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Characteristics of fruit ripening in tomato mutant *epi*[*]

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Abstract: The characteristics of fruit ripening and expression of ripening-related genes were investigated in *epi*, an ethylene overproduction mutant of tomato (*Lycopersicon esculentum* Mill.). The *epi* produces apparently more ethylene than its wild type VFN8 at every stage of vegetative and fruit growth and ripening; compared to VFN8, the *epi* fruit showed higher CO_2 evolution, faster descending of chlorophyll, slightly quicker increase of carotenoid and lycopene, and faster reduction in pericarp firmness during maturation and ripening; and the mRNAs of three ripening-related genes including *E8*, *pTOM5* and *pTOM6* were at higher levels in *epi*. The ripening-related characteristics changing of the fruit are consistent with the increase of ethylene production and ripening-related genes expression. These results suggest that *epi* mutation possibly did not affect the ethylene perception and signaling during fruit ripening, and that the modified characteristics of fruit ripening possibly resulted from the ethylene overproduction and increased expression of ripening-related genes.

Key words: *Epinastic* (*epi*) mutant, Ethylene overproduction, Ethylene signaling, Fruit ripening
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INTRODUCTION

As a gaseous phytohormone, ethylene plays an important role in plant growth and development. Ethylene can alter plant physiology and morphology due to its effect of regulating gene expression (Moctezuma *et al.*, 2003). Such regulation apparently depends on the effect of the normal ability of the plant tissues on ethylene perception and signal transduction. Impairment of the perception and signal transduction pathway(s) would thus ultimately lead to alterations in the plant's development (Guo and Ecker, 2004).

Epinastic (*epi*) is a natural mutant from the wild type of VFN8 tomato (Ursin, 1987). It is the first natural tomato mutant overproducing ethylene with strong epinastic growth of petiole. Its vegetative tissues produce ethylene several times that of its wild parent VFN8 (Fujino *et al*., 1988; Ying and Zeng, 1999; Wang and Ying, 2004). Incomplete triple response was observed in etiolated seedlings of *epi* with the inhibited apical hook formation (Wang and Ying, 2004). Inhibitors of ethylene synthesis and action were unable to rescue its phenotypes of vegetative growth (Fujino *et al*., 1989; Ursin and Bradford, 1989), leading to the suggestion that the mutation may result in a blockage of ethylene signaling pathway and constitutive ethylene response. This hypothesis is supported by the similarity in the *epi* seedling phenotype to that of the Arabidopsis *ctr1* mutant seedling whose phenotype also did not revert when grown on ethylene inhibitors (Kieber *et al*., 1993). Apart from epinasty, the *epi* phenotype is characterized by dark green leaves, thickened and shortened stems, apparent reduction in anthocyanin production, a shortened and highly branched root system, and a very erect and compact growth habit (Fujino *et al*., 1988; Wang and Ying, 2004). Barry *et*

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al.(2001) obtained a double mutant (*epi*/*epi*; *Nr*/*Nr*) containing both *epi* and *Nr* (never ripe) genes, and found that the double mutant has the same dark-grown seedling and vegetative phenotypes as *epi* but possesses the senescence and characteristics of never ripening. No significant differences were observed between *epi* and VFN8 in many aspects, including the leaf and petal senescence or abscission, the rate of fruit ripening and the time from anthesis to the onset of fruit ripening (Barry *et al*., 2001).

E8, a function unclear gene, expression is regulated during fruit ripening both by ethylene, and by ethylene-independent ripening signals (Deikman *et al*., 1992). *pTOM5* and *pTOM6* were confirmed to encode phytoene synthetase and polygalacturonase (PG) respectively, with both being ethylene-regulated (Bird *et al*., 1991; Grierson *et al*., 1986). Small increases in the transcript abundance of the three ethylene-regulated genes were observed in *epi* fruit compared to those in VFN8 throughout development and ripening (Barry *et al*., 2001).

However, we observed apparent delayed senescence of leaves and petals, accelerated petiole abscission in *epi* compared to its wild type (Wang and Ying, 2004). Here we aim to illustrate the effects of *epi* mutation on fruit ripening through the difference of ripening characteristics and ripening-related genes expression between *epi* and wild type VFN8.

MATERIALS AND METHEHODS

Fruit growth and ripening

The *epi* mutant and its wild-type VFN8 were grown in greenhouse conditions. Flowers were tagged at anthesis and the number of fruit was restricted to less than four per cluster. The fruits displaying first sign of colour change were identified as BK (breaker) stage. Average days from anthesis to BK were counted from 30 fruits. The fruits at 20 days before BK was marked as IM (immature green) and 5 d before BK as MG (mature green); The fruits on 3, 5, 7, 10 d after BK were noted as BK1, BK3, BK5, BK7, BK10 respectively. Each item of the measurements was conducted with fresh harvested fruits at every stage except for firmness determination. For RNA extraction the fruit pericarp were cut into 1 cm^2 piece, frozen in liquid nitrogen, and stored at –80 °C.

Measurements of respiration and ethylene production

For CO_2 determination, an infrared gas analyzer in A CIRAS-1 intellectual photosynthesis parameter analyzer (PP Systems Ltd., UK) and opened gas flow system with 163 ml/min gas flow rate was employed. At least five fruit were individually determined for each ripening stage at 20 °C.

For ethylene determination, at least five single fruits were sealed in an air-tight jar for 1 h at 20 °C, and 1 ml of headspace gas was injected into a gas chromatograph (Unicam 610) equipped with a flame ionization detector (FIA-21) and an activated alumina column kept at 90 °C, using nitrogen as the carrier gas. 10.44 μl/L of ethylene was used as a standard.

Determination of chlorophyll, carotenoid and lycopene in fruit

Cut each fruit into six pieces along the vertical axis. Three interval pieces were cut into very small pieces and mix well. Grind two grams fresh weight fruit slices in precooled mortar with 5 ml hexane and acetone (60:40) and small amount of acid washed sand. Transfer the upper organic layer into a capped tube on ice. Re-extract the remaining aqueous layer with 5 ml of the same solvent repeatedly and transfer the organic layer to the same tube until the aqueous layer becomes colorless. Take 1 ml from the total volume of the organic extract for determining the absorbance at 450 nm, 502 nm, 643 nm and 663 nm respectively (OD_{450}, OD_{502}, OD_{645} and OD_{663} respectively) on spectrophotometer (Tomes, 1963; Kirk, 1968; Davies, 1976). Calculate the amount of each pigment in 1 ml sample with the following equations.

$$\text{Chlorophyll } (\mu g/ml)=(20.2\times OD_{645})+(8.2\times OD_{663})$$

$$\text{Carotenoid } (\mu g/ml)=4\times OD_{450}$$

$$\text{Lycopene } (\mu g/ml)=3.12\times OD_{502}$$

Measurement of fruit firmness

Thirty fruit at the same full-red stage without softening were harvested from each variety and stored at 20 °C, 85% RH (relative humidity) for 15 d. Five fruit were randomly selected for the measurement on day 0, 5, 10, 15 of storage using M series texture analyzer (J.J. Lloyd Instrument, Canada) as described by Jackman *et al.*(1990). Ten mm×10 mm pericarp samples with epidermal tissue intact were excised

from the equatorial region of each tomato fruit and placed exocarp down on a steel plate and compressed with a 500-N load cell in conjunction with an 8-mm flat-ended cylindrical probe at a deformation speed of 5 mm/min. The bioyield force (F_m) (force to rupture) and deformation up to bioyield point (L_m) were obtained from force-deformation profiles. The firmness values were calculated as F_m/L_m.

RNA isolation and RNase protection assay (RPA)

Isolation of total RNA from tomato fruit was done according to Lashbrook *et al*.(1994). Template sequences for *E8* gene probe transcription were synthesized by PCR from *E8* gene cDNA (GenBank, No. X13437) as reported by Lincoln *et al*.(1987). Left and right Primers were 5′-TAGGAAAGCCCTAGAGTTG and 5′-TTAGATCTTGTAACGGGAC. Amplified 1092 bp fragments were inserted into vector PCR®2.1 with T7 RNA polymerase promoter. The templates for *pTOM5* (encoding phytoene) and *pTOM6* (encoding polygalacturonase) genes probe transcription were provided by BBSRC (University of Nottingham, UK). The plasmid containing cDNA templates was linearized with appropriate restriction enzymes. RNA probes were synthesized with the linearized plasmid templates, α-^{32}P labelled UTP and in vitro transcript system with T7 RNA polymerase (Amersham). Rnase ONE™ (Promega) was used for RPA performed according to the manufacturer's procedure.

Ten microgram of total RNA extracted from pericarp of each sample was employed for RPA. The band with the highest radioactive signal from each crossed probe was separately designated as a standard of 100% of each gene; Relative percentages of signal on the other bands were obtained by comparison with the standard of the same probe and calculated by Quantity One software (Bio-Rad).

RESULTS

Respiration and ethylene production

Before BK stage, there was no distinct difference of CO_2 production between *epi* and VFN8 fruits. Once the fruit came into ripening stage, CO_2 production of *epi* was constantly higher than that of VFN8 (Fig.1a). 50.9% more CO_2 production at peak stage was observed in *epi*. However, no advance of climacteric peak was observed in *epi* compared with its wild type VFN8.

At every stage of fruit growth from IM to BK10, endogenous ethylene production in *epi* was always higher than that in VFN8 (Fig.1b). Peak level of ethylene production in both varieties was synchronized, occurring at 5 d after BK in consistence with CO_2 production. At the peak stage, the *epi* fruit produced ethylene was two twice that of VFN8. This result integrating previous results further demonstrates that *epi* mutation overproduces ethylene throughout vegetative growth, fruit development and ripening (Wang and Ying, 2004).

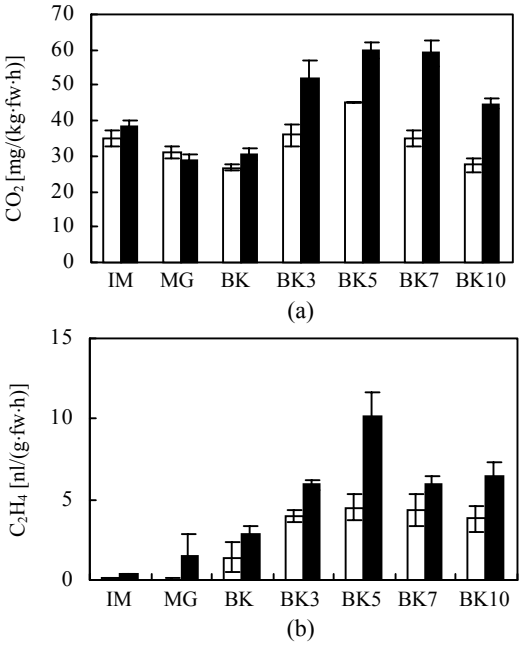

Fig.1 The production of CO_2 (a) and C_2H_4 (b) during fruit growth and ripening of *epi* (black bars) and VFN8 (white bars). Mean±*SE* values were determined for five fresh harvested fruit

Similar trends of ethylene and CO_2 production during fruit development and ripening and a synchronized respiration peak between the two varieties confirmed that ethylene can intensify the ripening response and accelerate the ripening process, but can not trigger the ripening of immature fruit.

Transformation of pigments in fruits

The transformation of main color components in fruit including chlorophyll, carotenoid, and lycopene conformed to the normal trend during development

and ripening of *epi* and VFN8 fruit. The decrease pattern of chlorophyll and increase pattern of carotenoid and lycopene in *epi* were not significantly different from those of its wild type. The amount of carotenoid and lycopene at every ripening stage was just slightly higher in *epi* than in VFN8 (Fig.2). However, higher level of chlorophyll in IM fruit of VFN8 decreased at higher rate post MG stage; and carotenoid and lycopene accumulated to higher levels at the last stage investigated in *epi* than in its wild type.

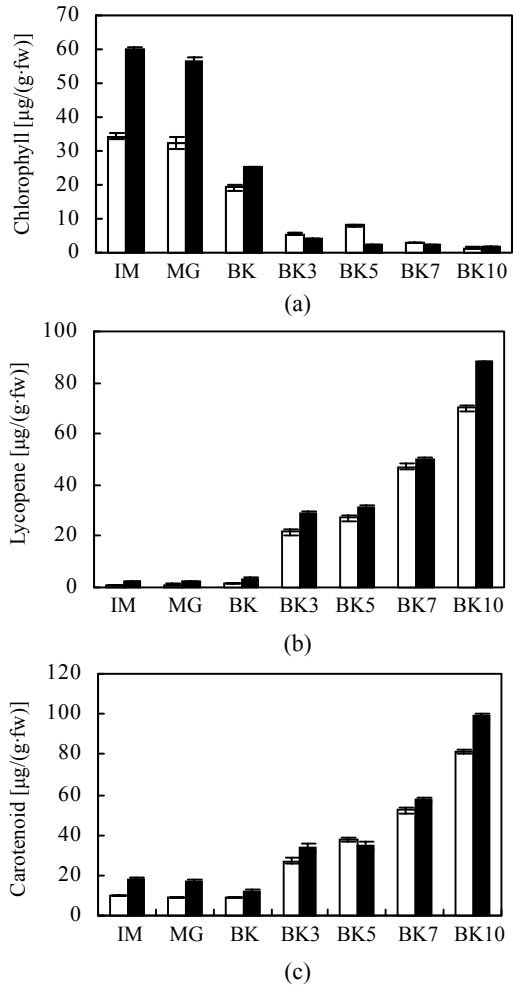

Fig.2 The production of chlorophyll (a), lycopene (b), carotenoid (c) in *epi* (black column) and VFN8 (white column) during fruit ripening. Mean±*SE* values were determined for five fresh harvested fruit

Decreasing of firmness

The faster (compared to VFN8) decrease of the bioyield point of full-red *epi* fruit to a lower level during storage at 20 °C indicated that the firmness of *epi* fruit decreased more rapidly. If decrease by 50% of bioyield point was designated as the end of standard shelf life, the shelf life of *epi* was 4.5 d, which is less than half that of VFN8 (more than 10 d) (Fig.3). The rate of firmness drop significantly demonstrated that the accelerated ripening of *epi* fruit was caused by the mutation.

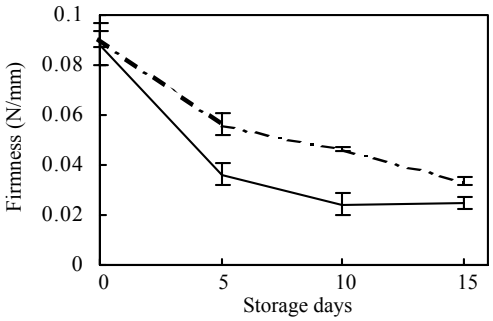

Fig.3 The firmness of *epi* (solid line) and VFN8 (broken line) fruits during 15 d of ripening at 22 °C. Mean±*SE* values were determined for five stored fruit

Expression of ripening-related gene

The expression of *E8*, *pTOM5* and *pTOM6* genes at mRNA level in *epi* and VFN8 showed similar pattern of rapid rise to a peak level from that before BK and then descending during fruit ripening. However, the timing of the peak level of mRNAs for each gene was not identical. The expression peaks of *E8* and *pTOM6* genes in *epi* and VFN8 were not synchronized while those of *pTOM5* in the two varieties were synchronized. Three genes' expressions at mRNA level in *epi* were all more than those in VFN8 at every ripening stage except IM and MG (Fig.4). The expression pattern of fruit ripening-related genes changes with fruit development and ripening and that of different genotypes are very consistent with the ethylene production (Fig.1b).

DISCUSSION

Indicative phenotypes of a whole-plant response to ethylene have previously been reported in tomato. For example, transgenic plants constitutively expressing high levels of the ACC synthase gene, *LeACS2*, produce elevated levels of ethylene, resulting in leaf epinasty and rapid senescence and abscission of flowers (Lee *et al*., 1997). Similarly, antisense inhibition of the ethylene receptor *LeETR4* results in a

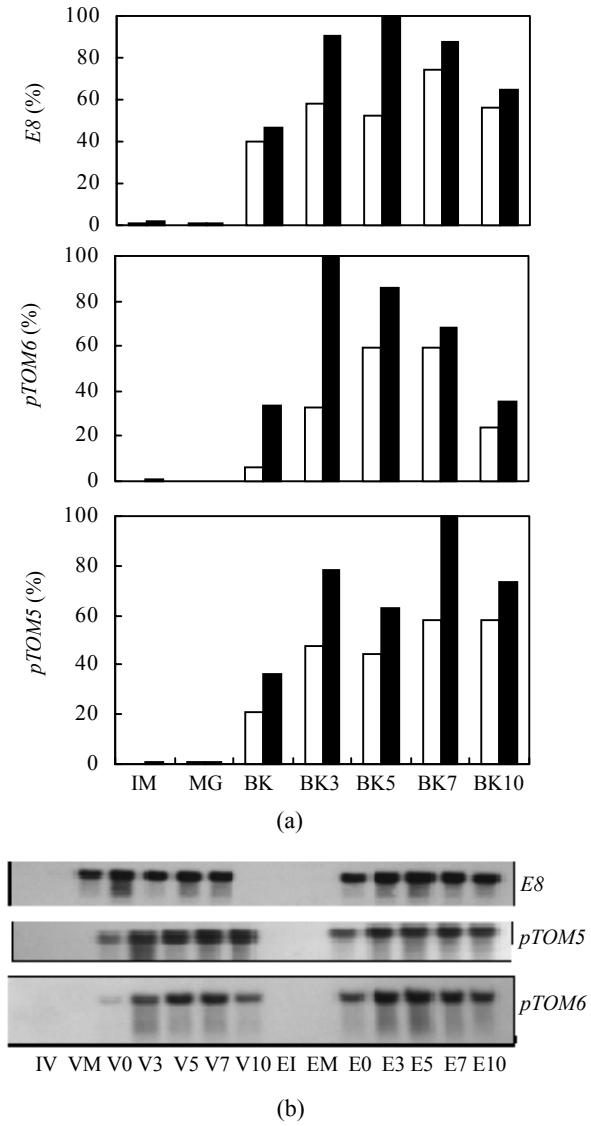

Fig.4 The expression of *E8*, *pTOM5* and *pTOM6* genes during VFN8 (white) and *epi* (black) fruit development and ripening (a) Relative percentage of expression (%); (b) Picture of hybridized bands exposure to X-film (V: VFN8; E: *epi*; I: Immature; M: Mature green; Number after V or E means days after BK stage)

constitutive ethylene response phenotype that includes leaf epinasty, premature senescence, abscission of flowers, and early fruit ripening (Tieman *et al*., 2000). However, the phenotype of *epi* differs from that of other ethylene-related mutants in that only a subset of ethylene responses appears to be modified.

In this study we found that some ripening-related processes of *epi* fruit were accelerated in contrast to its wild type VFN8. Climacteric peak represented by ethylene and carbon dioxide production wasn't advanced, but was significantly heightened (Fig.1). Coloration indicated by carotenoid and lycopene were speeded up (Fig.2) and firmness dropping represented by bioyield point (Fig.3) were notably accelerated. These changing trends were well consistent with the increase of ethylene production (Fig.1b). Furthermore, expression of some ripening-related genes, which were typically ethylene-regulated, were also enhanced (Fig.4). The expression peaks of *E8* and *pTOM6* genes in *epi* were advanced and *pTOM5* expression of the two varieties synchronized. The earlier expression of *pTOM6* gene is well correlated with the advance dropping of firmness. The lycopene and carotenoid accumulated to a higher level in *epi* than that in VFN8 until BK10, the last stage investigated, which is possibly related to the expression time of *pTOM5* in *epi*. The synthesis of lycopene and carotenoid possibly lag behind the expression of their encoding genes.

Two possible causes are possibly responsible for the accelerated ripening characteristics of *epi* fruit. One is the increased endogenous ethylene production enhancing the ethylene response. Another is impaired ethylene signaling branches leading to constitutive ethylene response.

Our previous study revealed that leaf senescence was significantly delayed, and apical hook formation in dark grown seedlings was partially inhibited (Wang and Ying, 2004). These phenotypes are not consistent with normal ethylene response caused by increased ethylene, which suggests a reduced ability of ethylene signaling. Some sub-branches in ethylene signal transduction pathways in these vegetative tissues were possibly blocked by the *epi* mutation. However, the *epi* fruit ripening was not delayed but accelerated to some degree, which indicates at least that the ability of ethylene perception and signaling was not affected by the mutation.

Accumulated evidences suggested that the *epi* mutation possibly produced pleiotropic effects on tomato responses to ethylene. Some were constitutively activated, some were inhibited, and some were not affected. So that several subsets of ethylene response pathway might exist throughout the tomato vegetative growth and fruit development and ripening, and different subsets of ethylene responses may be regulated through different mechanisms. However, more work is needed to elucidate the hypothesis.

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