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ORIGINAL ARTICLES

Inhibition of Ethylene Biosynthesis Enhances Vegetative Bud Formation without Affecting Growth and Development of Transgenic Tobacco Plants

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ABSTRACT

The role of ethylene in vegetative bud formation was investigated using transgenic tobacco plants expressing an antisense tomato 1-aminocyclopropane-carboxylic acid synthase (ACS) gene. Northern blot hybridization showed that the accumulation of ACS mRNA was strongly reduced in the bud-forming leaf explants of the transgenic plants. Consequently, these transgenic tissues exhibited low ACS enzyme activity, 1-aminocyclopropane-carboxylic acid (ACC) content and ethylene production, and at the same time the tissue capacity to generate buds was greatly enhanced. However, it was also noted that the antisense ACS gene did not inhibit the endogenous ACS gene expression in intact transgenic tobacco plants. The growth and development of the transgenic tobacco was almost identical to control plants with respect to height, internode number, leaf morphology, and flowering time. Furthermore, mature leaves of transgenic tobacco had similar chlorophyll content, stomatal conductance, photosynthetic ability, and transpiration rates compared to control plants. These results demonstrated that ethylene plays an important role in bud formation in tobacco tissue culture.

Key words: 1-Aminocyclopropane-carboxylic acid synthase; Antisense RNA; Transgenic tobacco; Vegetative bud formation

INTRODUCTION

Ethylene is a simple gaseous plant hormone that regulates many diverse plant processes, ranging from seed germination to organ senescence. Its biosynthetic rate varies with the developmental

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stage of the plant and the physiological status of different tissues, and it is responsive to environmental stresses. Ripening of climacteric fruits is accompanied by a massive synthesis of ethylene. The essential role of ethylene in fruit ripening has been demonstrated by physiological and transgenic approaches (Kende 1993; Alexander and Grierson 2002). The mechanism by which ethylene regulates plant growth and development is, however, very complicated and not well understood. Silencing ethylene biosynthesis gene expression demonstrated that ethylene was an essential hormone for ovule development in tobacco (Martinis and Mariani 1999). In recent years, evidence has accumulated for an inhibitory effect of ethylene on cell differentiation and shoot regeneration, and for a regulatory role in normal plant growth and development (Kumar and others 1998). Many of these studies, however, used inhibitors of ethylene production and action, for example, $AgNO_3$, aminoethoxyvinyl-glycine (AVG), norbornadiene, and aminooxyacetic acid (AOA), and as with all studies dependent on exogenous applications, have prompted arguments about confounding factors such as uptake, transport, and the actions of these exogenous compounds in plant tissues.

For the last decade, the two enzymes, 1-aminocyclopropane-carboxylic acid (ACC) synthase (ACS) and ACC oxidase (ACO), which are responsible for ethylene biosynthesis, have been investigated, and the genes encoding these enzymes have been cloned from several plants (Bleecker and Kende 2000). The significance of ethylene regulation of fruit ripening in climacteric fruits has been demonstrated by inhibition of ethylene production through antisense approaches. Expression of antisense RNA to ACS or ACO has been shown to reduce ethylene production and retard fruit ripening (Hamilton and others 1990; Oeller and others 1991). More recently, this approach has been used to study the role of ethylene in bud regeneration. Two research groups have reported that inhibition of ethylene production by the expression of an antisense *ACO* gene in transgenic mustard and melon enhanced *de novo* shoot regeneration (Pua and Lee 1995; Amor and others 1998). We have successfully produced transgenic tobacco plants expressing an antisense tomato *ACS* gene (Ma and Song 1997). The self-fertilized progeny of these plants have now been shown to be homozygous for the transferred gene. In this report, inhibition by antisense *ACS* of ethylene biosynthesis was analyzed in these plants, and its influence on tobacco vegetative bud formation and whole plant development was investigated.

Materials and Methods

Plant Materials and Nucleotide Acid Isolation

An ACS cDNA (*Le-ACS2*, GenBank accession number X59139) from tomato (*Lycopersicon esculentum* Mill.) was kindly provided by Prof A. Theologis (Plant Gene Expression Center, Berkeley, California, USA). A construct containing an antisense tomato *ACS* gene with a CaMV 35S promoter (*35S-anti-ACS*) was transferred into tobacco (*Nicotiana tabacum* L. cv Wisconsin 38) through *Agrobacterium*-mediated transformation as previously reported (Ma and Song 1997). Tobacco containing the empty pGA492 vector was used as control (CK), and the transgenic tobacco lines 7, 10, and 16 containing the antisense *ACS* construct (*35S-anti-ACS*) were used throughout this study (designated A7, A10, and A16). T_1, T_2, and T_3 plants were successive self-fertilized generations of lines A7, A10, and A16. Each generation of tobacco was germinated from seeds of the previous generation. The segregation of the integrated gene in the progeny was tested for kanamycin resistance by germinating seeds on agar medium containing kanamycin (150 $\mu g\ ml^{-1}$). T_2 generation seedlings homozygous for the *35S-anti-ACS* gene were transplanted to potting mix and plants allowed to develop to maturity in the greenhouse under a 16-h photoperiod and with day/night temperatures of 24°/16°C. T_2 generation plants were used for Southern blot experiments. After confirmation by Southern blot hybridization, six T_3-plants from these three T_2 lines were chosen for other experiments in this study.

Total RNA was isolated from tobacco tissues by TRI reagent (Molecular Research Center, Inc, Cincinnati, Ohio, USA) according to the manufacturer's instructions. $Poly(A)^+RNA$ was isolated using Poly-AT tract® mRNA Isolation Kit (Promega). Genomic DNA was purified from young tobacco leaf tissues according to the protocol described by Dellaporta and others (1983).

Isolation of Tobacco ACS Sequence for Use as a Probe

cDNA synthesis was based on the rapid amplification of cDNA ends method (Frohman and others 1988) using oligonucleotide primer 5′-GAC TCGAGTCGACATCGA$(T)_{17}$-3′. PCR was conducted using 5′-primer as 5′-GTGGATATTGCATTAGCGAG -3′ and 3′-primer as 5′-GTATATAACAATTAATTA TTTCTG-3′. These primers corresponded to tobacco ACS cDNA (GenBank no X65982) 3′ terminus. The PCR products were resolved on a 1.0% agarose gel

and purified by using a GlassMAXR DNA Isolation Kit (Gibco). The purified fragments were cloned into pGEM-T Easy vector (Promega). After sequencing, a clone of 320 bp that was identical to tobacco ACS cDNA was used as a probe for northern blot hybridization.

DNA and RNA Gel Blot Analysis

Genomic DNA from tobacco leaf tissues was digested with PstI plus KpnI and resolved on a 1.0% agarose gel. Total RNA (10 μg) was electrophoresed on 1.4% (w/v) formaldehyde agarose gels. DNA and RNA were blotted onto Hybond-N^+ membrane (Amersham) using established protocols (Sambrook and others 1989). The blots were hybridized at 42°C in 6× SSC, 5× Denhardt, 0.5% SDS, 100 μg ml^{-1} salmon sperm DNA with 50% formamide and washed with 0.1× SSC plus 0.1% SDS at 65°C. Probes were ^{32}P-labeled using a Ready-to-Go DNA Labeling Kit (Amersham). For DNA blot hybridization, the full-length of tomato *ACS* cDNA (GenBank accession number X59139) was used as probe. For RNA blot hybridization, tobacco *ACS* cDNA 3′ terminus (as isolated by RT-PCR showed above) was used as probe. Northern blots were quantified using Phosphor Image, and mRNA levels were normalized by comparison to a soybean 18S rRNA.

Ethylene Production Measurement

Ethylene was measured by gas chromatography. For the intact tobacco, leaf discs (10 mm diameter) were cut from the mature leaves from the fourth to sixth internode of tobacco plants in the greenhouse. Samples of 10 leaf discs were incubated on Whatman paper (No 3) in 35-ml flasks containing 1 ml of 10 mM morpholine sulfonate buffer (pH 6.1) at room temperature. For regenerating shoots, five bud-forming leaf explants that were incubated in MS9 medium for 5 to 15 days (see below) were moved into a 50-ml tube. The tube was sealed with a silicone cap for 10 h. Samples (1 ml) of the enclosed atmosphere were injected into a gas chromatograph (GC-103, Shanghai Analysis Equipment Factory, China) equipped with an activated alumina column (0.3 × 150 cm) and a flame ionization detector. The column was eluted with nitrogen at a flow rate of 30 ml min^{-1}. The column was heated at 80°C, and the flame ionization detector was set at 230°C. Samples were quantified against known amounts of an ethylene standard. Measurements were performed in triplicatez.

Assay of ACC Content and ACS Enzyme Activity

ACC content, enzyme isolation and determination of ACS enzyme activity were determined according to the method of Nara and Takeuchi (2002). Protein concentrations were determined by the Bradford assay (1976) using BSA as the standard.

Vegetative Bud Regeneration Analysis

T_2 generation seeds of A7, A10, and A16 plants were germinated in MS medium and seedlings 4–6 cm tall were subcultured in the same medium. One-month-old subcultured tobacco seedlings were used to evaluate bud regeneration. Leaves from these seedlings were cut into 1 × 2 cm pieces and grown in Petri dishes containing MS9 medium for bud regeneration (MS medium supplemented with 1 mg l^{-1} BA and 0.5 mg l^{-1} IAA) (Ma and Song 1997). All explants form buds under these conditions. The morphogenetic responses (number of buds per explant, weight of buds per explant) were investigated after 5–15 days of culture. When the fresh weight of the bud was determined, the leaf explant was not separated from the regenerating bud. As the weight of the leaf explant is very small (about 0.02 g), it has little influence on bud weight determination. These explants were also used for biochemical and gene expression analyses.

Phenotypic and Physiological Analyses

The T_3 generations of A7, A10, and A16 plus control tobacco grown in the greenhouse were used in these analyses. Tobacco plant height and the ratio of leaf length to width were determined when plants began to flower. Leaf number was counted from top to bottom. The number of days to emergence of the first flower was recorded as an indication of plant maturity.

The photosynthetic rate (Pn), stomatal conductance (Gs), and transpiration rate (Ev) of tobacco were measured with a portable photosynthetic analyzer system CIRAS-1 (ADC Inc., UK) on the fourth fully expanded leaf counted from the top of 2-month-old plants. Measurements were performed at 24°C, with light intensity of 60,000 lux, and a CO_2 concentration of 380 ppm. Each of the four positions within a sampled leaf was carefully placed in the cuvette until five stable measurements were obtained. Means represent measurements on six replicated plants.

Chlorophyll content was determined by spectroscopy. Leaves were ground in 3 ml of ice-cold AA

Table 1. Assessment of Transgenic Zygosity and Segregation of Kanamycin-resistant Phenotype in the T_1 and T_2 Progenies of the Transgenic Tobacco Lines A7, A10, and A16

Plants	Progeny	Km^R: Km^S (seed number)	Transgene copy number	Transgenic zygosity
A7	T_1	73:25	1	Hemizygous
	T_2	81:2	1	Homozygous
A10	T_1	65:20	1	Hemizygous
	T_2	71:0	1	Homozygous
A16	T_1	91:27	1	Hemizygous
	T_2	95:1	1	Homozygous

solution (80% acetone, 1% ascorbic acid). The homogenate was centrifuged at $3000 \times g$ for 10 min. The pellet was resuspended in 3 ml AA solution and centrifuged again. Both supernatants were combined and adjusted to 10 ml. The chlorophyll content was determined from the absorption coefficients by the protocol of Porra and others (1989). The probability value was estimated by Student's *t*-test.

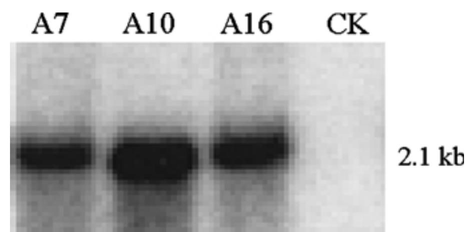

Figure 1. Southern blot hybridization to show the *35S-anti-ACS* gene in transgenic tobacco plants A7, A10, A16 and in control (CK). DNA (10 μg) was digested with PstI-KpnI and probed with tomato *ACS* cDNA.

Results

The Segregation and Inheritance of the *35S-anti-ACS* Gene

The inheritance of kanamycin resistance was analyzed in self-fertilized progeny of the transgenic tobacco. Resistance versus sensitivity to kanamycin segregated in a ratio close to 3:1 in the T_1 progeny of A7, A10, and A16 lines, indicating that probably one copy of T-DNA was integrated into the genome of these three lines (Table 1). The T_2 plants from A7, A10, and A16 exhibited nearly complete kanamycin resistance, suggesting that they were homozygous for the integrated gene.

The stable inheritance of the *35S-anti-ACS* gene was confirmed by Southern blot hybridization using tomato *ACS* cDNA as probe. A 2.1-kb band, which contained whole *35S-anti-ACS* gene in a plant expression vector, was detected in the T_2 transgenic tobacco plants of A7, A10, and A16 (Figure 1), indicating that the *35S-anti-ACS* gene had been retained in the progeny of the transgenic tobacco after meiosis. Six T_3-plants from these three T_2 lines were chosen for further analyses in this study.

Effects of Antisense Gene on Tobacco ACS mRNA Levels

The tomato *Le-ACS2* gene has 84% similarity with the tobacco *ACS* gene. It is therefore reasonable that the antisense construction from *Le-ACS2* should inhibit tobacco *ACS* gene expression. To examine the expression pattern of *ACS* in tobacco, total RNA was isolated from intact tobacco leaves and bud-forming leaf explants and hybridized with a probe of ^{32}P-labeled tobacco *ACS* 3′ fragment (see *Materials and Methods* for probe construction details). Results showed that *ACS* mRNA levels in intact plants were similar in transgenic lines A7, A10, and A16 compared with control tobacco (Figure 2A). However, in the bud-forming leaf explants after 1 week of culture in MS9 medium, *ACS* mRNA levels were markedly decreased in the A7, A10, and A16 lines compared with control plants (Figure 2B).

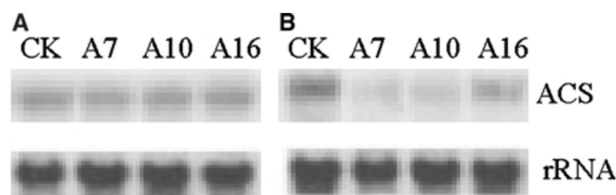

Figure 2. RNA gel blot analysis of *ACS* gene expression in tobacco tissues from plants A7, A10, A16 and control (CK). **A.** RNA from mature leaves; **B.** RNA from bud-forming leaf explants after 1 week of culture in MS9 medium. The different plants are shown in the top of the figure. Hybridization with a 18S rDNA probe has been included to confirm that the RNA preparations are undegraded and to serve as an internal control of variations in gel loading and blotting.

Table 2. ACS Enzyme Activity, ACC Content, and Ethylene Production in Mature Leaves of Intact Tobacco

Plants	ACS activity (pmol ACC h^{-1} mg^{-1} protein)	ACC content (pmol FW g^{-1})	Ethylene production (pmol FW g^{-1} h^{-1})
Control	5.2 ± 0.43	98.4 ± 8.5	28.8 ± 4.2
A7	4.9 ± 0.33	85.2 ± 9.8*	26.1 ± 5.1
A10	4.7 ± 0.30*	91.4 ± 7.6	24.5 ± 3.9
A16	5.0 ± 0.43	89.2 ± 6.8	25.5 ± 4.6

Values are means ± SD, n = 6.
Significant differences between the control and transgenic tobacco were tested using Student's t-test. Significant differences at the $P_{0.05}$ level between control and transgenic plants in the same column are marked by an asterisk (*).
ACS: 1-aminocyclopropane-carboxylic acid synthase; ACC: 1-aminocyclopropane-carboxylic acid.

Table 3. ACS Enzyme Activity and ACC Content in Bud-forming Leaf Explants of Tobacco

	ACS activity (pmol ACC h^{-1} mg^{-1} protein)			ACC content (pmol FW g^{-1})		
Plants	5 days	10 days	15 days	5 days	10 days	15 days
Control	25.3 ± 1.91	31.5 ± 1.52	14.1 ± 1.31	158.3 ± 10.2	168.2 ± 12.2	132.2 ± 9.2
A7	11.3 ± 1.12**	15.8 ± 1.26**	9.3 ± 1.18**	73.3 ± 6.9**	81.2 ± 11.2**	94.2 ± 8.2**
A10	10.8 ± 1.25**	13.8 ± 1.31**	10.1 ± 1.26**	79.3 ± 7.8**	71.3 ± 8.9**	103 ± 9.8**
A16	11.8 ± 1.05**	14.2 ± 1.21**	10.0 ± 1.06**	82.5 ± 8.5**	85.3 ± 10.4**	96.6 ± 7.6**

Data are the means of six replicates with 9–12 explants per replicate and are expressed as mean ± SD.
Significant differences at the P0.01 level between control and transgenic plants in the same column are marked by paired asterisks (**).

ACS Enzyme Activity, ACC Content, and Ethylene Production in Transgenic Tobacco

The ACS enzyme activity, ACC content and ethylene evolution in the transgenic plants were determined (Table 2). In the leaves from intact transgenic tobacco, ACS enzyme activity, ACC content, and ethylene evolution in transgenic A7, A10, and A16 lines were marginally lower than the control. This is consistent with the levels of *ACS* mRNA observed in northern blots (Figure 2A). During vegetative bud formation in leaf explants cultured for 5–15 days, however, ACS enzyme activity, ACC content, and ethylene evolution were markedly reduced compared to the control (Tables 3 and 4). All values reached maxima at day 10, which was also the time of the largest percentage reduction in these values.

Vegetative Bud Formation of Transgenic Tobacco

The effects of reduced ethylene biosynthesis on the morphogenesis of transgenic tobacco were evaluated. At 1 month, there was no observable difference in the growth and development of the transgenic and control plants when grown on MS medium. However, when leaf explants were transferred into bud-inducing medium, the number of buds formed by each explant was much higher in A7, A10, and A16 plants than in the control (Table 5). The fresh weights of total buds in explants of A7, A10, and A16 were also much greater than in the control, indicating bud growth in transgenic tobacco was much enhanced.

Growth and Development of Transgenic Tobacco

To test the effects of antisense *ACS* gene on intact tobacco, T_3 plants of A7, A10, A16, and the control plants were grown in the greenhouse. The gross morphology of the plants (height, leaf number, leaf width to length ratio) and days to first flowering were essentially identical, with the exception of A7, which flowered marginally earlier than the control or A10 and A16 (Table 6).

An analysis of some physiological functions of the mature leaves (photosynthetic rate, stomatal conductance, and transpiration rate) and chlorophyll content showed no significant difference between the transgenic plants and the control (Table 7).

Table 4. Ethylene Production in Bud-forming Leaf Explants of Tobacco

	Ethylene production (pmol FW g^{-1} h^{-1})		
Plants	5 days	10 days	15 days
Control	69.3 ± 7.2	87.5 ± 7.5	51.1 ± 4.2
A7	42.5 ± 6.1**	30.5 ± 4.1**	38.3 ± 7.8**
A10	49.5 ± 4.9**	40.4 ± 4.2**	45.1 ± 5.6**
A16	51.5 ± 5.3**	44.2 ± 3.8**	46.1 ± 4.8**

Data are the means of six replicates with 9–12 explants per replicate and are expressed as mean ± SD.
Significant differences at the $P_{0.01}$ level between control and transgenic plants in the same column are marked by paired asterisks (**).

Table 5. Vegetative Bud Formation from Leaf Explants, Cultured on Bud-inducing Medium (MS9), in Transgenic (A7, A10, and A16) and Normal (Control) Tobacco Plants

	Number of buds per explant			
Plants	Day 5	Day 10	Day 15	Fresh weight of buds per explant (g)
Control	0.0	2.8 ± 0.32	4.5 ± 0.35	0.31 ± 0.027
A7	0.0	4.1 ± 0.41**	10.2 ± 0.81**	0.89 ± 0.052**
A10	0.0	4.7 ± 0.29**	9.5 ± 0.52**	0.81 ± 0.064**
A16	0.0	3.8 ± 0.18**	8.4 ± 0.64**	0.75 ± 0.071**

Data are the means of six replicates with 9–12 explants per replicate and are expressed as mean ± SD.
Significant differences at the $P_{0.01}$ level between control and transgenic plants in the same column are marked by paired asterisks (**).

Table 6. Phenotype Analysis of Transgenic and Control Tobacco

Plants	Height (cm)	Leaf number	Ratio of leaf length to width of leaf	Days to first flower
Control	135 ± 15.7	16.3 ± 2.47	2.38 ± 0.415	95.2 ± 1.48
A7	137 ± 11.4	15.1 ± 3.92	2.34 ± 0.173	92.7 ± 2.56*
A10	132 ± 12.5	14.3 ± 3.25	2.27 ± 0.217	95.7 ± 1.25
A16	134 ± 10.2	14.9 ± 3.95	2.29 ± 0.187	94.1 ± 1.56

Values presented are expressed as mean ± SD; n = 6.
Significant differences at the $P_{0.05}$ level between control and transgenic plants in the same column are marked by an asterisk (*).

Table 7. Physiological Analysis of Mature Leaves in Transgenic and Control Tobacco

Plants	Pn (μmol CO_2 m^{-2} s^{-1})	Gs (mmol m^{-2} s^{-1})	Ev (mmol H_2O m^{-2} s^{-1})	Chl content (μg g^{-1})
Control	13.2 ± 1.15	105.3 ± 6.21	1.28 ± 0.18	525 ± 12.6
A7	12.3 ± 1.03	100.5 ± 5.26	1.19 ± 0.11	502 ± 14.4*
A10	12.8 ± 1.25	106.1 ± 7.32	1.24 ± 0.16	519 ± 18.9
A16	12.5 ± 1.46	103.1 ± 4.38	1.20 ± 0.22	507 ± 16.4

Values presented are expressed as mean ± SD; n = 6.
Significant differences at the $P_{0.05}$ level between control and transgenic plants in the same column are marked by an asterisk (*).
Pn: photosynthetic rate; Gs: stomatal conductance; Ev: transpiration rate; Chl: chlorophyll.

Discussion

Ethylene is a vital signaling molecule that regulates many facets of plant growth and development. The study of ethylene actions has been greatly aided by the ability to clone ethylene biosynthesis genes and the use of antisense RNA. In our previous report, an *ACS* cDNA from tomato was constructed in antisense orientation under the transcriptional control of the CaMV 35S promoter and introduced into tobacco by *Agrobacterium*-mediated transformation. Northern hybridization and RT-PCR analyses demonstrated the expression of this heterogeneous antisense gene in the transgenic tobacco, which caused a decrease in ethylene production. Furthermore, preliminary analysis showed that vegetative bud formation from leaf explants was promoted in the T_1 generation of transgenic tobacco (Ma and Song 1997). To further analyze the effects of the antisense *ACS* gene on tobacco growth and development, T_2 and T_3 plants were obtained by successive self-fertilizations from the transgenic lines A7, A10, and A16. We show here that the chimeric *35S-anti-ACS* gene was integrated into the transgenic tobacco line A7, A10, and A16 as a single locus (Table 1).

Southern hybridization showed that the *35S-anti-ACS* gene was stably inherited in the progeny of transgenic lines A7, A10, and A16 (Figure 1). Northern hybridization indicated that endogenous *ACS* mRNA levels were markedly decreased in bud-forming leaf explants after 1 week of culture in MS9 medium (Figure 2). The mRNA levels, however, did not significantly change in mature transgenic tobacco plants. The different inhibition effects by the antisense *35S-anti-ACS* gene in bud-forming leaf explants and intact plants were likely due to the different endogenous *ACS* mRNA levels. This can be observed not only from northern hybridization signals in the control tissues but also from ACS enzyme activity assays (Table 2 and 3). The same situation was also reported in other transgenic plants with antisense inhibition. For example, in transgenic tomato expressing the antisense *ACS* gene, the fruit tissues showed greater inhibition in *ACS* mRNA levels than that in vegetative tissues (Oeller and others 1991). In transgenic rice with an oat antisense arginine decarboxylase (ADC) gene, the transgenic callus tissues exhibited a significant reduction of ADC activity and a decrease in putrescine and spermidine content, but the regenerated plants (R0) displayed no variations in polyamine (PA) levels, whereas the progeny (R1) did (Capell and others 2000; Trung-Nghia and others 2003). In transgenic tobacco with an antisense S-adenosylmethionine decarboxylase (SAMDC) gene from *Datura stramonium*, SAMDC activity and PA levels were strongly altered relative to controls only in micropropagated shoots, but not in *in vivo*-grown plants (Torrigiani and others 2005). Therefore, the effects of antisense inhibition may depend on culture conditions, plant tissues, developmental stages, and even plant generation. Whether this conditional inhibition is related to a heterogeneous antisense gene is an issue that warrants further investigation.

Coinciding with the decreased *ACS* gene expression in tobacco bud-forming leaf explants, ACS enzyme activity in A7, A10, and A16 was decreased to 43%–50% of control tissues at day 5 and 10 of culture, which was parallel to the decrease in *ACS* mRNA levels. However, the difference in ACS enzyme activity at day 15 was much smaller between transgenic lines and control. This was mainly due to the reduction of ACS enzyme activity in control tissues at day 15 compared with day 5. Endogenous ACC levels in A7, A10, and A16 were decreased to 46%–51% of control at day 5 and 10 of culture. Ethylene production in the transgenic bud-forming leaf explants decreased to 61%–74% of control at day 5, and decreased more at day 10 to 35%–50% of control. This reflected the observed reduction of ACS enzyme activity and ACC content. Ethylene biosynthesis was promoted in the vegetative bud formation process in wild-type tobacco. This is in agreement with observations from many other plants (Kumar and others 1998). However, in the transgenic plants of A7, A10, and A16 this increase of ethylene production was negated, resulting in decreased ethylene biosynthesis compared with controls.

Concomitant with the decrease of ethylene biosynthesis, the vegetative bud formation from leaf explants was promoted in transgenic tobacco. The number of buds per explant increased by 36%–68% in A7, A10, and A16 compared with control at day 10 of culture, and 87%–127% at day 15. The fresh weight of buds per explant of A7, A10, and A16 lines increased by 142%–187% at day 15 compared with control. This indicated that the growth of the transgenic buds was also faster than in controls.

In contrast to the process of vegetative bud formation, ethylene biosynthesis in normal growing A7, A10, and A16 plants showed little difference to that of the control with marginal decreases in ACS enzyme activity (3.9%–9.6%), ACC content (7.1%–13.4%) and ethylene production (9.4%–14.9%). The growth and development of transgenic tobacco was almost identical to that of control plants (plant height, internode number, leaf morphology, and

flowering time). Furthermore, mature leaves of transgenic tobacco showed similar physiological functions to that of control plants (chlorophyll content, stomatal conductance, photosynthetic capacity and transpiration rate). This demonstrates that small variations in ethylene biosynthesis will not affect plant growth and development in tobacco.

In plants, various genes have been successfully suppressed by homologous antisense RNA. There are only a few reports of partial inhibition using heterologous antisense RNA. For example, a petunia chalcone synthase (chs) antisense gene was reported to inhibit *chs* gene expression in tobacco and potato (Mol and others 1990). The overall sequence similarity of *chs* genes among these three kinds of plants was 75% or more. Also, the expression of the glutamine synthetase (GS) gene of tobacco can be inhibited by the alfalfa antisense *GS* gene. This gene has 81% similarity to that of tobacco (Temple and others 1993). The tomato *Le-ACS2* gene has 84% similarity at the nucleotide level to the tobacco *ACS* gene. It is reasonable that antisense construction from *Le-ACS2* should inhibit tobacco *ACS* gene expression and this was demonstrated by our northern hybridization and the observed level of ACS enzyme activity. However, the degree of inhibition is less than the homologous antisense RNA. It was reported that antisense *GS* did not lower the steady-state level of endogenous *GS* transcripts in heterologous tobacco plants but inhibited GS protein levels. It was likely that inhibition occurred at the translation level in that system (Temple and others 1993). However, in our system, the inhibition caused by the antisense ACS gene was presumably at the level of the endogenous tobacco *ACS* transcript. It is likely that the antisense message binds by homology to the endogenous transcript, and the double-stranded RNA molecules are targeted for degradation. This is in agreement with more thoroughly investigated homologous antisense RNA systems.

Although the role of ethylene in organogenesis and bud formation has been extensively studied, the use of ethylene inhibitors has given inconsistent results. For example, addition of AVG, an ethylene biosynthesis inhibitor, inhibited shoot elongation and bud induction in poplar (González and others 1997). However, the presence of $AgNO_3$ or AVG in the medium enhanced shoot regeneration in cabbage, cauliflower, and broccoli (Pua and others 1999). Torrigiani and colleagues have used tobacco thin layers to study ethylene and polyamine actions in vegetative bud formation (Biondi and others 1998; Scaramagli and others 1999; Torrigiani and others 2003; 2005). Their results indicated that treatment with AVG, which strongly inhibited ethylene production, provoked a large increase in the formation of meristemoids early in culture and the appearance of anomalous (''twin'') buds. Furthermore, AVG treatment increasingly inhibited *ACS* and *ACO* transcript accumulation during culture from day 2 to day 15. This is in agreement with our results using a transgenic approach. However, Torrigiani and colleagues also reported that treatments with an ethylene antagonist, silver thiosulfate (STS), or an ethylene-releasing compound, 2-chloroethylphosphonic acid (CEPA), decreased the number of buds regenerated compared to controls. Silver thiosulfate and CEPA were detrimental to meristemoid initiation and formation. These inconsistent results are likely to arise from different physiological functions or side effects of these compounds. Experiments on mustard and melon with antisense *ACO*, however, gave the identical results (Pua and Lee 1995; Amor and others 1998). The present study used another ethylene biosynthetic gene, *ACS*. Our results indicated that vegetative bud formation was promoted by inhibition of ethylene biosynthesis in tobacco. Therefore, it is likely that ethylene has similar actions in vegetative bud formation and shoot regeneration across a range of plants.

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