plants at the end of the growing season of (November) 2015. Field biomass index was estimated using (height × diameter^2) (Wei et al., 2007). Data were reported as the mean of all events. Comparisons of means between transgenic and wild type poplar plants were conducted using two-tailed Student's t-test with the pooled variance (Steel et al., 1997).

Quantitative real-time PCR analysis of *DT-A* expression in transgenic poplar plants

The two *rPTAG2I::DT-A* transgenic poplar lines with retarded vegetative growth, four representative *rPTAG2I::DT-A* transgenic poplar lines with normal growth, and wild type plants were vegetatively propagated and grown in greenhouse. A 1.5-cm shoot from the apex of two-month-old greenhouse-grown poplar plants were collected for RNA isolation. Total plant RNA was extracted using the RNeasy Plant Mini Kit including RNase-Free DNase set (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Richmond, CA) was used to synthesize cDNA, and cDNA products were utilized for quantitative real-time PCR assays using SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories, Richmond, CA) on a CFX96™ Real-Time PCR detection system (Bio-Rad Laboratories, Richmond, CA). The primer pair, DTA-F (5'-GAGTTTATCAAAAGGTTCGGT-3') with DTA-R (5'-TTCGCCTGTTCCCAGTTATT-3') was used for analysis of *DT-A* transcripts, and the pair, UBQ-F (5'-GTTGATTTTTGCTGGGAAGC-3') with UBQ-R (5'-GATCTTGGCCTTCACGTTGT-3') was used to amplify cDNA of the internal reference gene, ubiquitin. Data were analyzed using CFX Manager™ software version 2.0. The *DT-A* gene expression in each sample was normalized using the expression level of the ubiquitin gene in the same sample. Three biological replicates were performed with the wild type and each independent transgenic line.

Chapter 3 Elevated auxin and reduced cytokinin contents in rootstocks improve their performance and grafting success

3.1 Abstract

Plant grafting is an important technique for horticultural and silvicultural production. However, many rootstock plants suffer from undesirable lateral bud outgrowth, low grafting success rates, or poor rooting. Here we used a root-predominant gene promoter (*SbUGT*) to drive expression of a tryptophan-2-mono oxygenase gene (*iaaM*) from *Agrobacterium tumefaciens* to increase auxin levels in tobacco. The transgenic plants, when used as a rootstock, displayed inhibited lateral bud outgrowth, enhanced grafting success rate, and improved root initiation. However, root elongation and biomass of *SbUGT::iaaM* transgenic plants were reduced compared to that of wild-type plants. In contrast, when we used this same promoter to drive *CKX* (a cytokinin degradation gene) expression, the transgenic tobacco plants displayed enhanced root elongation and biomass. We then made crosses between the *SbUGT::CKX* and *SbUGT::iaaM* transgenic plants. We observed that overexpression of the *CKX* gene neutralized the negative effects of auxin overproduction on root elongation. Also, the simultaneous expression of both the *iaaM* and *CKX* genes in rootstock did not disrupt normal growth and developmental patterns of non-transgenic scions. Our results demonstrate that expression of both the *iaaM* and *CKX* genes predominantly in roots of rootstock inhibits lateral bud release from rootstock, improves grafting success rates, and enhances root initiation and biomass. The use of non-transgenic scions has potentials to eliminate the spread of transgenes via flowers and fruits.

3.2 Introduction

Grafting is an essential tool in horticulture and silviculture that is most commonly used in asexual propagation of commercially grown plants. In tree fruit production, grafting of scions to rootstocks is used to produce dwarf trees, enhance disease resistance, increase fruit yield and quality, combine production of multiple varieties on a single tree, and enhance fertilization (Artlip et al., 2016). More recently, grafting has been extended from fruit trees to vegetables for enhancing resistances to biotic and abiotic stress factors, improving water and nutrient uptake, or increasing yield (Melnyk and Meyerowitz, 2015; Nakamura et al., 2016; Warschefsky et al., 2016; Zhao and Song, 2014).

However, many woody plant species with excellent rootstock characteristics are difficult to root from stem cuttings. For instance, it is difficult to induce adventitious rooting on stem cuttings from the apple cultivar 'M.9', which is commonly used as a dwarfing rootstock (Pawlicki and Welander, 1995; Zhu et al., 2001). A dwarf pear cultivar, BP10030, is cold hardy and graft compatible with most pear varieties but it is also difficult to root from stem cuttings (Zhu et al., 2003).

Also, the undesirable outgrowth of lateral buds from rootstocks after grafting is a common phenomenon. If lateral shoots from rootstock are not suppressed or removed, healing of the graft union can be adversely affected and rootstock's lateral shoots compete with scions for light and nutrients, reducing the rate of grafting success and scion growth (Daley and Hassell, 2014). Chemical treatments or manual removal may be used to eliminate lateral shoots from rootstock but these procedures are time consuming and expensive (Daley and Hassell, 2014).

Traditional breeding efforts have made impressive progress toward improving rootstock performance in numerous plant species, but continued improvement remains limited to selection of existing traits within the gene pool of rootstock cultivars (Cousins, 2005). In contrast to traditional breeding, transgenic plant technology can be used to introduce completely new traits into rootstock lines and at a much faster rate, sometimes within months (Gambino and Gribaudo, 2012).

Lateral branching in plants is regulated by interactions between the phytohormones indole-3-acetic acid (IAA, auxin), cytokinin, and strigolactone (Ferguson and Beveridge, 2009). It has been reported that apically-derived auxin inhibits lateral bud outgrowth and cytokinin directly or indirectly stimulates bud outgrowth (Müller and Leyser, 2011). Insertion of the *Agrobacterium* gene *iaaM* gene that encodes a tryptophan-2-monoxygenase into plants has been shown to convert tryptophan to indole-3-acetamide. Indole-3-acetamide is then slowly converted by endogenous hydrolases to the active phytohormone indole-3-acetic acid (Sitbon et al., 1992). Cytokinin oxidase (CKX) degrades the phytohormone cytokinin. Here we report the use of a root-predominant gene promoter sequence (*SbUGT*) to drive the expression of *iaaM* gene and an *Arabidopsis* cytokinin oxidase/dehydrogenase gene (*AtCKX2*, abbreviated as *CKX*) using tobacco as a model plant. The transgenic plants, when used as rootstock, displayed inhibited lateral bud outgrowth, enhanced grafting success rate, and improved root initiation and biomass. The combined use of the auxin overproducing and cytokinin inactivating genes in roots represents an excellent strategy for rootstock improvement.

3.3 Results

3.3.1 The *SbUGT::iaaM* expression inhibited the outgrowth of lateral buds following decapitation

The *SbUGT::GUS* fusion gene (**Figure 3.1**) was predominantly active in roots of transgenic tobacco plants (**Figure 3.2**). The *SbUGT* promoter sequence was used to control the expression of *iaaM* (**Figure 3.1**). Of 58 *SbUGT::iaaM* tobacco lines produced, more than 75% of these plants showed no difference in growth and developmental patterns in the above ground organs when compared to wild-type plants (**Figure 3.3**). The remaining 25% showed a weak but visible auxin overproducing phenotype characterized by slight downward-curled and epinastic older leaves (**Figure 3.4**, *SbUGT::iaaM*-39 plant on the right). In contrast, the expression of the *iaaM* under the control of a *small auxin up RNAs* (*SAUR*) gene promoter, that is highly active in shoots and leaves (Li et al., 1992; Li et al., 1994), resulted in stunted shoot growth and strong leaf epinasity (see Guilfoyle et al., 1992). Expression of *iaaM* in shoots appeared to inhibit lateral bud release in rootstocks following decapitation, as *iaaM* expression levels (**Figure 3.5A**) positively correlated with bud release delays of six weeks in *SbUGT::iaaM*-39, four weeks in *SbUGT::iaaM*-24, and one week in *SbUGT::iaaM*-15 lines.

SbUGT::GUS

SbUGT::iaaM

Figure 3.1 T-DNA regions of the *SbUGT::GUS* and *SbUGT::iaaM* constructs. LB: left border sequence of T-DNA. RB: right border sequence of T-DNA. *tNOS*: nopaline synthase terminator. *NptII*: neomycin phosphotransferase gene. *pNOS*: nopaline synthase gene promoter sequence. *SbUGT*: the -102 to +86 gene fragment of flavonoid glycosyltransferase gene. *GUS*: the coding sequence for the β-glucuronidase gene. *iaaM*: a tryptophan-2-mono oxygenase gene from *Agrobacterium tumefaciens.*

Figure 3.2 Analysis of *SbUGT* promoter activity in transgenic tobacco by GUS histochemical assay. Histochemical staining of GUS activity in a *SbUGT::GUS* tobacco T_1 seedling, showing that the *SbUGT* promoter was predominantly active in roots.

Figure 3.3 Phenotype of wild-type and *SbUGT::iaaM* tobacco. Four-month-old wild-type and *SbUGT::iaaM*-15 tobacco plants, showing that expression of the *SbUGT::iaaM* gene did not affect growth and developmental patterns of leaves and shoots.

Figure 3.4 Phenotype of wild-type and *SbUGT::iaaM* tobacco after decapitation. Three weeks after decapitation, wild-type plants released numerous lateral buds while the *SbUGT::iaaM*-39 plants had no lateral buds released from the decapitated shoots, the arrow heads indicate the decapitated shoots of wild-type (left) and *SbUGT::iaaM*-39 (right) tobacco.

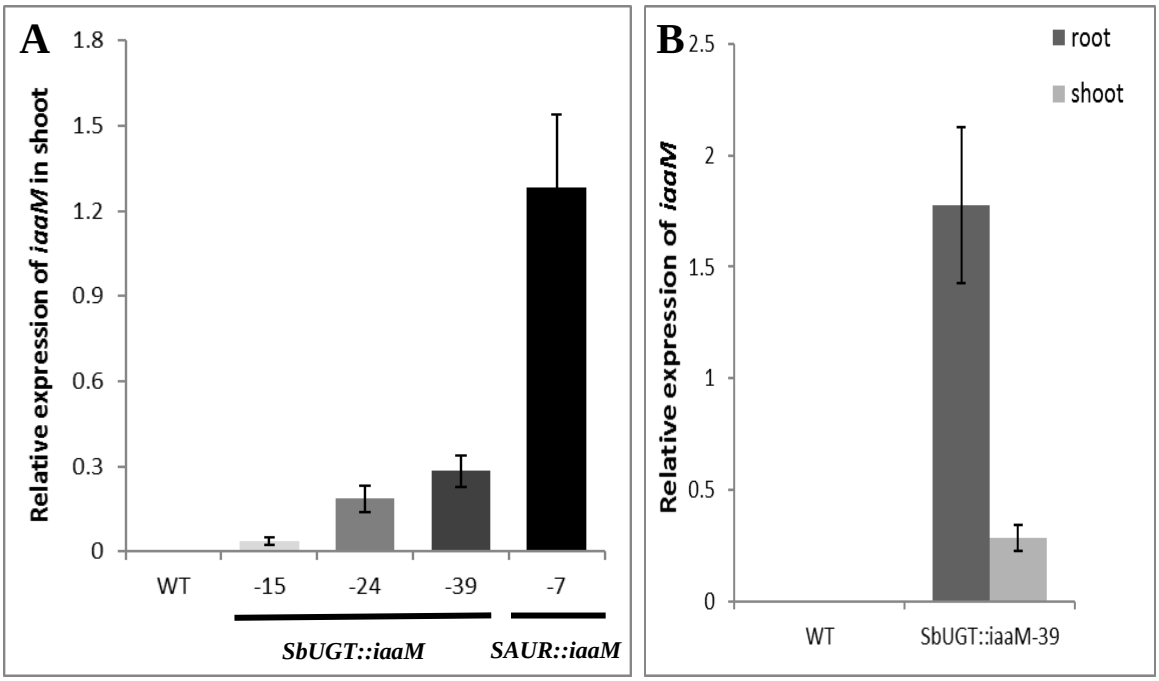


Figure 3.5 Relative expression levels of the *iaaM* gene in *SbUGT::iaaM* and *SAUR::iaaM* tobacco plants. (A) Expression of *iaaM* in shoots appeared to inhibit lateral bud release in rootstocks following decapitation, as *iaaM* expression levels positively correlated with bud release delays. (B) Relatively high expression of the *iaaM* gene in roots but low expression in shoots were observed in the *SbUGT::iaaM*-39 plant line. Expression levels of the tobacco elongation factor 1α gene in each biological replicate were used as an internal reference (Schmidt and Delaney, 2010). Data represent means from three independent biological replicates. Bars show standard errors.

In *SbUGT::iaaM*-39 transgenic plants, *iaaM* was highly expressed in roots, but was also detectable in shoot tissues (**Figure 3.5B**). This result is slightly differently from histochemical staining of GUS activity in young seedlings (**Figure 3.2**), which suggests the activity of the *SbUGT* promoter could be developmentally regulated. In *SbUGT::iaaM*-39 roots, free IAA levels increased about 3-fold compared to wild type (**Table 3.1**), and expression of the endogenous auxin responsive gene *GRETCHEN HAGEN 3* (*GH3*) was also increased (Li et al., 1999) (**Figure 3.6**). Free IAA level in *SbUGT::iaaM*-39 shoots also increased about 2-fold compared to that of wild-type plants, with 414.1ng/g dry weight for *SbUGT::iaaM*-39 and 156.2ng/g dry weight for wild-type plants. Based on these observations, the *SbUGT::iaaM*-39 line was selected for further experimentation.

Table 3.1 Endogenous auxin contents in roots of WT, *SbUGT::iaaM*-39 (*iaaM*), *SbUGT::CKX*-64 (*CKX*), and the *SbUGT::iaaM*-39/*SbUGT::CKX*-64 (*iaaM+CKX*) hybrid plants

Plants	Root IAA content (ng/g DW) (mean ± SE)
Wild type	351.9±7.6 c
iaaM	946.4±20.3 a
CKX	172.2±15.8 d
iaaM+CKX	830.4±33.4 b

Data represent the average of three biological replicates. Each replicate consists of the pooled root samples from 10 plants. Values followed by the different letter are significantly different at $p < 0.05$ (ANOVA; LSD). SE, standard errors.

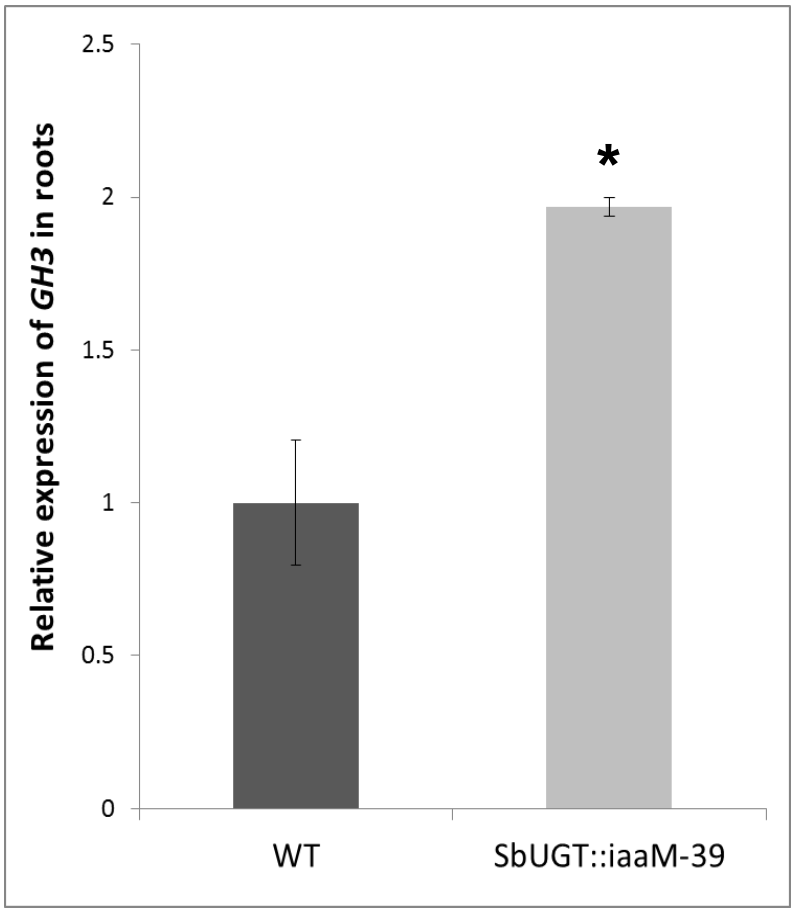


Figure 3.6 Expression levels of the auxin responsive *GH3* gene were enhanced in roots of the *SbUGT::iaaM*-39 line. Asterisk (*) represent significant differences between wild-type and *SbUGT::iaaM*-39 tobacco using two-tailed Student's t test with the pooled variance ($p < 0.05$). Bars represent standard errors.

3.3.2 *SbUGT::iaaM* gene expression suppressed rootstock's lateral bud release and improved grafting success rates

After wild-type tobacco scions were grafted onto wild-type plant rootstocks (abbreviated as WT/WT), lateral shoots began to develop from the rootstock within two weeks of grafting and the growth of scions was reduced (**Figure 3.7A**). When lateral buds were removed from wild-type rootstock, vigorous scion growth was observed (**Figure 3.7B**). However, no lateral bud release from the rootstocks of WT/*SbUGT::iaaM-39* (abbreviated as WT/*iaaM*) grafts were observed, and scion growth was also vigorous (**Figure 3.7C**). This was quite unlike scion growth of WT/WT grafts, which was vigorous only if the lateral buds of the rootstock were removed (**Figure 3.8**). These results demonstrate that there is no need to remove lateral buds from rootstock thus eliminating costs associated with this procedure.

When WT plants were used as rootstock, a 24% grafting success rate was observed if lateral buds were not removed from the rootstock (**Table 3.2**). After manual removal of buds from the WT rootstock, the grafting success rate increased to 68%. On the other hand, when the *iaaM* plants were used as rootstocks under the identical experimental conditions, we observed no lateral bud release and the grafting success rate reached 91%, demonstrating that expression of the *SbUGT::iaaM* in rootstock significantly enhanced grafting success rates.

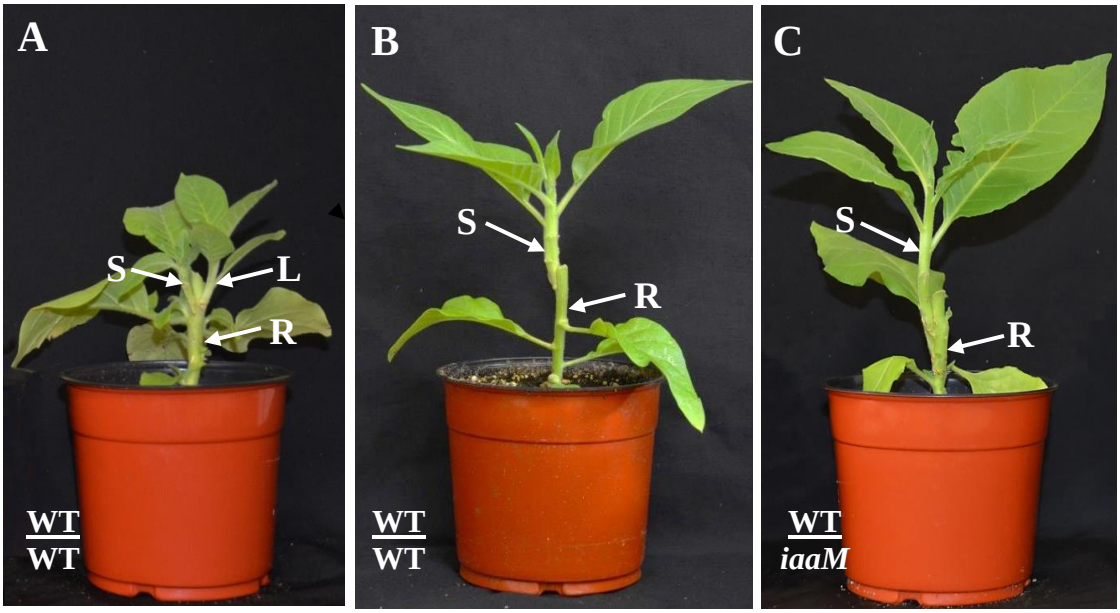


Figure 3.7 Using *SbUGT::iaaM*-39 plants as rootstock leads to inhibited lateral bud release from rootstock stumps and vigorous scion growth. (A) Two weeks after grafting, WT/WT grafts had released lateral buds and scion growth was inhibited. (B) If WT/WT grafts' lateral buds were manually removed from the rootstock stumps, scions grew vigorously. (C) WT/*iaaM* grafts had no lateral buds released from the rootstock stumps, and scions grew vigorously. S: Scion; L: Lateral bud; R: Rootstock.

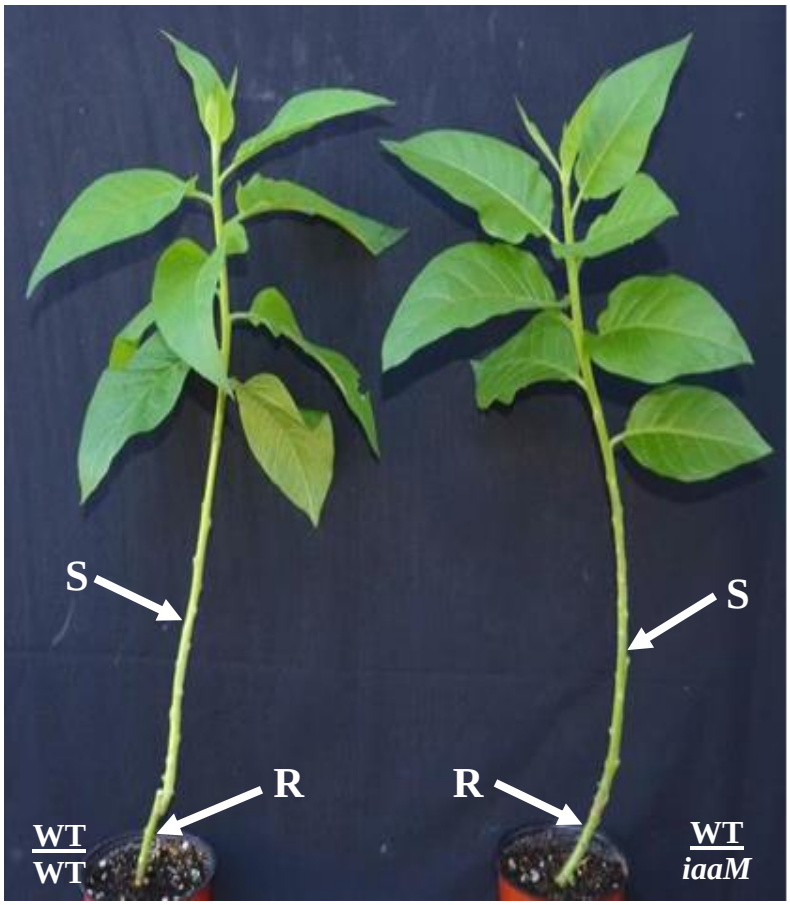


Figure 3.8 Scion growth on wild-type and transgenic rootstock. Two months after grafting, WT/*iaaM* grafts had normal scion growth similar to WT/WT with lateral buds removed from rootstock stumps. S: Scion. R: Rootstock.

Table 3.2 Grafting success rates of grafts with or without removing lateral buds from rootstock stumps

Grafting method	Grafting success rates (mean ± SE) (%)[a]			
	WT/WT (with lateral buds intact on rootstocks)	WT/WT (with lateral buds removed from rootstocks)	WT/*iaaM* (with lateral buds intact on rootstocks)	WT/*iaaM*+*CKX* (with lateral buds intact on rootstocks)
Decapitated plants as rootstock	24±4a	68±5b	91±1c	89±2c

[a] Grafts with more than 2-cm increase in scion growth were considered as successful grafting. Data were collected three weeks after grafting. Each type of grafting has three replicates. For each replicate, 8-11 grafts were performed.

Values with the different letters indicate significantly different at $p < 0.05$ (ANOVA; LSD). SE, standard errors.

We also examined growth performance of WT/WT and WT/*iaaM* grafts under field conditions (**Table 3.3**). While lateral bud release was observed from the rootstock of the WT/WT grafts 10 days after planting, no lateral buds were released from the rootstock of the WT/*iaaM* grafts, demonstrating that the use of *iaaM* rootstocks can eliminate the need for lateral buds removal under field conditions. With lateral buds removed from the rootstock of the WT/WT grafts, the scions grew more vigorously, as indicated by height and dry biomass, than the WT/WT grafts for which lateral buds were left intact. Scion growth in the WT/*iaaM* grafts that exhibited no lateral bud release was similar to that of the WT/WT grafts after manual lateral bud removal from the rootstock. Finally, lateral bud release from the scions of the WT/*iaaM* grafts was similar to that of the WT/WT grafts two weeks following apical shoot excision (**Figure 3.9**), demonstrating that the *iaaM* rootstock had minimal effects on the branching behavior of scions.

Table 3.3 Growth performance of scions of field-grown grafts

Grafts (scion/rootstock)	Height on day 60 (cm)[a] (mean ± SE)	Height on day 90 (cm)[b] (mean ± SE)	Dry scion biomass (g)[c] (mean ± SE)
WT/WT (lateral buds intact on rootstock)	66.4±1.7a	99.8±3.0a	69.9±2.0a
WT/WT (lateral buds removed from rootstock)	76.3±1.9b	128.4±3.1b	83.8±2.7b
WT/*iaaM* (lateral buds intact on rootstock)	81.2±3.3b	127.8±5.9b	81.5±3.2b

[a] Height on day 60: plant height after 60 days in the field.

[b] Height on day 90: plant height after 90 days in the field.

[c] Dry scion biomass includes all stem and branch biomass above the graft union (excluding leaves), data were collected after 90 days in the field.

Data were collected from 10 individual plants and presented as averages. Values in the same column followed by the different letter are significant different at $p < 0.05$ (ANOVA; LSD). SE, standard errors.

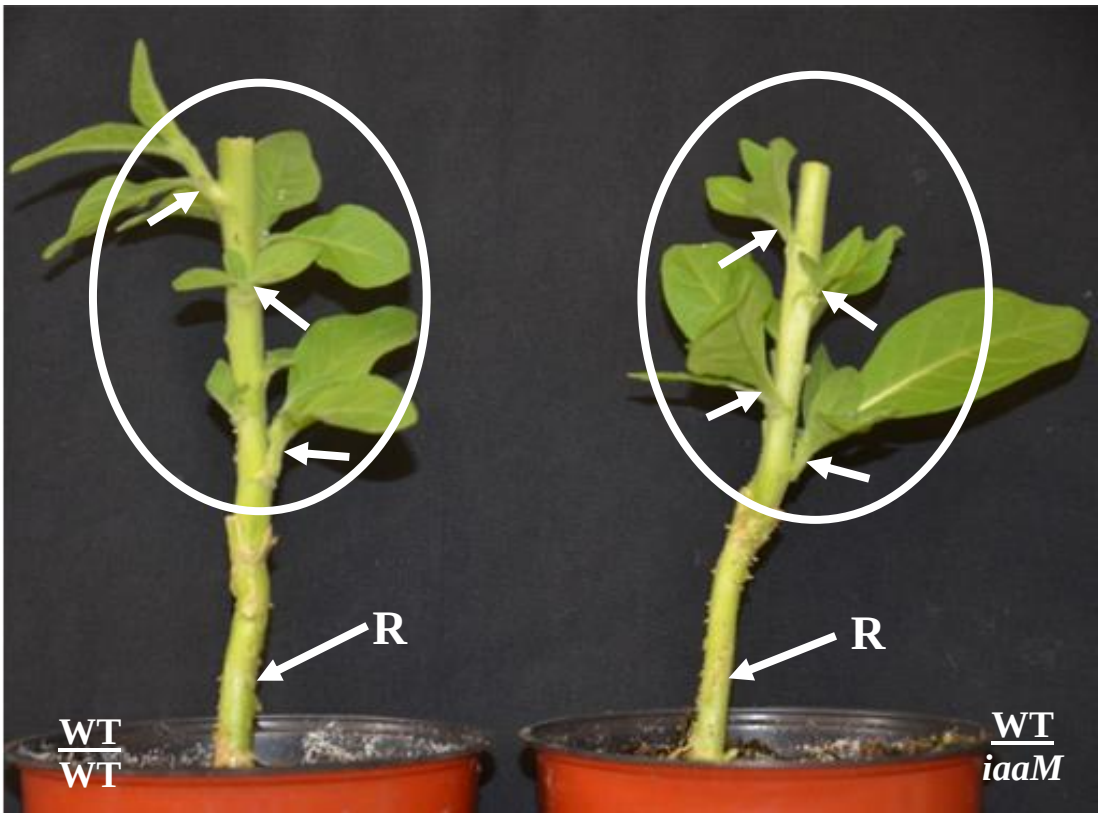


Figure 3.9 Lateral buds release on wild-type scions of wild-type and *iaaM* rootstock following decapitation. Two weeks following decapitation, scions of WT/*iaaM* had normal lateral buds release as the scions of WT/WT grafts, showing that increase of auxin in rootstock did not affect normal growth and development of the scions. Circles show scions. Arrow heads indicate released lateral buds. R: Rootstock.

3.3.3 The reduction in root growth observed in *SbUGT::iaaM* rootstock can be compensated by *SbUGT::CKX* expression

One concern about application of the *SbUGT::iaaM* expression as a practical technology was an observed reduction in root growth. Although root initiation in *iaaM* cuttings was more rapid compared to that of the wild-type plants, root elongation and root biomass were reduced (**Figures 3.10, 3.11, Table 3.4**). To circumvent the negative effects of *iaaM* gene expression on root growth, we over-expressed an *Arabidopsis* cytokinin oxidase/dehydrogenase gene (*AtCKX2*, abbreviated as *CKX*) in roots. In general, *SbUGT::CKX* tobacco plants displayed improved root elongation and increased root biomass. Although this phenomenon was observed in multiple *CKX* overexpression lines, one line, *SbUGT::CKX-64* was selected for further experiments. The roots of *SbUGT::CKX-64* plants had significantly reduced endogenous cytokinin content compared to wild-type plants (**Figure 3.12**), and expression of the a cytokinin-responsive reporter *ARR5*::GUS (Romanov et al., 2002) was significantly reduced (data not shown), providing additional evidence that expression of the *SbUGT::CKX* gene was effective at reducing cytokinin levels in the roots of transgenic plants.

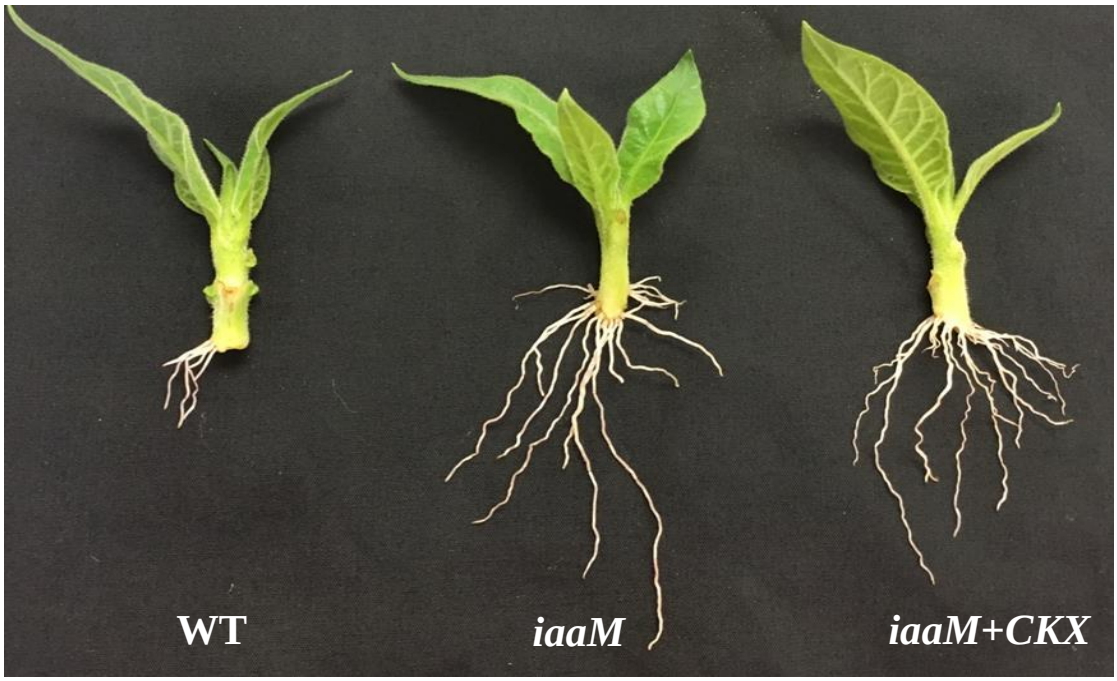


Figure 3.10 Rooting initiation of *iaaM* and *iaaM+CKX* tobacco. Shoot tips of wild type, *SbUGT::iaaM*-39 (*iaaM*), and *SbUGT::iaaM*-39/*SbUGT::CKX*-64 (*iaaM+CKX*) hybrid plant that were rooted in fritted clay medium for 10 days, showed that the *iaaM* gene expression promoted root initiation.

Figure 3.11 Root growth of *iaaM* and *iaaM+CKX* tobacco. Six-week-old (C) *iaaM+CKX* plants had more root growth compared to the (A) wild-type or (B) *iaaM* plants.

Table 3.4 Root growth characteristics of stem cuttings of *SbUGT::iaaM*-39 (*iaaM*) and *SbUGT::iaaM*-39/*SbUGT::CKX*-64 (*iaaM+CKX*) hybrid plants under greenhouse conditions

Plants	Root number (mean ± SE) [a]	Root length (cm) (mean ± SE)[b]	Dry root biomass (mg) (mean ± SE)[b]
WT	4.4 ± 0.5	28.2 ± 0.7	543.6 ± 14.6
iaaM	13.8 ± 1.1*	24.5 ±0.9*	345.3 ± 23.9*
iaaM+CKX	14.4 ±1.2 *	33.7 ± 1.0*	688.3 ± 57.3*

[a] The average number of emerged roots per stem after being rooted in fritted clay medium for 10 days.

[b] Data were collected after being rooted in fritted clay medium for six weeks.

Data were collected from eight replicates and presented as averages. Asterisks (*) represent significant differences compared to wild type using two-tailed Student's t test with the pooled variance ($p < 0.05$). Bars represent standard error.

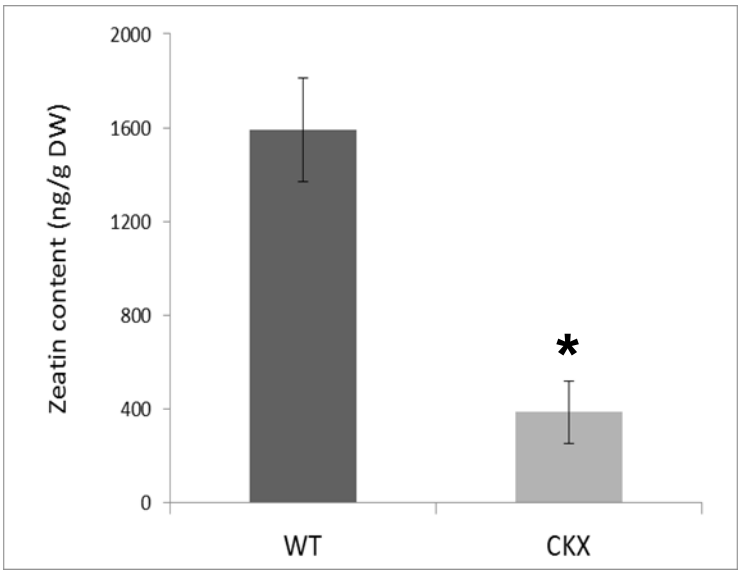


Figure 3.12 Endogenous zeatin contents in roots of wild-type and *CKX* plants. Asterisk (*) represent significant differences between wild-type and *SbUGT::CKX* tobacco using two-tailed Student's t test with the pooled variance ($p < 0.05$). Bars represent standard errors.

Crosses of *SbUGT::iaaM*-39 with *SbUGT::CKX*-64 produced hybrids (*iaaM+CKX*) with both the *iaaM* and *CKX* transgenes present in progeny plants. Analysis of IAA content in root tissues of plants from both the *SbUGT::iaaM*-39 and *iaaM+CKX* hybrid transgenic lines showed that overexpression of the *iaaM* gene led to significant increases in auxin concentration but overexpression of the *CKX* gene reduced auxin concentrations with or without overexpression of the *iaaM* gene (**Table 3.1**), similar to previously reported in *Arabidopsis* (Jones et al., 2010). These results demonstrate that expression of the *SbUGT::CKX* gene results in reduced IAA content in roots. The *SbUGT::CKX* overexpression-mediated reduction in root auxin content may contribute to the improvement in root elongation and root biomass in *iaaM+CKX* hybrid plants.

Improvement of root elongation was observed in the seedlings derived from *iaaM+CKX* hybrid seeds (**Figure 3.13**). Rooting of shoot cuttings from *iaaM+CKX* hybrid plants after 10 days was also improved compared to wild-type plants (**Figure 3.10**). Six weeks after rooting, the *iaaM+CKX* hybrid plants produced longer roots than both *SbUGT::iaaM-39* and wild-type plants (**Figure 3.11**). Dry root biomass of the *iaaM+CKX* hybrid progeny plants was significantly greater compared to those of wild-type plants (**Table 3.4**). These results demonstrate that reducing cytokinin levels in roots can neutralize the negative effects of root length and root biomass caused by the *iaaM* gene expression, and act synergistically with auxin to promote root initiation.

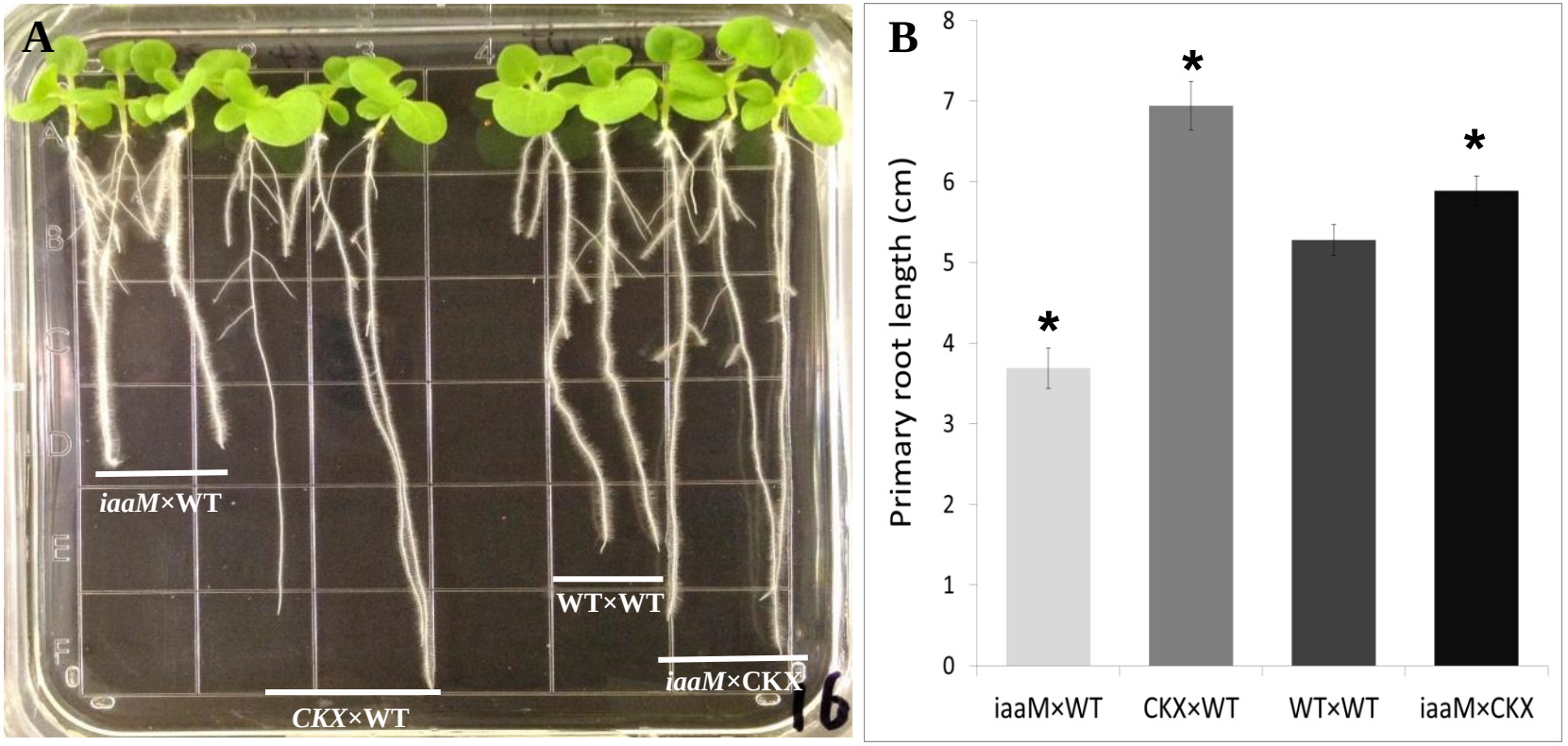


Figure 3.13 Negative effects of the *SbUGT::iaaM* gene expression on root growth of rootstock can be compensated with expression of the *SbUGT::CKX* gene. (A) Eight-day-old progeny seedlings from the crossings of: *iaaM* or *CKX* to wild type, self-crossed wild type, and *iaaM* to *CKX*. The results showed that auxin-mediated reduction in root growth was neutralized with expression of the *CKX* gene in roots. (B) Effects of the *iaaM* and *CKX* gene expression on primary root length. Asterisks (*) represent significant differences compared to wild type using two-tailed Student's t test with the pooled variance ($p < 0.05$). Bars represent standard errors.

3.3.4 Simultaneous expression of *SbUGT::iaaM* and *SbUGT::CKX* genes suppressed lateral bud release from rootstock and improved grafting success rates

If wild-type scions were grafted on to wild-type, *SbUGT::iaaM*-39, *SbUGT::CKX*-64, and *SbUGT::iaaM+ SbUGT::CKX* rootstock, respectively, lateral buds were released from both the wild-type rootstock (**Figure 3.14A**) and the *CKX* rootstock (**Figure 3.14D**), but not from the *iaaM* or the *iaaM+CKX* rootstock (**Figure 3.14B, C**). When lateral buds were released from the wild-type or *CKX* rootstock, scion growth was inhibited (**Figure 3.14A, D**) but scion growth was vigorous when grafted onto the *iaaM* or the *iaaM+CKX* hybrid rootstock (**Figure 3.14B, C**). We also determined grafting success rates when *iaaM+CKX* hybrid plants were used as rootstock. Similar to that of the *iaaM* overexpressing rootstock, grafting success rate was dramatically improved when *iaaM+CKX* hybrid plants were used as rootstock relative to grafting success observed with wild-type rootstock (**Table 3.2**). These results demonstrate that expression of both the *SbUGT::iaaM* and *SbUGT::CKX* genes in rootstock plants repressed lateral bud release from the rootstock and improved grafting success rate, similar to the effects of *SbUGT::iaaM* gene.

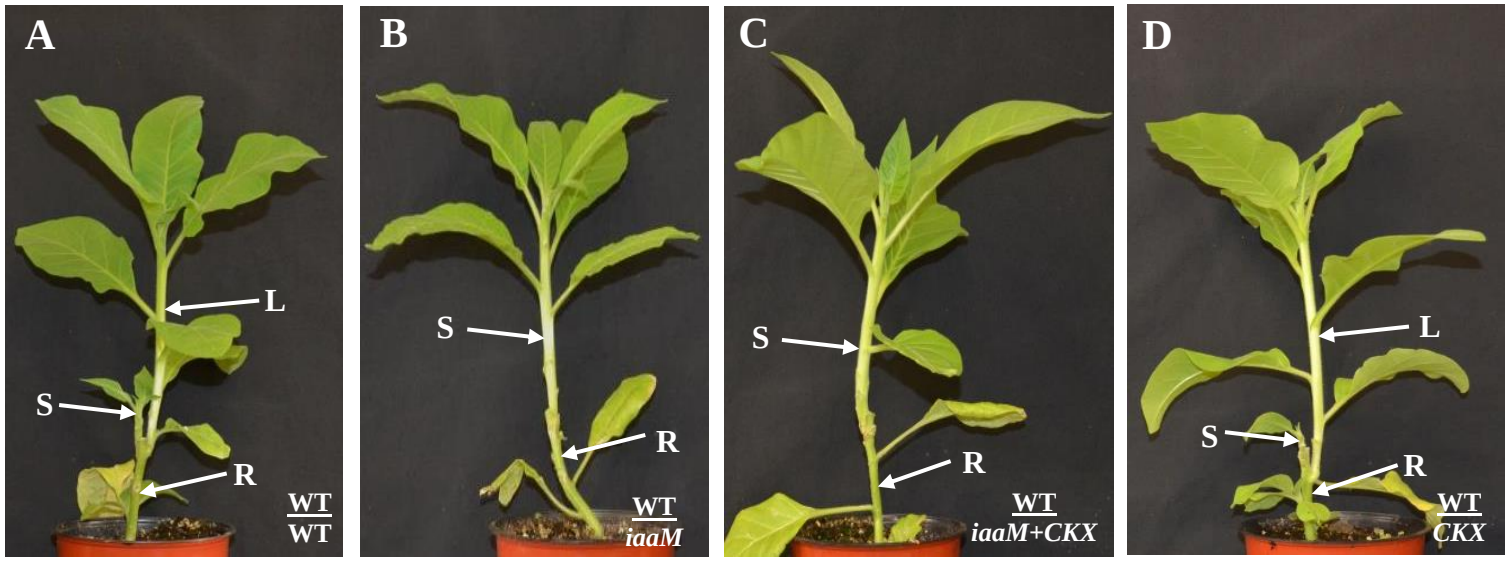


Figure 3.14 The *SbUGT::iaaM*-39/*SbUGT::CKX*-64 hybrid plant (*iaaM+CKX*) used as rootstock inhibited lateral bud release from the stumps and enhanced scion growth. (A-D) Three weeks after grafting, (A) WT/WT grafts had little scion growth because of outgrowth of a lateral shoot; (B) WT/*iaaM* and (C) WT/*iaaM+CKX* had no lateral buds released from the rootstock stumps and vigorous scion growth; and (D) WT/*CKX* had lateral shoot outgrowth with little scion growth. S: Scion. L: Lateral bud. R: Rootstock.

3.4 Discussion

This study demonstrates that root-predominant expression of an *iaaM* gene, whose product catalyzes biosynthesis of an auxin precursor, results in a series of improved rootstock characteristics. However, root elongation and root biomass of *iaaM* rootstock were adversely affected compared to those of the wild-type rootstock. We have further shown that over-expression of a cytokinin degradation gene (*CKX*) compensated the negative effect of the *iaaM* gene expression on root elongation and biomass of rootstocks. Our results have demonstrated that increases in auxin level and reductions in cytokinin concentration predominantly in roots can produce several beneficial characteristics including inhibited lateral bud release from the rootstock, improved grafting success rates, and enhanced root initiation and root biomass. This technology may also be useful in other woody plants for improving the quality of rootstock because effects of auxin and cytokinin on plant growth and development are basically the same in higher plants. As such, the technology presented in this manuscript should be applicable for many economically important woody plants, such as apple and pear (Zhu et al., 2001; Zhu et al., 2003). However, we also recognize that there are some differences in anatomical structure, developmental mechanisms, and physiological characteristics among different plant species. Therefore, it is also possible that the effects of the root predominant expression of the *iaaM* and *CKX* genes may be somewhat different in woody plants than in tobacco.

Grafting success is largely dependent on the rapid formation of a graft union, where scion and rootstock fuse to form a chimeric plant. Yin et al. (2012) used an auxin responsive reporter gene (*DR5::GUS*) to show that endogenous auxin may accumulate in the graft

union and therefore suggested a role for auxin in graft union formation. The increased auxin levels in the graft joint zone suggested by Yin et al (2012) were most likely due to auxin accumulation in the basal end of the scion, as we have previously demonstrated (Li et al., 1999). However, more direct experimental evidence is needed to determine whether auxin plays an important role in grafting success rates. Using rootstock that overexpress the *SbUGT::iaaM* gene, we have demonstrated that the WT/*iaaM* grafts had a much higher grafting success rate (91%) than the WT/WT grafts on which lateral buds were manually removed (68%). These results provide additional evidences that auxin plays a critical role in grafting success.

Lateral buds released from rootstock have been shown to negatively affect scion growth, and thus grafting success. Lateral buds originating from the rootstock are usually removed manually (Daley and Hassell, 2014). Thimann and Skoog (1934) reported that prior to grafting, exogenously applied auxin to the rootstock could block the release of lateral buds. Chemical or manual removal of lateral buds from rootstock have also been used to eliminate lateral shoot development but these methods are time-consuming and expensive (Choi et al., 2002; Memmott and Hassell, 2009). The transgenic approach presented here may provide an excellent tool to suppress lateral bud release from rootstock, improve grafting success rates, and also reduce the costs associated with chemical or manual removal of lateral buds.

Fast initiation and establishment of adventitious roots from shoot cuttings are important traits for rootstock plants. However, many plant species or cultivars having a number of excellent rootstock characteristics are difficult to root. Dwarf apple rootstock varieties that have been commonly used for grafting are difficult to root from shoot cuttings (Pawlicki and

Welander, 1995). Zhu et al. (2001) successfully used the *RolB* gene from *A. Rhizogenes* to enhance rooting of dwarf apple and pear varieties. The biochemical or molecular functions of the *RolB* gene in plants are still the subject of debate but some believe that the RolB protein may be involved in altering either hormone concentrations or signaling (Arshad et al., 2014). Similar to the results reported with *RolB* rootstock (Zhu et al., 2001), our auxin overproducing transgenic rootstock lines also displayed enhanced rooting ability. However, inhibition of lateral buds outgrowth from rootstock and improved grafting success rates were not reported with the use of *RolB* transgenic rootstock.

Reduction in cytokinin level has been shown to promote adventitious root initiation and elongation (Bellini et al., 2014). In *Arabidopsis*, transgene-mediated reduction in endogenous cytokinin concentration, or mutations that alter the expression of cytokinin receptor genes, enhances adventitious root initiation (Riefler et al., 2006; Werner et al., 2003). Cytokinin may modify the expression of auxin transport genes such as *PIN* genes, reducing the formation of the auxin gradient required for the root initiation (Laplaze et al., 2007). Consistent with these previous reports, we have also observed that overexpression of the *SbUGT::CKX* gene alone or in combination with the *SbUGT::iaaM* gene enhanced root initiation and growth.

It has been reported that elevated auxin or cytokinin content in plants can effectively inhibit root elongation (Cary et al., 1995; Eliasson et al., 1989), while reductions in tissue cytokinin concentrations can promote root elongation (Werner et al., 2010). Werner et al. (2010) reported that a root-specific reduction of the cytokinin concentration resulted in the development of longer primary roots. Rootstock plants with improved root length exhibited

increased resistance to drought and nutrients stresses (Warschefsky et al., 2016). It will be interesting to determine whether the *SbUGT::CKX* rootstock plants also have enhanced tolerance to drought or nutrient stresses. Analyses for plant hormone concentrations in *SbUGT::iaaM* and *SbUGT::CKX* transgenic plants revealed that overexpression of the *CKX* gene resulted in reduced auxin levels. Jones et al. (2010) showed that cytokinins can enhance the expression of several *PIN* genes that are involved in cell-to-cell auxin transport, thus leading to altered auxin levels in the cell. However, whether the expressions of *PIN* genes in the *CKX* overexpressing transgenic plants are reduced still needs to be experimentally determined. It is, however, possible that lower auxin levels may have contributed to the improvement in root growth that we observed in the *CKX* over-expressing rootstock.

In this study, we have demonstrated that a differential expression of the *iaaM* and *CKX* genes can result in inhibition of lateral bud release from the rootstock, improved grafting success rates, and enhanced root initiation and root biomass. Although transgenic technology provides a powerful tool for crop improvement, gene flow and food safety concerns over transgenic plants have impeded its utilization in the horticultural and forestry industries (Kausch et al., 2010; Li et al., 2016; Ye et al., 2016). The use of non-transgenic scions and transgenic *SbUGT::iaaM* and *SbUGT::CKX* rootstock may encounter less public opposition because fruits, seeds and pollen grains produced from scion shoots are non-transgenic.

3.5 Materials and Methods

Plasmid construction

The *SbUGT* promoter sequence, −102 to +86 relative to the transcription start site of a flavonoid glycosyltransferase gene from Scutellaria barbata (Chiou et al., 2010), was synthesized and inserted upstream of the *GusPlus* coding region in a pCAMBIA-*GusPlus-nptII* plasmid (Chen et al., 2006) to create the *SbUGT::GUS* construct. The *SbUGT* promoter sequence as well as the coding region of *iaaM* (a tryptophan-2-mono oxygenase gene from *Agrobacterium tumefaciens*) (Sitbon et al., 1992) or *AtCKX2* (*Arabidopsis* cytokinin oxidase 2 gene) (Werner et al., 2003) were synthesized as one fragment, and subcloned into a pCAMBIA-*GusPlus-nptII* plasmid to create the *SbUGT::iaaM* or *SbUGT::CKX* construct, respectively.

Tobacco transformation and molecular confirmation of transgenic plants

Plasmid vector of *SbUGT::GUS, SbUGT::iaaM* or *SbUGT::CKX* construct was introduced into *Agrobacterium tumeficiens* strain EHA105 and the resulting bacteria were used to transform *Nicotiana tabacum* cv. Xanthi. Tobacco leaf disc transformation was performed as described previously (Zheng et al., 2007).

Genomic DNA was extracted from the leaves of putative transgenic plants using a modified CTAB protocol (Porebski et al., 1997). Extracted DNA was fractioned on a 0.8% (w/v) agarose gel in order to separate genomic DNA from any potential contamination from Ti-plasmids. The purified genomic DNA was gel extracted and then used as template for PCR (Chen et al., 2006). The primer pair iaaM-F (5’-TTCTCCGAAGCACAACTA -3’) and iaaM-R (5’-GCCCACCTAATGTCTCC -3’) was used to amplify a 797bp fragment from the

iaaM gene within the T-DNA region of the Ti-plasmid. The primer pair CKX-F (5'-CGTTATGGGTGGATGTG-3'), CKX-R (5'-TAAGCCAAGGATGAGGA-3') was used to amplify a 711bp fragment of the *CKX* gene within the T-DNA region of the Ti-plasmid. PCR reaction solution was 20 µl aliquot containing 1× PCR buffer (Takara, Japan), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2µl e2TAK DNA polymerase (Takara, Japan), 0.25 µM of each primer and 500 ng DNA. The amplification started with an initial denaturation step at 98℃ for 5 min, followed by 35 cycles of 98℃ for 10 s, 60-65 ℃ for 5 s, and 72℃ extension plus a final extension at 72℃ for 10 min.

Histochemical GUS activity assays

T_0 *SbUGT::GUS* tobacco plants were self-pollinated to produce T_1 progeny seeds. Five-day-old T_1 seedlings were incubated in X-gluc solution at 37℃ overnight for histochemical GUS activity staining. The histochemical assay staining solution contained 100 mM potassium phosphate buffer, pH 7.0, 10 mM Na_2EDTA, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, 0.1% triton X-100, 1 g/l X-gluc (5-bromo-4-chloro-3-indolyl-β-d-glucuronic acid). Seedlings were treated with successive ethanol solutions, with increasing ethanol concentrations, to gradually remove chlorophylls and other pigments, after which they were then visually inspected and photographed.

Quantitative real-time PCR analysis

Shoot or root RNAs were extracted from two-month-old *SAUR::iaaM*, *SbUGT::iaaM*, or wild-type tobacco plants using the RNeasy Plant Mini Kit including RNase-Free DNase set (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Richmond, CA) was used to synthesize cDNA,

after which cDNA was used as a template for quantitative real-time PCR analysis using SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories, Richmond, CA) on a CFX96™ Real-Time PCR detection system (Bio-Rad Laboratories, Richmond, CA). Primer sequences for all genes analyzed are as follows:

iaaM forward: 5'-TGGATTTCTCCGAAGCACA-3',

iaaM reverse: 5'-CCCGGTAACGCATTTCAT-3',

GH3 forward: 5'-GGATTATGCAATTTCAAGG-3',

GH3 reverse: 5'-ACGATGGGCTAAAGTGTCT-3',

EF1α forward: 5'-GCTGCTCAGAAGAAGAAATG -3',

EF1α reverse: 5'-GAGCTGGTTCCAGACATACAC -3'.

The tobacco *GH3* gene sequence was identified based on the deduced amino acid sequences from the *Arabidopsis* and soybean *GH3* gene sequences. EF1α was used to amplify cDNA of the internal reference gene, elongation factor 1α (Schmidt and Delaney, 2010). Data were analyzed using CFX Manager™ software version 2.0. The gene expression levels in each sample were normalized using the expression level of the elongation factor 1α gene in the same sample. Three biological replicates were performed with all treatments.

Evaluation of *SbUGT::iaaM*-39 rootstock in the greenhouse

The *SbUGT::iaaM*-39 and wild-type tobacco plants were vegetatively propagated and grew in greenhouse for one month before grafting. Scion and rootstock were jointed using the cleft graft technique (Lee and Oda, 2010). Parafilm was used to wrap the graft union for at least one week. A total of 20 WT/WT and 10 WT/iaaM grafts were used for each experiment. Among the 20 WT/WT grafts, 10 were left with the lateral buds intact on the rootstock and the other 10 had the lateral buds manually removed from the rootstock. The

growth of the grafted plants was recorded two months after grafting. Two months after grafting, apical portions of the scions were removed, and lateral bud release from scion shoots was recorded after two weeks.

Field evaluation of grafts

The *SbUGT::iaaM*-39 and wild-type tobacco plants were vegetatively propagated and grafted in the greenhouse as described above. A total of 20 WT/WT grafts, 10 WT/iaaM-39 grafts were used for the experiment. Three weeks after grafting, all grafted plants were randomly planted in a field lot on the UConn depot campus in Storrs, Connecticut, USA in July, 2015. The 20 WT/WT grafts were divided into two groups: 10 with lateral buds on the rootstock intact and 10 with the rootstock lateral buds manually removed. Initial shoot heights of plants were recorded at time of transplanting in the field, and then again at 60 and 90 days (October 2015). All scions above the graft union were harvested at Day 90. After removing leaves, scion shoot tissues were oven-dried at 70°C for 10 days and then weighed. Shoot biomass was determined for each graft. Data were reported as means of all 10 replicates. Analysis of variance among field-grown graft combinations was performed using IBM SPSS 19.0 (IBM Corp., Somers, NY, USA). When sufficient differences ($P < 0.05$) were observed, Fisher's protected least significant difference test ($P = 0.05$) was performed to calculate differences between different treatments.

Crosses between *SbUGT::iaaM*-39 and *SbUGT::CKX*-64 plants and hybrid progeny evaluation

Wild-type, *SbUGT::iaaM*-39, and *SbUGT::CKX*-64 tobacco plants were vegetatively propagated. During flowering, wild-type pollen were used to pollinate wild-type,

SbUGT::iaaM-39, or *SbUGT::CKX*-64 plants which had anthers removed before maturity to prevent undesired self pollination. *SbUGT::CKX*-64 pollen was used to pollinate *SbUGT::iaaM*-39 flowers in the same way. Paper bags were used to wrap pollinated flowers, in order to reduce undesired pollination. The progeny seeds were germinated and grown on MS medium. Genomic DNA was extracted from leaves of seedlings using a modified CTAB method (Porebski et al., 1997). The primer pairs, iaaM-F and iaaM-R or CKX-F and CKX-R, (primer sequence information has been listed before), were used to confirm the presence of the *iaaM* or *CKX* genes in hybrid plants, respectively. Detailed information about primers have been described above. Eight days after germination, photographs and primary root length data were collected. Data were recorded on an average of 30 seedlings. Means between wild-type and transgenic plants were compared using the two-tailed Student's t-test with the pooled variance (Steel and Dickey, 1997).

Root growth evaluation under greenhouse conditions

The *SbUGT::iaaM-39*, wild-type, and one representative *iaaM+CKX* hybrid plant were vegetatively propagated and planted in pots with fritted clay medium in greenhouse. Ten days after planting, root number of each plant was recorded. Six weeks after rooting, shoot height of each plant was recorded. All plants were carefully dug out from medium. Root length was determined for each plant. Shoot and root tissues were oven-dried at 70°C for 10 days and then weighed. Data were reported as means of all eight replicates. Means between wild-type and transgenic plants were compared using the two-tailed Student's t-test with the pooled variance (Steel and Dickey, 1997).

Evaluation of *iaaM+CKX* hybrid rootstock in greenhouse

The *SbUGT::iaaM-39*, wild-type, and one representative *iaaM+CKX* hybrid plant were used as rootstock, and wild-type scions were grafted as described above. One group of WT/WT grafts has lateral buds intact on the rootstock and the other group has the rootstock lateral buds manually removed. Three weeks after grafting, grafting success rates were recorded. Grafts with more than a 2-cm increase in scion's height growth were considered as successful grafts. For each rootstock/scion and lateral bud removal treatment, 8-11 grafts were performed as one replicate. Data were reported as means of three biological replicates. Analysis of variance on grafting success rates between different grafts was performed using IBM SPSS 19.0 (IBM Corp., Somers, NY, USA). When sufficient differences ($P < 0.05$) were observed, Fisher's protected least significant difference test ($P = 0.05$) was performed to calculate differences between groups.

Quantification of IAA and zeatin content

Hormone extractions were handled in the same manner as described (Krishnan et al., 2016; Krishnan and Merewitz, 2015). About 50 mg frozen-dried root or shoot samples from two-month-old *SbUGT::iaaM-39*, *SbUGT::CKX-64*, one representative *iaaM+CKX* hybrid plant, or wild-type samples were ground to a fine powder in liquid nitrogen using a mortar and pestle. IAA or Zeatin content analysis was carried out using an ultra-high-performance LC-tandem mass spectrometer (UPLC/MS/MS) (Quattro Premier XE ACQUITY Tandem Quadrupole; Waters, Milford, MA). Samples from 10 plants were pooled for each replicate, and data were reported as a mean of three biological replicates. Analysis of variance was performed on IAA content data using IBM SPSS 19.0 (IBM Corp., Somers, NY, USA).

When sufficient differences ($P < 0.05$) were observed, Fisher's protected least significant difference test ($P = 0.05$) was performed to calculate differences between groups. Means of Zeatin content between *SbUGT::CKX-64* and wild-type plants were compared using the two-tailed Student's t-test with the pooled variance.

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