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Using a Seedling Hypocotyl Elongation Assay as a Genetic Screen for Ethylene Sensitivity of Seedling Geranium Cultivars

David G. Clark,¹
Christopher Dervinis,
James E. Barrett, and
Terril A. Nell

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Environmental Horticulture Dept., University of Florida, Gainesville, FL 32611-0670.

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¹To whom correspondence should be addressed.

SUMMARY. Experiments were conducted to determine if the seedling hypocotyl elongation and petal abscission assays could be used to identify differences in ethylene sensitivity among seedling geranium (*Pelargonium xhortorum*) cultivars. When seedlings of six geranium cultivars were germinated and grown in the dark in the presence of the ethylene biosynthetic precursor 1-aminocyclopropane-1-carboxylic acid (ACC) at various concentrations, they exhibited the triple response (measured as reduced hypocotyl length). While seedlings from all six cultivars were sensitive to ACC, 'Scarlet Elite' seedlings were most sensitive, and 'Multibloom Lavender', 'Elite White' and 'Ringo 2000 Salmon' seedlings were the least sensitive when germinated and grown on 20 mM [2022 mg·L⁻¹ (ppm)] ACC. Florets representing three developmental stages of each of the six cultivars were exposed to 1 µL·L⁻¹ of exogenous ethylene for 0, 30, or 60 min to determine if differences in cultivar sensitivity could be determined for petal abscission. Of the six cultivars tested, 'Ringo 2000 Salmon', 'Multibloom Lavender' and 'Elite White' were the least ethylene sensitive. Florets were also self-pollinated to test for cultivar differences in ethylene synthesis and subsequent petal abscission. Ethylene production and petal abscission were both promoted in self-pollinated florets compared to nonpollinated florets. 'Ringo 2000 Salmon', 'Multibloom Lavender' and 'Elite White' florets produced similar amounts of ethylene as all other cultivars, but abscised fewer petals after pollination. Our results indicate that the seedling hypocotyls elongation assay may be used to identify geranium cultivars with reduced sensitivity to ethylene. The data also suggest that genetic variability exists among geraniums for both ethylene sensitivity and biosynthesis.

Ethylene induces premature floral abscission in numerous plant species (Abeles et al., 1992). Diploid zonal geraniums are among the most sensitive species with regard to ethylene-induced petal abscission; exposure of florets to 1 µL·L⁻¹ ethylene can result in complete abscission in as little as 1 h (Evensen et al., 1993; Clark et al., 1997). Exposure to trace amounts of ethylene during post-harvest transit and marketing can cause petal drop and significantly reduce the visual quality of geraniums (Armitage et al., 1980) and other important floriculture crops.

Additionally, ethylene plays a role in the rapid abscission of the corolla following pollination (Abeles et al., 1992). A rapid increase in ethylene production by floral tissues after pollination, followed by accelerated corolla senescence and abscission, was reported for carnation (*Dianthus caryophyllus*) (Nichols, 1977), digitalis (*Digitalis purpurea*) (Stead and Moore, 1979, 1983), petunia (*Petunia hybrida*) (Pech et al., 1987; Whitehead et al., 1984), and geranium (Clark et al., 1997). Petal abscission by pollinated geranium florets is completely but reversibly blocked by 2,5 norbornadiene, an ethylene action inhibitor, indicating that endogenous ethylene perception is essential for the abscission response (Clark et al., 1997).

Ethylene also has a regulatory effect on the growth of seedlings following germination. Dark-grown etiolated pea (*Pisum sativum*) seedlings exposed to exogenous ethylene exhibited: 1) decreased elongation of the hypocotyl (Arshad and Frankenburger, 1988; Koch and Moore, 1990); 2) increased hypocotyl diameter; and 3) exaggerated plumular hooks (Goeschl et al., 1966). This triple response of seedlings was used to identify ethylene-insensitive mutants as a means to elucidate the mechanism of ethylene perception and action at the molecular level in arabidopsis (*Arabidopsis thaliana*) (Guzman and Ecker, 1990; Harpam et al., 1991) and tomato (*Lycopersicon esculentum*) (Lanahan et al., 1994). The effect of ethylene on hypocotyl elongation appears to be concentration-dependent; as exogenous ethylene concentration is increased, hypocotyl length is decreased up to a point of saturation (Goeschl and Kays, 1975; Harpam et al., 1991).

Zonal geranium cultivars differ

for the magnitude of flower petal abscission they display (Armitage et al., 1980). It is not known if cultivar differences exist for the production of ethylene and subsequent petal abscission in response to pollination. In geranium, ethylene biosynthesis in response to pollination occurs primarily in the pistil (Clark et al., 1997). Ethylene biosynthesis is regulated by the enzymes ACC synthase, which converts S-adenosylmethionine (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC), and ACC oxidase, which converts ACC to ethylene (Yang and Hoffman, 1984). Any differences in the production of these enzymes by different geranium cultivars could lead to variation in rates of pollination-induced ethylene production and subsequent abscission.

Flowers are often exposed to ethylene in the postharvest shipping and marketing environment. They can also produce endogenous ethylene due to accidental pollination during transit. Due to the reflexed structure of the geranium stigma, it is easy for florets to become self-pollinated when disturbed, and only a few grains of pollen are sufficient to induce endogenous ethylene production (Clark et al., 1997). Because geraniums are highly susceptible to petal abscission when exposed to ethylene, it would be advantageous for breeders to develop cultivars that either produce less ethylene or are ethylene-insensitive. In this report, we present the responses of six geranium cultivars to ethylene as it affects seedling hypocotyl elongation and flower petal abscission. We also discuss the effect of pollination on ethylene production and petal abscission of the six cultivars tested. Our objectives were to determine if the hypocotyl elongation component of the seedling triple response could be used as an assay to detect differences in ethylene sensitivity among geranium cultivars, and to see if these differences would correlate to differences in petal abscission as induced by pollination and exogenous ethylene. Furthermore, we wanted to observe if rates of ethylene synthesis by geranium florets after pollination vary between cultivars, and if these rates reflect the sensitivity of these cultivars to petal abscission. Information obtained from results of these experiments are directly applicable to flower breeders who may be searching for a screen to determine the amount of

genetic variability they have for ethylene sensitivity in their breeding lines, or to develop ethylene insensitive cultivars via mutation breeding.

Materials and methods

EXPERIMENT 1: EFFECT OF ACC ON SEEDLING HYPOCOTYL ELONGATION. To observe the effect of ACC on elongation of geranium seedling hypocotyls, 20 seeds of the geranium cultivars 'Orbit Red', 'Pinto Pink', 'Ringo 2000 Salmon' (Sluis & Groot-Novartis, Summit, N.J.), 'Multibloom Laven-der', 'Elite White', and 'Scarlet Elite' (Goldsmith Seeds, Inc., Gilroy, Calif.) were plated onto medium containing 0.8% agar and ACC at 0, 10, 20, 40, or 80 mM [0, 1011, 2022, 4044, or 8088 mg·L⁻¹ (ppm)], then placed in the dark at 21 °C (70 °F). After 1 week, seedling hypocotyl lengths were measured, and this experiment was repeated twice. Data for the percent reduction of hypocotyl length were analyzed via analysis of variance (ANOVA) using SAS 6.12 statistical software. Where ANOVA indicated significant differences in the treatment means, Tukey's mean separation test was used to compute an honestly significant difference (HSD) at $\alpha = 0.05$ by which treatment means were compared.

PLANT GROWTH. For all subsequent experiments, plants of all six cultivars were grown from seed in a commercial growing medium (#2, Fafard Inc., Agawam, Mass.) and transplanted to 3.8-L (1-gal) plastic pots. Plants were hand-watered as needed with a solution containing 150 mg·L⁻¹ nitrogen from Peter's 20N-4.4P-16.6K (Scotts-Sierra Horticultural Products Co., Marysville, Ohio). Experiments were conducted in February to April 1998, and florets at various stages of development were collected. Stage 1, florets were open with the stigma partially closed and not yet receptive to pollen; Stage 2, florets had open stigmatic lobes and were receptive for pollination; Stage 3, florets had stigmatic lobes that were fully reflexed.

EXPERIMENT 2: EXOGENOUS ETHYLENE TREATMENT AND PETAL ABSCISSION. To observe the effect of ethylene treatment on geranium petal abscission, experiments were conducted to determine if different cultivars showed differential sensitivity to exogenous ethylene or self-pollination-induced ethylene production. Florets at Stage 1, Stage 2, and Stage 3 were collected

from each of the six cultivars. Immediately after collection, the pedicel of each floret was placed into 1.5 mL microcentrifuge tubes filled with deionized distilled water. Florets were then placed inside sealed 40-L (10 gal) glass tanks. Ethylene was injected through a septum in the lid of the tank to a final concentration of 1.0 $\mu\text{L}\cdot\text{L}^{-1}$. The ethylene concentration inside the tanks was verified by testing a sample via a gas chromatograph equipped with a flame ionization detector and a column of activated alumina (model 5890 Series II; Hewlett Packard, Kennett Square, Pa.). Three ethylene treatment durations (0 min ethylene, 30 min ethylene, 60 min ethylene) were established: 1) control florets (0 min) were held in ethylene-free air for 60 min, 2) florets were treated with 30 min of ethylene followed by 30 min in air (30 min), or 3) florets were treated with 60 min of ethylene (60 min). Florets from all treatments were then tested for petal abscission 60 min after the start of ethylene treatment. To test for petal abscission, florets were held under a hand held blow drier (model IPD-2D; Clairol, Stamford, Conn.) on the high-speed, low-heat setting. Florets were held 30 cm (12 inch) from the drier nozzle for 5 s, and the number of abscised petals was recorded. The experiment consisted of a factorial arrangement of six cultivars, three floret development stages, and three ethylene durations. Five florets per cultivar/stage/duration combination were used, and the experiment was repeated three times on different days.

EXPERIMENT 3: POLLINATION INDUCED ETHYLENE PRODUCTION AND PETAL ABSCISSION. To observe the effect of pollination on petal abscission, geranium florets of all six cultivars were collected at stage 2 (receptive to pollination) and placed in microfuge tube trays filled with deionized distilled water. Florets were subsequently self-pollinated or not pollinated, and then placed at 21 °C (70 °F) under fluorescent light (30 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) for 2 h. Florets were then tested for petal abscission as described above, and the number of abscised petals was recorded. The experiment consisted of a factorial arrangement of treatments with six cultivars and two pollination types (pollinated or not pollinated). Ten florets per treatment were used, and the experiment was repeated three times on different days.

To determine the effect of pollination on ethylene production by geranium florets, florets were harvested at Stage 2 (receptive to pollination) and were either self-pollinated or not pollinated. Immediately following treatment, all petals and sepals were removed, and the gynoecium and petiole of each floret was placed in a 6 mL glass test tube filled with 1 mL of deionized distilled water. The test tubes were sealed with a rubber septum, and the floral tissues were held inside for 2 h in light at room temperature. Subsequently, the ethylene concentration of 1 mL of the headspace of each tube was measured via gas chromatography using a gas chromatograph (model 5890; Hewlett-Packard, Palo Alto, Calif.) equipped with a flame ionization detector and a column of activated alumina. The experiment consisted of a factorial arrangement of six cultivars and two pollination treatments (pollinated or not pollinated). Ten florets per cultivar/treatment combination were used, and the experiment was repeated three times on different days. All data for percent petal abscission and ethylene production in Expts. 2 and 3 were analyzed via ANOVA using SAS 6.12 statistical software. Where ANOVA indicated differences in the treatment means, Tukey's mean separation test was used to calculate an HSD at $\alpha = 0.05$, by which treatment means were compared.

Results and discussion

EFFECT OF ACC ON SEEDLING HYPOCOTYL ELONGATION. A wide variety of

plant species including arabidopsis (Bleecker et al., 1988), tomato (Lanahan et al., 1994) and pea (Goeschl et al., 1975) show a concentration-dependent reduction of etiolated seedling hypocotyl elongation when grown in the dark in the presence of ethylene or ACC (the biosynthetic precursor to ethylene). In recent years, researchers have used this response as a genetic screen to help select mutant plants with altered ethylene synthesis and perception (Bleecker et al., 1988; Guzman and Ecker, 1990; Lanahan et al., 1994). Results from these experiments led us to investigate whether geranium seedlings displayed the seedling triple response, and if cultivar differences in ethylene sensitivity could be detected at the seedling level. In our experiments, we chose to germinate seeds on a medium containing ACC instead of growing them in the presence of ethylene because ACC oxidase (which converts ACC to ethylene) genes were expressed in roots of seedling plants (Clark et al., 1997). Seedlings of all geranium cultivars used in these experiments showed a concentration dependent reduction in hypocotyl length when plated on media containing ACC (Table 1). For all cultivars, ACC was effective in reducing hypocotyl elongation at a concentration of 10 mM compared to controls. Hypocotyl length further decreased as ACC concentration was increased, with maximal effect in all cultivars at 40 mM ACC (Table 1). No additional effect on inhibition of hypocotyl elongation was observed with 80 mM ACC, which suggests that 40 mM ACC is sufficient

to produce enough endogenous ethylene to completely saturate the ethylene binding sites of each cultivar.

For ACC induced hypocotyl length reduction, 'Elite Scarlet' was the most ethylene sensitive. It was the only cultivar that displayed a complete hypocotyl length reduction when germinated and grown on 10 mM ACC (Table 1). 'Pinto Pink' and 'Orbit Red' were considered to be moderately sensitive to ethylene because they showed complete hypocotyl length reduction on 20 mM ACC (Table 1). 'Ringo 2000 Salmon', 'Multibloom Lavender' and 'Elite White' were less ethylene sensitive than all other cultivars because they did not show complete hypocotyl reduction on 20 mM ACC (Table 1). The seedling triple response has been used to identify ethylene-insensitive mutants in various sensitive species (Guzman and Ecker, 1990; Harpam et al., 1991; Lanahan et al., 1994), but variation in ethylene sensitivity among commercial geranium seedlings has not been reported. To our knowledge, geranium seedlings have not been previously shown to display the seedling triple response. Our results indicate that seedling geraniums, like other ethylene-sensitive species, display the seedling triple response phenotype in the presence of ACC, and cultivar differences exist for the magnitude of this response at the seedling level. It is logical to suggest that these cultivar differences are due to differential ethylene sensitivity, but it is impossible to rule out whether the seedlings observed in this study produced differen-

Table 1. Hypocotyl length of etiolated seedlings of six geranium cultivars grown for 1 week on agar containing 10, 20, 40 or 80 mM 1-aminocyclopropane-1-carboxylic acid (ACC). Values in the table represent the mean percentage hypocotyl length compared to control hypocotyls grown on ACC-free media. Mean percentages were calculated by dividing the mean hypocotyl length at a given ACC concentration by the mean hypocotyl length of seedlings on ACC-free media X 100.

Cultivar	Hypocotyl length (% of control)				
	[ACC] (mM)				
	0	10	20	40	80
Orbit Red	100	36.5	21.5	20.0	14.0
Ringo 2000 Salmon	100	44.0	34.0	20.0	21.0
Multibloom Lavender	100	70.5	44.5	25.0	23.0
Elite White	100	53.5	46.5	32.0	33.0
Pinto Pink	100	76.0	37.0	37.0	32.0
Elite Scarlet	100	21.0	15.5	11.5	13.0
Factorial analysis	$P > F$				
Cultivar (C)	*				
ACC	*				
C × ACC	*(HSD _{0.05} = 13.0)				

Table 2. Analysis of variance of petal abscission by florets of six geranium cultivars at three stages of development. Florets were treated 60 min in air, 30 min with 1 $\mu\text{L}\cdot\text{L}^{-1}$ ethylene followed by 30 min in air, or 60 min with 1 $\mu\text{L}\cdot\text{L}^{-1}$ ethylene. Petal abscission was assessed 1 h after the onset of treatment.

Factorial analysis	<i>P</i> > <i>F</i>
Cultivar (C)	*
Ethylene duration (ED)	*
Stage of development (SD)	*
C $\times$ ED	*(HSD _{0.05} = 12.3)
C $\times$ SD	NS
ED $\times$ SD	*(HSD _{0.05} = 19.5)
C $\times$ ED $\times$ SD	NS

*F value significant at the 5% level. HSD calculated by a Tukey's test at the 5% level.

NSF value not significant at the 5% level.

Table 3. Percent petal abscission of geranium florets at three stages of development. Florets were treated 60 min in air, 30 min with 1 $\text{mL}\cdot\text{L}^{-1}$ ethylene followed by 30 min in air, or 60 min with 1 $\text{mL}\cdot\text{L}^{-1}$ ethylene. Petal abscission was assessed 1 h after the onset of treatment.^z

Stage of development (all cultivars)	Petal abscission (%)		
	Ethylene duration (min)		
	0	30	60
1	1.1	4.2	14.0
2	1.6	20.7	38.7
3	21.7	58.5	68.7

^zHSD_{0.05} = 19.5 as calculated by a Tukey's test at the 5% level.

Table 4. Mean percent petal abscission of florets from six geranium cultivars. Florets were treated 60 min in air, 30 min with 1 $\text{mL}\cdot\text{L}^{-1}$ ethylene followed by 30 min in air, or 60 min with 1 $\text{mL}\cdot\text{L}^{-1}$ ethylene. Petal abscission was assessed 1 h after the onset of treatment.^z

Cultivar (all stages)	Petal abscission (%)		
	Ethylene duration (min)		
	0	30	60
Orbit Red	8.0	33.3	56.0
Ringo 2000 Salmon	8.0	15.1	35.6
Multibloom Lavender	9.3	14.3	23.6
Elite White	8.0	23.1	28.9
Pinto Pink	11.6	49.3	60.0
Elite Scarlet	4.0	32.4	48.7

^zHSD_{0.05} = 12.3 as calculated by a Tukey's test at the 5% level.

tial amounts of ethylene within the seedlings. Regardless of the mechanism, it is possible that geranium seedlings showing reduced ethylene sensitivity or biosynthesis could be effectively isolated by germinating them on 20 mM ACC and subsequently analyzing them for lack of reduction in hypocotyl length.

EXOGENOUS ETHYLENE TREATMENT AND PETAL ABSCISSION. For geranium florets exposed to exogenous ethylene, stage of development affected how a floret responded to duration of ethylene treatment for the amount of petal abscission (Tables 2 and 3). Among florets held in ethylene-free air, petal abscission by Stage 1 and

Stage 2 florets was negligible, while Stage 3 florets abscised about 20% of their petals (Table 3). Exposure to 1.0 $\mu\text{L}\cdot\text{L}^{-1}$ ethylene for 30 min increased petal abscission of Stage 2 and Stage 3 florets, with more abscission occurring in Stage 3 florets than in Stage 2 florets (Table 3). Treatment with ethylene for 60 min increased petal abscission of florets at all stages of development compared to florets held in air (Table 3). As floret age increased, petal abscission increased. After 60 min of ethylene treatment, Stage 1 florets abscised 14% of their petals, Stage 2 florets abscised 39%, and Stage 3 florets abscised 69% (Table 3). These observations suggest that the sensitiv-

ity of diploid geranium florets increases with floret age, and thus lower concentrations of ethylene are required to induce petal abscission as the floret ages. A similar age-dependent floral response to ethylene has been reported with florets of regal geranium (Evensen 1991) and digitalis (Stead and Moore, 1983).

The six cultivars also differed in percent petal abscission as ethylene exposure duration increased, regardless of the stage of floret development (Tables 2 and 4). All cultivars had similar low levels of abscission when held in ethylene-free air (Table 4). After exposure to 1.0 $\mu\text{L}\cdot\text{L}^{-1}$ ethylene for 30 min, florets of all cultivars except for 'Ringo 2000 Salmon' and 'Multibloom Lavender' showed increased petal abscission compared to florets of the same cultivar held in air (Table 4). At an ethylene exposure duration of 60 min, 'Ringo 2000 Salmon', 'Multibloom Lavender' and 'Elite White' florets abscised fewer petals than the other cultivars tested (Table 4), and thus appear to be less sensitive to exogenous ethylene for petal abscission. This observation is consistent with the seedling triple response data described above, suggesting that the cultivars 'Multibloom Lavender' 'Elite White' and 'Ringo 2000 Salmon' are less sensitive to ethylene than the other cultivars tested. These results indicate that ethylene sensitivity is a genetically variable character in geranium. This observation is consistent with the findings of Deneke et al. (1990), who observed cultivar differences for petal abscission by regal geranium florets after treatment with 0.5 $\mu\text{L}\cdot\text{L}^{-1}$ ethylene for 2 h.

POLLINATION INDUCED ETHYLENE PRODUCTION AND PETAL ABSCISSION. All cultivars were tested for the effect of self-pollination on floret ethylene production and subsequent petal abscission. Ethylene production was promoted by self-pollination, and the amount of ethylene produced was influenced by cultivar (Table 5). 'Orbit Red' and 'Scarlet Elite' produced less ethylene after self-pollination than 'Pinto Pink', while all other cultivars produced similar amounts of ethylene. Petal abscission was also promoted by self-pollination and the extent of the abscission response was affected by cultivar (Table 5). 'Multibloom Lavender' and 'Elite White' responded differently by abscising fewer petals

Table 5. Petal abscission and ethylene production of pollinated florets of six geranium cultivars 2 h after pollination (+), compared to unpollinated (-) florets.

Cultivar	Pollination	Petal abscission (%)	Ethylene production (nL·h ⁻¹ per pistil)
Orbit Red	+	25.3	1.1
	-	1.3	0.2
Ringo 2000 Salmon	+	10.0	1.4
	-	0.0	0.3
Multibloom Lavender	+	9.3	1.3
	-	2.7	0.1
Elite White	+	2.7	1.2
	-	0.0	0.5
Pinto Pink	+	19.3	1.6
	-	5.3	0.2
Elite Scarlet	+	16.7	1.1
	-	5.3	0.2

P > F		
Factorial analysis	Petal abscission (%)	Ethylene production
Cultivar (C)	NS	NS
Pollination (P)	*	*
C × P	*(HSD _{0.05} = 9.9)	*(HSD _{0.05} = 0.5)

*F value significant at 5% level. HSD calculated by Tukey's test at 5% level.

NSF value not significant at 5% level.

than 'Orbit Red' and 'Pinto Pink' after self-pollination. There was no apparent relationship between the amount of ethylene produced and the amount of petal abscission observed. However, 'Elite White' and 'Multibloom Lavender' were both screened out as least sensitive to ethylene in seedling and floret assays. When combined, the data presented in these experiments suggest that differences in petal abscission observed in geraniums after ethylene treatment and self-pollination are more dependent on differences in ethylene sensitivity than ethylene biosynthesis.

Based on the hypocotyl elongation and petal abscission data discussed above, all geranium cultivars tested appear to be sensitive to ethylene. All cultivars display the seedling triple response (as measured by reduced hypocotyl length) and petal abscission when florets are exposed to exogenous ethylene or pollinated. 'Multibloom Lavender' 'Ringo 2000 Salmon' and 'Elite White' appear to have reduced ethylene sensitivity compared to the other cultivars tested. Seedlings of these cultivars required a higher concentration of ACC to achieve the maximal response to ethylene (reduced hypocotyl length) compared to the other cultivars. Florets of these cultivars were less sensitive to exogenous ethylene application for flower petal abscission. When

florets of these cultivars were self-pollinated, they produced roughly similar amounts of ethylene as the other three cultivars, but abscised fewer petals.

While the extent of variation for ethylene sensitivity was minimal for the cultivars tested, the observations reported here suggest that the hypocotyl elongation component of the seedling triple response may be used as an assay to identify diploid geranium cultivars that are less susceptible to ethylene-induced petal abscission. Perhaps a more practical option would be to employ this assay to screen mutant geranium seedling populations to identify individuals displaying higher degrees of ethylene-insensitivity. This technique has been used successfully with arabidopsis (Guzman and Ecker, 1990; Harpam et al., 1991) and tomato (Lanahan et al., 1994) to isolate and breed ethylene insensitive plants. In both arabidopsis and tomato ethylene insensitive mutants, one of the associated phenotypes is significantly delayed flower senescence. By using mutagenesis combined with the seedling triple response as a screen, it may be possible to develop ethylene insensitive geraniums with reduced petal abscission via conventional breeding methods. It may also be possible for breeders to quickly assay their existing breeding stock to see if variation exists for ethylene sensitivity. Recent obser-

vations in our laboratory have demonstrated that other important floriculture crops such as snapdragon and petunia show decreased seedling hypocotyl elongation in response to ethylene (Clark, unpublished results). It is likely that assays similar to the one described here can be developed for use on other seed propagated ethylene sensitive species as well. In using this type of genetic approach it is likely that breeders will want to screen for partial ethylene insensitivity rather than complete ethylene sensitivity because high degrees of ethylene insensitivity have been shown to lead to undesirable phenotypes at the whole plant level such as reduced adventitious root formation (Clark et al., 1999). Performance trials will be of utmost importance in finding the appropriate combination of ethylene sensitivity and horticultural production characteristics.

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