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Characteristics of transgenic tomatoes antisensed for the ethylene receptor genes *LeETR1* and *LeETR2*[*]

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Abstract: Two stable transformed lines containing antisense *LeETR1* or *LeETR2* sequences and their hybridized line were investigated to determine the effect of *LeETR1* and *LeETR2* specificity in the ethylene receptor family in tomato (*Lycopersicon esculentum* Mill.) on ethylene signaling. The transgenic line *ale1* containing antisense *LeETR1* displayed shorter length of seedling grown in the dark and adult plant in the light, severe epinastic petiole, and accelerated abscission of petiole explant and senescence of flower explant, compared with its wild type B1. The transgenic line *ale2* containing antisense *LeETR2* also exhibited shorter hypocotyls and slightly accelerated abscission. The phenotypes of cross line *dale* of *LeETR1* and *LeETR2* were close to *ale1* in many aspects. These results suggested that *LeETR1* probably plays a relatively important role in ethylene signaling of tomato growth and development.

Key words: Antisense transformation, Ethylene receptor, Ethylene response, Tomato
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INTRODUCTION

Ethylene is a gaseous plant hormone affecting many developmental processes and fitness responses, including germination, flower and leaf senescence, fruit ripening, leaf abscission, root nodulation, programmed cell death, and responsiveness to stress and pathogen attack (Bleecker and Kende, 2000; Johnson and Ecker, 1998).

According to the negatively regulating model, ethylene receptors combining with CTR1 negatively regulate ethylene response. Triple or quadruple loss-of-function mutants display a constitutive ethylene response phenotype (Bleecker and Kende, 2000; Chang and Stadler, 2001). It was reported that single or double loss-of-function receptor mutants displayed ethylene response phenotypes (Hua and Meyerowitz, 1998). Carefully analysis of receptor null mutants revealed that loss of even one ethylene receptor, specifically *ETR1*, results in a significant increase in ethylene responsiveness in *Arabidopsis* (Cancel and Larsen, 2002).

In tomato, the ethylene receptor gene family presently consists of six members *LeETR1~LeETR6* (Wilkinson *et al*., 1995; Zhou *et al*., 1996a; 1996b; Lashbrook *et al*., 1998; Tieman and Klee, 1999; Klee and Tieman, 2002). The proteins encoded by these genes are structurally diverse and at worst, are less than 50% identical. Each receptor gene has a distinct pattern of expression throughout development and in response to external stimuli. *LeETR1* and *LeETR2* are constitutively expressed in all tissues throughout development with *LeETR1* expression about 5-fold that of *LeETR2*. In contrast, expression patterns of the other four genes are highly regulated. *NR* (*never-ripe*) is constitutively expressed, but highly expressed in pedicel abscission zone and during fruit development (Wilkinson *et al*., 1995; Lashbrook *et al*., 1998). As

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fruits approach maturity, expression of several receptors increases. *NR*, *LeETR4* and *LeETR5* are induced by fruit ripening, organ senescence and abscission and pathogen infection, and are at a lower level in vegetative tissue (Tieman *et al*., 2000; Ciardi *et al*., 2000; Tieman and Klee, 1999). The pathogen inducibility of *LeETR4* is associated with increased ethylene synthesis following infection (Ciardi *et al*., 2001).

Loss of *LeETR4* results in dramatic morphological changes associated with increased ethylene responsiveness. Strongly epinastic petiole, accelerated senescence of flower and delayed ripening of fruit were observed in the transgenic tomato mutant resulting from transcript reduction of *LeETR4* (Tieman *et al*., 2000). Some expected and unexpected phenotypes related to ethylene response, such as shorter internodes and delayed abscission, resulted from the ethylene receptor gene *LeETR1* transgenic tomato (Whitelaw *et al*., 2002) and reduced expression of *LeETR1* in tomato.

The increased ethylene responses of *ETR1* loss-of-function mutant in *Arabidopsis* indicated that *ETR1* may play more prominent role than the other receptors in ethylene signaling. However, previous result of *LeETR1* antisense expression is in part against the negative regulation model of ethylene receptors in ethylene signaling, such as delayed abscission (Whitelaw *et al*., 2002), which needs to be explained by more research information. The amino acid sequences of LeETR1 and LeETR2 protein are most closely related to ETR1 of *Arabidopsis* and are highly similar to each other (Zhou *et al*., 1996a; 1996b; Lashbrook *et al*., 1998). They constitutively express in all tomato tissues throughout development (Lashbrook *et al*., 1998). But their functional significance in ethylene receptor family is still unclear. Two transgenic lines of tomato, *ale1* and *ale2*, were prepared by introducing the antisense copy of *LeETR1* or *LeETR2* separately. A crossed line *dale* was obtained by crossing the *ale1* and *ale2*.

MATERIALS AND METHEODS

The regions from 2351~2664 bp of *LeETR1* (GeneBank, U41103) and 2125~2390 bp of *LeETR2* (GeneBank, AF043085) genes were separately cloned into vector PCR®2.1 with *Bam*HI and *Xba*I. The cloned sequence was then reversely subcloned into the intermedium expressional vector pPZP111 between the sites of *Hin*dIII and *Eco*RI. The antisense sequence containing cauliflower mosaic virus (CaMV) 35S promoter, *NPT*II and *GUS* gene at the pPZP111 (Hajdukiewicz *et al*., 1994) was transferred into tomato (*Lycopersicon esculentum* Mill.) line B1 through *Agrobacterium tumefaciens* LAB4404 (Zheng *et al*., 2001). Two transformed lines, *ale1* and *ale2*, respectively containing antisense copy of 3′-untranslated portion of *LeETR1* or *LeETR2* were obtained through three generations screen by kanamycin resistance, PCR (polymerase chain reaction) and GUS (β-glucuronidase gene) assay. The line *dale* was from the hybridization of *ale1* with *ale2*.

The T4 of *ale1* and *ale2*, T0 of *dale* and wild line were grown in a plastic canopy, every plant in each line was assayed for the marked gene *NPT*II by PCR (Klimyuk *et al*., 1993) with 5′-CCA CCA TGA TAT TCG GCA AG-3′ (AF234316: bases 9168~9187) as the left primer and 5′-GTG GAG AGG CTA TTC GGC TA-3′ (AF234316: bases 9717~9698) as the right primer (Wang *et al*., 1993).

For the etiolated seedlings experiment, seeds were surface-sterilized with 10% bleach then stored at 4 °C for 4 d in the dark to synchronize germination. Seeds were then sown in plant nutrient agar medium in vials with 0.5% (w/v) Suc, 0.5% (w/v) agar and other nutrient elements (Lincoln *et al*., 1992). Seedlings were germinated for 5 d in the dark in air with or without 10 μl/L ethylene at 24 °C. Lengths and diameters of hypocotyls were measured. Thirty seeds were in each vial with three vials used for each line. Ten plants in each vial and a total of thirty in each line were measured.

Leaf abscission zone explants used for the experiment consisted of a stem segment with one petiole still attached and leaflets removed. Flower explants for the senescence experiment contained a single freshly opened flower and its pedicel. Stem sect or flower pedicel base were inserted into 0.8% sterilized agar contained in a sealed 250 ml-bottle with a cap embedding a rubber plug in the center. A pre-determined quantity of ethylene, necessary to achieve a concentration of 10 μl/L was injected into the sealed bottle through the rubber plug. Fifteen samples were in each bottle and three bottles were

used for each line. The numbers of abscission or senescence samples were recorded at certain time in each bottle.

About 10~15 adult plants in each line were randomly selected for the determination of stem length and diameter.

RESULTS

Transgenic lines were ascertained by PCR and Southern-blot analysis

Each plant of *ale1*, *ale2*, and *dale* were further identified for the presence of *NPT*II gene by PCR. Positive results were obtained from all assayed plants, which confirmed the homozygosity of the antisense transgenic lines (Fig.1)

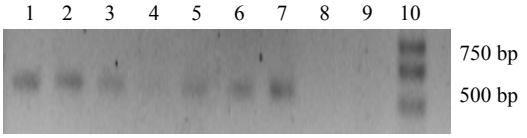

Fig.1 The *NPT*II gene in transgenic lines was ascertained by PCR amplifying 553 bp fragment
1~3: *ale1*; 5~6: *ale2*; 4, 8~9: B1; 10: Marker

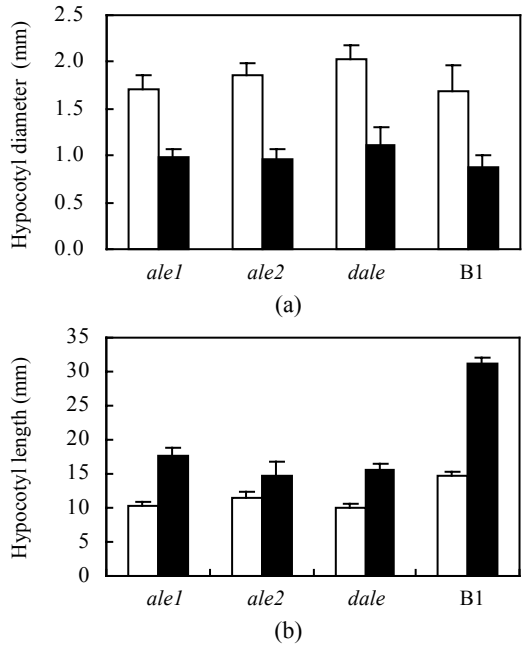

Fig.2 Dark-grown transgenic seedlings either in air (black column) or in ethylene (white column) have thicker (a) and shorter (b) hypocotyl compared with their wild type. Mean±*SE* values were determined from 30 seedlings

Length of etiolated seedlings and adult plant

All hypocotyls of etiolated seedlings of *ale1*, *ale2* and *dale*, exposed to air either or not containing 10 μl/L of ethylene, were shorter and thicker than those of their wild type B1 (Fig.2). All of the three positive transgenic lines were shortened by at least 43% compared with wild type.

Adult plants of *ale1*, *ale2* and *dale* were also shortened and thickened to different extents (Fig.3). *ale1* displayed the shortest length and was shortened by 36.4% in relation to that of B1; *ale2* and *dale* were also shortened to some degree. The diameter of *ale1* and *dale* stems increased by 19.2% and 20.8% respectively compared with B1.

Abscission of petiole explants

The abscission of petiole explants of *ale1*, *ale2* and *dale* were significantly accelerated, not only in 10 μl/L ethylene but also in air (Fig.4a). The abscission rates of all transgenic lines rapidly increased within the first 24 h and were much higher than those of contrast line B1 throughout the investigating period. *ale1* and *dale* showed significantly higher abscission level than *ale2* did during the period of investigation.

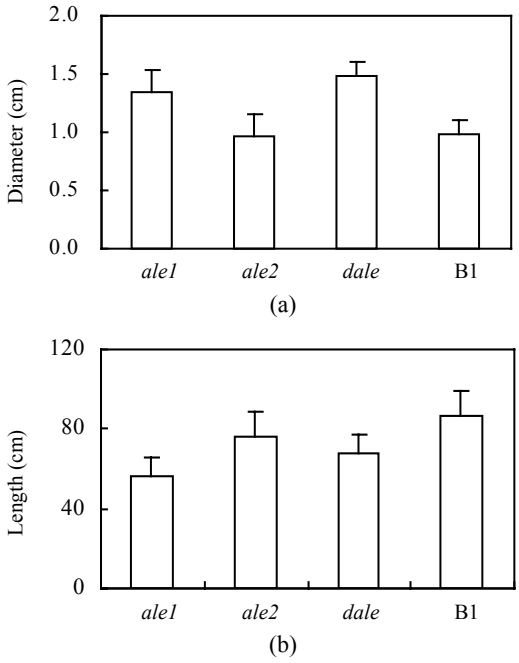

Fig.3 Stem diameter (a) and length (b) were measured from adult plants. Mean±*SE* values were determined from 10~15 plants of each lines

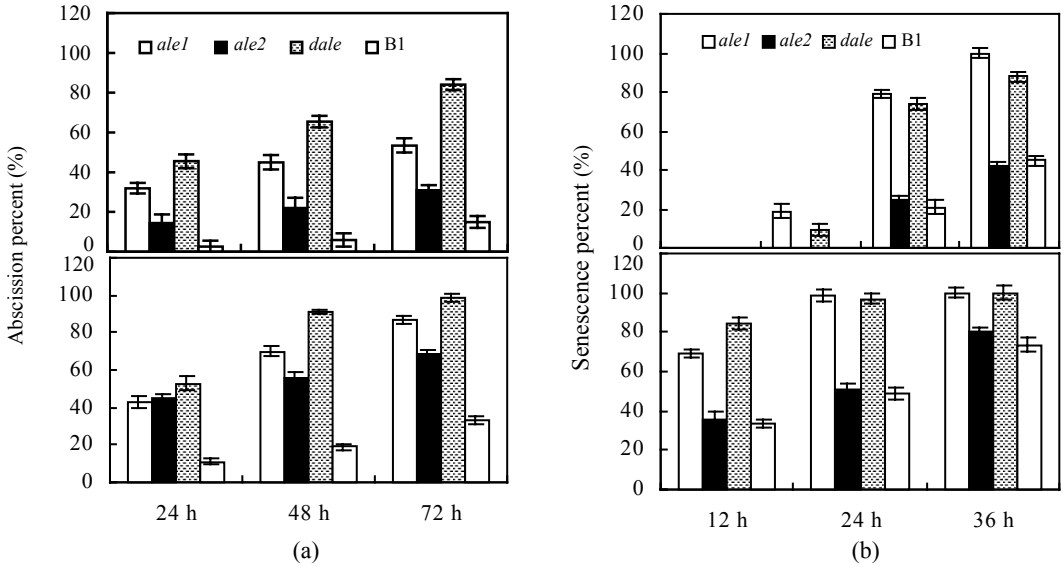

Fig.4 The abscission percent of petiole (a) and the senescence percent of flower (b) exposed to air (upper) and ethylene (lower). Error bars mean±*SE* values

Senescence of flowers

Accelerated senescences of flower explants of *ale1* and *dale* were observed when they were exposed to air or ethylene (Fig.4b). All *ale1* flower explants senesced within 48 h when exposed to air without ethylene and with 24 h when exposed to ethylene. The samples of *ale1* and *dale* displayed much higher senescence rate than other lines did. The result suggested that both sensitivity and response to ethylene of *ale1* and *dale* flower senescence might be increased.

DISCUSSION

According to the negative regulating model, single or double loss-of-function ethylene receptor mutants showed no significant ethylene response phenotype. Triple or quadruple loss-of-function mutants displayed a constitutive ethylene response phenotype. However the loss-of-function mutants in the ethylene receptor *ETR1* caused enhanced sensitivity and exaggerated response to ethylene in *Arabidopsis*. An apparent growth defect resulted from enhanced response to ethylene, and increased sensitivity was also observed (Cancel and Larsen, 2002). Some expected and unexpected phenotypes in relation to ethylene response, such as shorter internodes and delayed abscission, resulted from the ethylene receptor *LeETR1* transgenic tomato (Whitelaw *et al*., 2002). Strongly epinastic petioles, accelerated senescence of flowers and delayed ripening of fruit were observed in the transgenic tomato mutant of transcript reduction of *LeETR4* (Tieman *et al*., 2000).

In this study, more typical phenotypes in relation to ethylene response, such as the shortened and thickened hypocotyls of etiolated seedlings and adult plants, and accelerated abscission of petioles and senescence of flowers, were observed in the transgenic lines *ale1*, *ale2* and their crossed lines *dale*. These phenotypes suggest that either sensitivity or response to ethylene is enhanced in these transgenic lines.

LeETR1 and *LeETR2* in tomato are highly homologous. The *ale1* and *ale2* displayed some similar ethylene response phenotypes, but *ale1* showed more intensive ethylene response in many aspects than the *ale2* did. The characteristics of *dale* were more close to those of *ale1*. From the significant effects, especially accelerated abscission resulting from *LeETR1* transgenic expression, it is supposed that *LeETR1* possibly plays more important role on the ethylene signaling pathway *LeETR* family.

It may also be inferred that each member in *LeETR* family has some specific functions and is not totally redundant from this study and previous reports (Tieman *et al*., 2000; Whitelaw *et al*., 2002). There may be special or temporal specific function for different receptor.

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