

# Lines of lettuce selected for ethylene insensitivity at the seedling stage displayed variable responses to ethylene or wounding as mature heads

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## Abstract

Hypocotyl elongation in germinating lettuce (*Lactuca sativa* L., cv. Diana) M<sub>2</sub> seedlings from an ethyl methanesulfonate treated population was used to select lines that were less sensitive to ethylene (C<sub>2</sub>H<sub>4</sub>) than the progenitor line. Individuals were identified that had hypocotyls at least twice as long in the presence of hormonal C<sub>2</sub>H<sub>4</sub> levels (> 1 μl l<sup>-1</sup>) as wild-type plants. This phenotype was confirmed in M<sub>3</sub> seedlings for three independent mutants. The phenotype was normal in terms of growth for the mutant lines, so there were no obvious confounding growth defects or effect of other induced mutations. Changes in response to C<sub>2</sub>H<sub>4</sub> and wounding (chlorophyll loss, induced respiration, induced phenylalanine ammonia-lyase (PAL) activity) in mature heads of lettuce were variable among the three mutant lines. Selection for C<sub>2</sub>H<sub>4</sub> insensitivity at the seedling stage did not produce mature plants that were consistently less sensitive to C<sub>2</sub>H<sub>4</sub>-induced increases in PAL activity or CO<sub>2</sub> production. However, all the selected mutated plants did have lower rates of C<sub>2</sub>H<sub>4</sub>-induced chlorophyll loss. All of the mutants differed from the wild-type in at least 3 of the 7 phenotypes measured, while one line differed in all the measured phenotypes. The apparent random distribution of induced changes observed in the mutant lines suggests that screening additional mutants may identify lines that combine a number of useful horticultural traits.

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## 1. Introduction

The quality and shelf-life of harvested lettuce (*Lactuca sativa* L.) is adversely affected by exposure to hormonal levels of C<sub>2</sub>H<sub>4</sub> (e.g. > 1 μl l<sup>-1</sup>) in air (Saltveit, 1999). Some of the symptoms associated with C<sub>2</sub>H<sub>4</sub> exposure are premature senescence with loss of chlorophyll

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and yellowing, tissue browning, and elevated respiration. Ethylene and wounding both promote the activity of the phenylpropanoid pathway (e.g. phenylalanine ammonia-lyase (PAL) activity) with the accompanying accumulation of phenolic compounds and tissue browning (Tomás-Barberán et al., 1997). Storage of crisphead lettuce in  $> 5 \mu\text{l C}_2\text{H}_4 \text{ l}^{-1}$  air at  $5^\circ\text{C}$  induces PAL activity and the formation of small ( $\sim 2$  mm diameter) brown lesions in achlorophyllous (i.e. white) mid-rib tissue (i.e. russet spotting) (Ke and Saltveit, 1988; Ritenour et al., 1995).

The small amounts of  $\text{C}_2\text{H}_4$  produced by harvested lettuce are transiently increased when tissue experiences a biotic or abiotic stress. For example, wounding (i.e. cutting leaf tissue into  $2 \times 3 \text{ cm}^2$  pieces) increased  $\text{C}_2\text{H}_4$  production by crisphead lettuce held at  $20^\circ\text{C}$  threefold from  $0.2$  to  $0.6 \mu\text{l g}^{-1} \text{ FW h}^{-1}$  (Ke and Saltveit, 1989). However, wound-induced rates of  $\text{C}_2\text{H}_4$  production from lettuce are very low compared to other species, and they returned to non-stressed control levels within 24 h. Cutting used in the preparation of fresh-cut lettuce also induces phenylpropanoid activity and the synergistic action of wounding and  $\text{C}_2\text{H}_4$  can drastically reduce the quality and shelf life by inducing tissue browning (Lopez-Galvez et al., 1996; Saltveit, 1996). Although both wounding and  $\text{C}_2\text{H}_4$  can increase phenylpropanoid activity, and wounding induces a transitory increase in  $\text{C}_2\text{H}_4$  production, a kinetic study showed that wound-induced  $\text{C}_2\text{H}_4$  does not appear to be responsible for the increase in phenylpropanoid metabolism following wounding (Ke and Saltveit, 1989).

In contrast to the small effect endogenously produced  $\text{C}_2\text{H}_4$  may have on harvested lettuce, the postharvest environment is often inadvertently polluted with hormonal levels of  $\text{C}_2\text{H}_4$  that can have significant deleterious effects on the storage life and quality of harvested lettuce (e.g. russet spotting). Presently, the only way to control the adverse effects of  $\text{C}_2\text{H}_4$  on whole or processed lettuce is to eliminate it from the storage environment, to hold the tissue as close to  $0^\circ\text{C}$  as possible to minimize physiological activity, and to increase the concentration of inhibitors of  $\text{C}_2\text{H}_4$  action (e.g.  $\text{CO}_2$ ) in the storage atmosphere (Salt-

veit, 1999). New inhibitors of  $\text{C}_2\text{H}_4$  action (e.g. 1-methylcyclopropene; Fan and Mattheis, 2000) and incorporation of specific genes (Hua et al., 1995) can both render plants insensitive to  $\text{C}_2\text{H}_4$ , but the use of chemicals and genetic engineering may be objectionable to a significant number of consumers. A more acceptable method may be to select lines of lettuce for  $\text{C}_2\text{H}_4$  insensitivity that have been generated through traditional breeding or induced mutation.

Ethylene insensitive lettuce could be used in an identical manner to normal lettuce, providing there were no deleterious pleiotropic effects. Ethylene contamination of the postharvest atmosphere would no longer be a concern. Ethylene insensitive lettuce may not only affect the postharvest life of harvested lettuce, but could affect the plant's response to other abiotic and biotic stresses (e.g. disease resistance, drought and salt tolerance). Both positive and negative effects have been reported on disease resistance in other plants (Stepanova and Ecker, 2000). In addition, if  $\text{C}_2\text{H}_4$  insensitivity reduced the plant's response to abiotic stress, it might result in greater uniformity of crops growth under stressful conditions.

In sensitive plants such as lettuce, exposure to hormonal levels of  $\text{C}_2\text{H}_4$  (e.g.  $> 1 \mu\text{l C}_2\text{H}_4 \text{ l}^{-1}$  air) reduces hypocotyl elongation in germinating hypogeal seeds (Abeles et al., 1992). This response is often log-linear with a log increase in  $\text{C}_2\text{H}_4$  concentration producing a linear decrease in hypocotyl elongation. Seedlings with significantly longer hypocotyls in the presence of  $\text{C}_2\text{H}_4$  levels that inhibit elongation in normal seedlings could be selected for their reduced  $\text{C}_2\text{H}_4$  sensitivity. This selection procedure allows the screening of large numbers of  $\text{M}_2$  seeds with minimal effort (Waycott et al., 1995), and was used to identify  $\text{C}_2\text{H}_4$  insensitive *Arabidopsis thaliana* plants from mutated populations (Bleecker et al., 1988).

Research reported in this paper was undertaken to examine the possibility of decreasing  $\text{C}_2\text{H}_4$  sensitivity in horticulturally mature heads of harvested lettuce by selecting for  $\text{C}_2\text{H}_4$  insensitivity in  $\text{M}_2$  seedlings following treatment with ethyl methanesulfonate (EMS). Nine parameters of quality, and  $\text{C}_2\text{H}_4$ -induced and wound-induced metabolic changes were used to ascertain how

the mutant lines would differ from their progenitor in their growth, and in responses to exogenously applied  $C_2H_4$  and wounding. We show that quality was unaffected in the mutated lines, and that responses to  $C_2H_4$  and wounding among the lines were variable and seemingly unrelated to one another.

## 2. Materials and methods

### 2.1. Selection of $C_2H_4$ -insensitive mutants of lettuce

Seeds of the lettuce butterhead cultivar ‘Diana’ were agitated for 12 h in aerated solutions of 0.5 or 0.05% (v/v) EMS (Sigma, St. Louis, MO), with 0.025% (v/v) Tween 20 detergent (Fisher Scientific, Pittsburgh, PA). They were then washed once in distilled water for ~30 min and rinsed in distilled water four times.  $M_1$  seeds were planted and grown to maturity in the greenhouse, generating over 3000  $M_2$  families (Okubara et al., 1997). The higher dosage of 0.5% (v/v) EMS resulted in weak plants that did not produce abundant seed and so was not used subsequently.

Approximately 20 seeds from each of about 250  $M_2$  families resulting from the 0.05% EMS treatment were germinated on moist filter paper in  $25 \times 100$  mm<sup>2</sup> diameter plastic Petri dishes, and grown at 20 °C in air supplemented with  $10 \mu\text{l l}^{-1}$   $C_2H_4$  for 5 days. Mutant seedlings that had hypocotyls at least twice the length of normal plants were easily identified, grown to maturity, and the  $M_3$  seed collected. Progeny from the mutant plants were screened to verify the heritable nature of the  $C_2H_4$  insensitivity. Three mutant lines (i.e. 242, 243, and 244) were selected for further study.

### 2.2. Plant material

Plants were grown from parental (‘Diana’) and mutated lines (242, 243, 244) in 4-L plastic pots under standard cultural practices during the spring in a greenhouse at Davis, CA. Three separate plantings were seeded about 3 weeks apart. Whole heads were harvested, the outer leaves removed,

and the heads washed, dried and stored at 5 °C until used. Leaf discs (1.7 cm diameter) were excised from fully expanded, mature leaves with a stainless steel cork borer and placed in  $15 \times 100$  mm<sup>2</sup> diameter plastic Petri dishes.

### 2.3. Ethylene- and wound-induced metabolic changes

To study wound-induced changes, excised leaves were cut into  $2 \times 2$  cm<sup>2</sup> pieces or 1.7-cm diameter leaf discs were excised and stored in a flow-through system in  $C_2H_4$ -free air at 10 °C. To study  $C_2H_4$ -induced changes, 2 whole heads per replicate were stored in a flow-through system in air or air plus  $C_2H_4$  ( $80 \pm 10 \mu\text{l l}^{-1}$ ) for 3–5 days at 10 °C.

Carbon dioxide and  $C_2H_4$  production from whole heads, cut pieces, and leaf discs was periodically analyzed as previously described (Saltveit, 1982; Saltveit and Strike, 1989). Leaf tissue was analyzed for chlorophyll content as previously described (Inskeep and Bloom, 1985; Jaime and Saltveit, 2002). PAL was extracted from leaf tissue and its activity assayed as previously described (Ke and Saltveit, 1989; Campos-Vargas and Saltveit, 2002).

### 2.4. Statistical analysis

Each experiment had at least two replicates of each treatment and all experiments were run at least twice with similar results. Measurements from all the replicates were combined and treatment effects subjected to an ANOVA, and 5% LSD values calculated when significant treatment differences were detected.

## 3. Results

Hypocotyls of the wild-type seedlings elongated to  $33.0 \pm 2.3$  mm after 7 days at 20 °C in the absence of  $C_2H_4$  (Fig. 1). Exposure to  $C_2H_4$  ( $10 \mu\text{l l}^{-1}$ ) reduced hypocotyl elongation  $77 \pm 6\%$  from  $33.0 \pm 2.3$  to  $7.6 \pm 0.7$  mm. Screening the  $M_2$  families resulted in the identification of three mutants. Seedlings grown from these selected

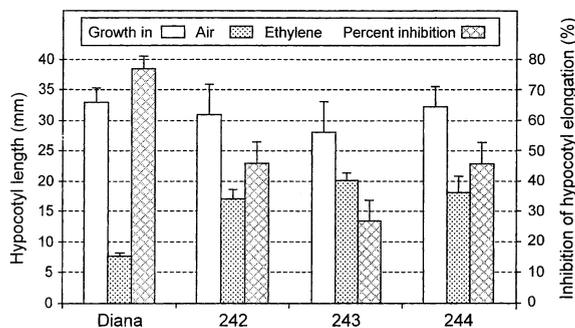


Fig. 1. Effect of ethylene exposure on hypocotyl growth on progenitor ('Diana') and mutant (242, 243, and 244) lines of lettuce seedlings. Hypocotyl growth under air and  $10 \mu\text{l l}^{-1}$  ethylene in air for 7 days at  $20^\circ\text{C}$ , and the percent inhibition of hypocotyls grown in ethylene versus air. The lines atop the bars represent the standard error of the means ( $n = 6$ ).

mutant lines had an average hypocotyl of length of  $30.2 \pm 5.2$  mm in air and  $18.2 \pm 1.4$  mm in the  $\text{C}_2\text{H}_4$  atmosphere. The average  $\text{C}_2\text{H}_4$  inhibition of hypocotyl elongation for the mutants (i.e.  $39 \pm 11\%$ ) was therefore about half that of the wild-type. Mutants 242 and 244 were inhibited 46%, while line 243 was inhibited 28%. Genetic complementation analysis indicated that  $\text{C}_2\text{H}_4$  insensitivity was conferred by a single independent locus in each of these mutants (Saltveit et al., unpublished).

The mutant lines were similar to the wild-type progenitor in head weight and leaf thickness. At the time of harvest, whole cleaned heads of cv. Diana weighed  $0.35 \pm 0.11$  kg, while the mutants weighed  $0.34 \pm 0.06$ ,  $0.40 \pm 0.17$ , and  $0.32 \pm 0.07$  kg for 242, 243, and 244, respectively. Leaf disks cut from mature, fully expanded leaves weighed  $21.8 \pm 1.2$  mg  $\text{cm}^{-2}$  for 'Diana' and  $23.3 \pm 1.7$ ,  $24.6 \pm 1.3$ , and  $24.4 \pm 1.6$  mg  $\text{cm}^{-2}$  for mutants 242, 243, and 244, respectively.

In the absence of  $\text{C}_2\text{H}_4$ , PAL activities in the non-mutated and mutated lines were similar ( $0.14 \pm 0.6$  vs.  $0.17 \pm 0.04$   $\mu\text{mol cinnamic acid g}^{-1} \text{h}^{-1}$ ) (Fig. 2). Exposure to  $80 \pm 10 \mu\text{l l}^{-1}$   $\text{C}_2\text{H}_4$  in air for 3 days at  $10^\circ\text{C}$  increased PAL activity by  $47 \pm 21\%$  (43% for 'Diana' and 40, 28, and 76% for mutants 242, 243, and 244, respectively). These were small increases in comparison to the average threefold increase induced by wounding. Cutting the leaves into  $2 \times 2 \text{ cm}^2$  pieces

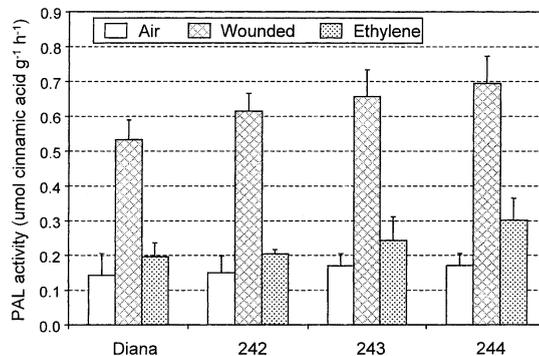


Fig. 2. Activity of PAL in wild-type ('Diana') and mutant (242, 243, and 244) lines of lettuce held in air, air plus  $80 \pm 10 \mu\text{l l}^{-1}$  ethylene for 3 days at  $5^\circ\text{C}$ , or wounded (cut into  $2 \times 2 \text{ cm}^2$  pieces) and held for 1 days at  $10^\circ\text{C}$ . The lines atop the bars represent the standard error of the means ( $n = 6$ ).

increased PAL activity in 'Diana' after 24 h by 2.8-fold ( $0.53 \pm 0.6$   $\mu\text{mol cinnamic acid g}^{-1} \text{h}^{-1}$ ), and by 3.2-, 2.7-, and 3.1-fold ( $0.62$ ,  $0.66$ , and  $0.69$   $\mu\text{mol cinnamic acid g}^{-1} \text{h}^{-1}$ ) for mutated lines 242, 243, and 244, respectively (Table 1). Therefore, while there was no significant reduction among the mutants, or between the mutated lines and the wild-type with regard to wound-induced PAL activity, there were differences in  $\text{C}_2\text{H}_4$ -induced PAL activity. Mutant 243 had significantly less  $\text{C}_2\text{H}_4$ -induced PAL activity (a 28% increase) and 244 had significantly more  $\text{C}_2\text{H}_4$ -induced PAL activity (a 76% increase) than the control (a 43% increase) or 242 (a 40% increase) (Table 1).

Wounding also stimulated  $\text{C}_2\text{H}_4$  production. Excised 1-cm diameter leaf disks from 'Diana' produced  $\text{C}_2\text{H}_4$  at  $0.72 \pm 0.07$   $\mu\text{l kg}^{-1} \text{h}^{-1}$ , while wound-induced  $\text{C}_2\text{H}_4$  production from mutants 242, 243, and 244 was  $1.10 \pm 0.06$ ,  $1.50 \pm 0.19$ , and  $0.68 \pm 0.10$   $\mu\text{l kg}^{-1} \text{h}^{-1}$ , respectively. The increase in  $\text{C}_2\text{H}_4$  production over the non-wounded control was 120% for 'Diana', and 110, 105, and 161% for 242, 243, and 244, respectively (Table 1). Wounding significantly increased  $\text{C}_2\text{H}_4$  production in line 244 compared to increases in the other genotypes.

Respiration ( $\text{CO}_2$  production) was stimulated in whole heads of all the lettuce lines by exposure to a flow of  $80 \pm 10 \mu\text{l l}^{-1}$   $\text{C}_2\text{H}_4$  in air at  $10^\circ\text{C}$  (Fig. 3). The maximum stimulation occurred

Table 1

Comparison between the progenitor (cv. Diana) and mutant (242, 243, and 244) lines of lettuce to exposure to hormonal levels of ethylene and wounding

Parameter measured	Lines			
	Diana	242	243	244
Weight of whole head (% of Diana)	100	97	114	91
Leaf thickness (% of Diana)	100	107	113	112
Change induced by ethylene (% of non-ethylene treated tissue)				
Inhibition of hypocotyl elongation	77 <sup>a</sup>	46 <sup>b</sup>	28 <sup>c</sup>	46 <sup>b</sup>
Increase in PAL activity	43 <sup>b</sup>	40 <sup>b</sup>	28 <sup>c</sup>	76 <sup>a</sup>
Increase in CO <sub>2</sub> production	47 <sup>a</sup>	40 <sup>a</sup>	49 <sup>a</sup>	10 <sup>b</sup>
Increase in chlorophyll loss	48 <sup>a</sup>	2 <sup>c</sup>	20 <sup>b</sup>	3 <sup>c</sup>
Change induced by wounding (% of non-wounded tissue)				
Increase in PAL activity	280 <sup>b</sup>	320 <sup>a</sup>	270 <sup>b</sup>	310 <sup>a</sup>
Increase in C <sub>2</sub> H <sub>4</sub> production	120 <sup>b</sup>	110 <sup>b</sup>	105 <sup>b</sup>	161 <sup>a</sup>
Increase in CO <sub>2</sub> production	108 <sup>b</sup>	120 <sup>b</sup>	90 <sup>c</sup>	145 <sup>a</sup>

Values are the mean of two replicates from each of two independent experiments. Values in a row with the same letter are not significantly different at the 5% level.

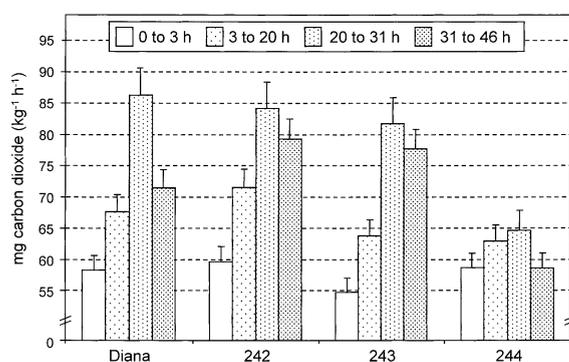


Fig. 3. Effect of duration of exposure to ethylene ( $80 \pm 10 \mu\text{l l}^{-1}$ ) in air on respiration ( $\text{CO}_2$  production) of whole heads of mutant (242, 243, and 244) or wild-type progenitor (cv. Diana) lines. The lines atop the bars represent the standard error of the means ( $n = 6$ ).

between 20 and 31 h of exposure and ranged from 47% for 'Diana', to 40, 49, and 10% for the mutants 242, 243, and 244, respectively (Table 1). Interestingly, C<sub>2</sub>H<sub>4</sub>-induced respiration was significantly lower in mutant 244.

Wounding was much more effective at stimulating respiration (CO<sub>2</sub> production) in all the tested genotypes than was exposure to C<sub>2</sub>H<sub>4</sub>. At 10 °C, whole, harvested heads produced CO<sub>2</sub> at  $57.9 \pm 2.1 \text{ mg kg}^{-1} \text{ h}^{-1}$  (Fig. 3). Excised 1-cm diameter mid-rib tissue from 'Diana' produced

CO<sub>2</sub> at  $122 \pm 18 \text{ mg kg}^{-1} \text{ h}^{-1}$ , while discs from mutants 242, 243, and 244 produced CO<sub>2</sub> at  $131 \pm 19$ ,  $105 \pm 25$ , and  $144 \pm 41 \text{ mg kg}^{-1} \text{ h}^{-1}$ , respectively. The increases ranged from 108% for 'Diana', to 120, 90, and 145% for mutants 242, 243, and 244, respectively (Table 1). As with C<sub>2</sub>H<sub>4</sub>-induced PAL activity, wound-induced CO<sub>2</sub> production was lowest in mutant 243 and highest in mutant 244.

The chlorophyll content of leaves from the progenitor and mutant lines were similar at harvest ( $0.77 \pm 0.04 \text{ mg g}^{-1}$ ), and after 5 days in air without C<sub>2</sub>H<sub>4</sub> at 10 °C ( $0.57 \pm 0.03 \text{ mg g}^{-1}$ ). However, compared to the air controls, exposure to  $80 \mu\text{l l}^{-1}$  C<sub>2</sub>H<sub>4</sub> in air for 5 days at 10 °C resulted in the loss of 48% of the chlorophyll from 'Diana' leaves, and 2, 20 and 3% from mutants 242, 243, and 244, respectively (Table 1).

#### 4. Discussion

Mutant lines of lettuce selected at the seedling stage for significantly reduced sensitivity to C<sub>2</sub>H<sub>4</sub> produced mature heads that were variable in their response to C<sub>2</sub>H<sub>4</sub> exposure (Table 1). None of the parameters measured at the mature heading stage

showed a consistent change for all three mutants tested. For example, C<sub>2</sub>H<sub>4</sub> inhibition of hypocotyl elongation and C<sub>2</sub>H<sub>4</sub> induction of PAL activity were both lowest in line 243, but lines 242 and 244 had much lower rates of chlorophyll loss, and line 244 had the lowest increase in CO<sub>2</sub> production while the other mutants were similar to the wild-type progenitor.

Wound-induced changes exhibited the same variability, but the differences between the parent and mutated lines were smaller than observed for C<sub>2</sub>H<sub>4</sub>-induced changes. For example, there was a five-fold difference in the C<sub>2</sub>H<sub>4</sub>-induced increase in CO<sub>2</sub> production among the lines, but only a 60% difference in wound-induced CO<sub>2</sub> production. Likewise, there was a 19% difference in wound-induced PAL activity among all the lines, while there was a 170% difference in C<sub>2</sub>H<sub>4</sub>-induced PAL activity. Selecting for C<sub>2</sub>H<sub>4</sub> insensitivity at the seedling stage did not produce mature plants that were consistently less sensitive to C<sub>2</sub>H<sub>4</sub>-induced increases in PAL activity or CO<sub>2</sub> production. However, all the selected mutated plants did have lower rates of C<sub>2</sub>H<sub>4</sub>-induced chlorophyll loss, yet reduction in chlorophyll loss in line 242 was accompanied by no change in C<sub>2</sub>H<sub>4</sub>-induced PAL activity, while C<sub>2</sub>H<sub>4</sub>-induced PAL activity in line 244 was significantly higher than control.

A number of studies have shown that the response of plant tissue to a variety of stresses (e.g. chilling, C<sub>2</sub>H<sub>4</sub>) varies with the tissue and its stage of development. For example, selecting the fastest germinating tomato seeds under low temperature stress (11 ± 0.5 °C) was highly effective and significantly improved the germination performance of the progeny (Foolad and Lin, 1998). Selection for rapid germination under one stress (e.g. cold or salt) also improved germination under other stresses as well as in non-stress environments (Foolad et al., 1999). However, using the rate of tomato seed germination under chilling stress as the criteria for selecting chilling tolerant lines did not produce mature chilling tolerant plants (Foolad and Lin, 2000).

Mutational analysis of C<sub>2</sub>H<sub>4</sub> sensitivity in other plant species, particularly *Arabidopsis*, has identified multiple independent genes determining this phenotype (Stepanova and Ecker, 2000). At least

five families of genes and more than a dozen loci have been identified in the C<sub>2</sub>H<sub>4</sub>-signaling pathway. Clearly our identification of three independent mutants in lettuce has not saturated the pathway and other mutants and phenotypes are possible. Different members of the gene families show different patterns of expression (Johnson and Ecker, 1998). Therefore, it is possible that further screening of additional lettuce mutants may identify lines with different horticultural characteristics.

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