Front. Agric. China 2009, 3(1): 55–59
DOI 10.1007/s11703-009-0017-y

RESEARCH ARTICLE

Chunli SONG, Junlian MA, Xia TANG, Zide ZHANG, Pingping ZHOU, Zhixia HOU

Cloning of strawberry *FaEtr2* gene and its plant expression vector construction for antisense RNA

© Higher Education Press and Springer-Verlag 2009

Abstract An ethylene receptor *FaEtr2* gene was amplified by polymerase chain reaction (PCR) from ripening strawberry fruit. A 1049-bp PCR product (All Star-*Etr*2) was cloned. Sequence analysis showed that the All Star-*Etr2* nucleotide sequence had 100% identity with Chandler-*Etr2* from the GenBank. A pair of primers containing restriction enzyme sites were designed and used to amplify the sequenced plasmid. The PCR product was digested by the corresponding restricted enzymes and inserted between the CaMV 35S promoter and NOS terminator of expression vector pBI121 directionally. The constructed expression vector was transformed into *Agrobacterium fumefeciens* LBA4404 in the follow-up research to silence a ripening-related ethylene receptor *FaEtr2* gene in strawberry fruits.

Keywords strawberry, ethylene receptor, *FaEtr2* gene, plant expression vector

1 Introduction

Cultivated strawberry (*Fragaria ananassa* Duch.) is a highly-valued fruit crop species. Strawberry fruits are appreciated for their unique flavor and nutritious quality, but the fleshy fruits becoming less firm with progressive ripening are liable to post-harvest deterioration that impairs their preservation, resulting in great losses. Therefore, efforts have long been made to understand the regulatory mechanisms of fleshy fruit ripening. Dihydroflavnol 4-reductase and pectate lyase gene, two transcripts previously shown to be associated with anthocyanin synthesis and fruit firmness, respectively, were identified in a subtractive library from ripening fruit (Medina-Escobar et al., 1998). Jimenez-Bermudez et al. (2002) transformed strawberry with antisense pectate lyase constructs driven by the CaMV 35S promoter. Antisense plants with demonstrated repression of pectate lyase gene expression exhibited the decreased fruit set, yet there were no differences in general fruit qualities. A gene encoding cystathionine γ-synthesis, an enzyme required for methionine synthesis, was also characterized in developing strawberry fruits. The gene was identified via its abundant transcript in *F. vesca* fruit (Marty et al., 2000). This report indicated that the transcript and protein levels were high in ripening fruits and that levels were influenced by auxin application. The unusual correlation of accumulation of this protein led the authors to posit that it may be important in the ripening process. A cell with a gene encoding an endo-1, 4-β-glucanase was also cloned from ripening fruit (Harpster et al., 1998).The corresponding transcript was present only in ripening fruit, with increasing steady-state levels as the tissue progressed towards reddening and ripening. The expression of two other members of the endo-1, 4-β-glucanase gene family (*FaEG1* and *FaEG3*) was studied, indicating that the transcripts were both present during ripening (Trainotti et al., 1999). Both genes were used to produce antisense plants, yet neither had an effect on fruit softening (Wooley et al., 2001).

Despite the conspicuous influence of achenes and auxin on strawberry fruit ripening and coincident gene expression, little is known about the role of ethylene in the strawberry ripening process. Although strawberry is a non-climacteric fruit, ethylene is quite abundant in green strawberry fruit (Knee et al., 1977). Two cDNAs coding for enzymes of the ethylene biosynthetic pathway (i.e. *FaACO1* and *FaACO2*), and three cDNAs coding different ethylene receptors have been isolated recently. The

Received July 19, 2008; accepted September 17, 2008

Chunli SONG, Pingping ZHOU
College of Chinese Veterinary, Agricultural University of Hebei, Dingzhou 073000, China

Junlian MA, Xia TANG, Zide ZHANG (✉)
College of Food Science and Technology, Agricultural University of Hebei, Baoding 071001, China
E-mail: zhangzde@heinfo.net

Zhixia HOU
The Key Laboratory for Silviculture and Conservation of Ministry of Education, Beijing Forestry University, Beijing 100083, China

receptor *FaEtr2*, with a degenerate histidine-kinase domain belonging to type-II, (Cancel et al., 2002) is mostly expressed in ripening strawberries. Perhaps even the little ethylene produced by ripening strawberries might be sufficient to trigger ripening-related physiological responses (Trainotti et al., 2005). So a specific PCR product of *FaEtr2* was obtained and an antisense expression vector was constructed for further research on gene function to improve the varieties of strawberry through transgenic technology.

2 Materials and methods

2.1 Materials

TIAN gel Midi Purification Kit and pGEM-T cloning kit were products from TIANGEN BIOTECH (BEIJNG) CO., LTD., Taq DNA polymerase and T4 DNA ligase were purchased from SLE. Biology Tech. (Baoding) Co., Ltd., various restriction enzymes were purchased from TakaRa Biotechnology (Dalian) Co., Ltd., and *E. coli* DH5*α* and pBI121 plant expression vector were stored in our laboratory. Primer synthesis and sequencing were done by Beijing Sunbiotech Co., Ltd. *Agrobacterium* strain LBA4404 was provided by Dr. Wang Lei from the Institute of Biotechnology, Chinese Academy of Agricultural Sciences.

2.2 Methods

2.2.1 Cloning of *FaEtr2* gene

Specific primers (sense 5'-TGCTTACCCAAGAGAT-TCGC-3', antisense 5'-ATCATTCCTCCCCTGACTTCC-3') were designed according to the reported cDNA sequence of Chandler *FaEtr2*, and a partial sequence of *FaEtr2* was obtained. The PCR product was purified and ligated with pGEM-T vector, then transformed into *E. coli* DH5*α* competent cells. White colonies were screened in the medium with X-gal/IPTG. The plasmid was digested with *Eco*R I to select the correct recombinant plasmid. Bacterium liquid with recombinant plasmid was sent to Beijing Sunbiotech Co., Ltd. and sequenced. The sequencing results were blasted in NCBI and analyzed with Omiga software.

2.2.2 Amplification of reverse fragments

One pair of primers was designed according to the restriction enzyme sites of the amplified fragments and the pBI121 vector. The primers were 5'-TGGATC-CAGTGGTGATTCTGGTGAG-3' for sense (with *Bam*H I restriction site) and 5'-CCACTATCCTTGTCTAGAT-TATCCA-3' for antisense (with *Xba* I restriction site). Using a sequenced plasmid as template, the PCR product of the reverse fragment was obtained.

2.2.3 Construction of plant expression vector

The expression vector of pBI121 and the PCR product of the reverse fragments were double-digested with *Xba* I and *Bam*H I and linked (T4 DNA ligase 16°C for 16 h) directionally. The linked product was transformed into *E. coli* DH5*α* competent cells. Positive clones were screened on solid LB medium containing kanamycin (100 μg·mL^{-1}). A positive plasmid was identified by restriction enzyme digestion and the recombinant plasmid was recorded as pBI121-anti-*Etr2*.

2.2.4 Transformation of plant expression vector into agrobacterium

Plant expression vector was transformed into agrobacterium LBA4404 competent cells by the freeze-thaw method. Positive colonies were screened on solid YEB medium containing Kan (100 μg·mL^{-1}) and rif (50 μg·mL^{-1}). Using pBI121-anti-*Etr2* plasmid (positive control), non-transformed *Agrobacterium* liquid (negative control) and transformed *Agrobacterium* liquid as the template, transformation was verified by PCR amplification with specific primers.

3 Results and analysis

3.1 Cloning of *FaEtr2* gene

It was inferred that the PCR product should be about 1.0 kb, according to the sites of primers on the *FaEtr2* gene. Analysis of PCR products by 1% agarose gel electrophoresis showed that the fragment was about the same size as that expected (Fig. 1).

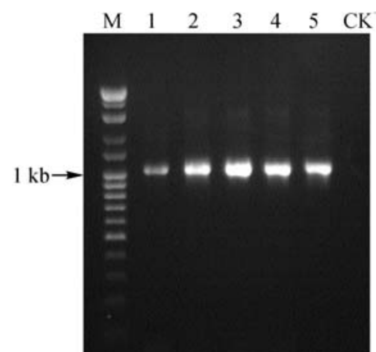

Fig. 1 Agarose gel electrophoretogram of PCR products
Note: M: 100 bp DNA ladder-3K marker; 1—5: PCR products; CK^{-}: negative comparison.

The purified fragment was linked with pGEM-T vector and transformed into the competent *E. coli* DH5*α*. The

recombinant fragment was obtained by blue-white spots screening. *Eco*R I was chosen to digest the recombinant plasmid according to the multi-cloning sites at both ends of the vector. If the recombinant plasmid contained an inserted fragment, it should be digested into a small fragment of about 1.0 kb and a 3.0 kb fragment of the T-vector. Digestion results as seen on agarose gel electrophoresis detection were consistent with expectations, which indicated that a DNA fragment of about 1.0 kb was inserted into the pGEM-T vector (Fig. 2).

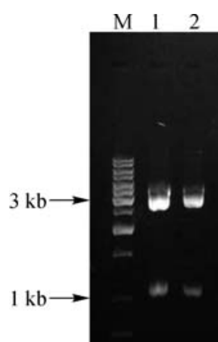

Fig. 2 Electrophoresis pattern of recombinant plasmid digested by *Eco*R I
Note: M: 1 kb ladder marker; 1, 2: recombinant plasmid digested by *Eco*R I.

Positive clones were sent to Beijing Sunbiotech Co., Ltd. and sequenced. The results showed that the sequencing PCR amplification product was 1049 bp (Fig. 3), containing the primer sequences and no intron sequences. Sequence analysis showed that the All Star–*Etr2* nucleotide sequence had 100% identity with Chandler-*Etr2* (AJ297513) in the GenBank.

3.2 Amplification of reverse fragment

We used the diluted sequencing plasmid as a template to amplify the reverse fragment. The amplified products should be 516 bp according to the site of the primers on the *FaEtr2* gene. The result seen on electrophoresis was consistent with expectations (Fig. 4).

3.3 Construction and identification of antisense expression vector

3.3.1 Double-digestion and purification of plant expression vector and PCR product

The PCR product and pBI121 plant expression vector were digested with *Xba* I and *Bam*H I. The sizes of the fragments did not change significantly, with only a few bases cut (Figs. 5, 6).

TGCTTACCCA	AGAGATTCGC	AAGTCCCTTG	ATAGACATAC	AATATTGTCC	50
ACAACCCTTT	TTGAGCTATC	TGAGACATTG	GGTTTGCAGT	ACTGTGCAGT	100
TTGGATGCCT	AATGAAATTA	AAACAGAGAT	GATCCTGACC	CATGAGTTGA	150
AAGGGAAGAA	CTATTCTAAT	ATGTACAACT	TTTCTATACC	AATTGGCGAT	200
CCAGATGTTG	TACTTATTAA	AGGGAGTGAT	GGGGTCAACA	TTCTTCGGCC	250
AGATTCAGCA	CTTGTATGTG	GAAGCAGTGG	TGATTCTGGT	GAGCCGGGAC	300
CAGTAGCTGC	AATTCGGATG	CCAATGCTTC	GGGTTTCCAA	TTTTAAAGGG	350
GGGACTCCTG	AGTTGATCCA	GACTTGTTAT	GCGATATTGG	TTCTGGTTCT	400
ACCTGGTGGA	GAGCCTAGAT	CTTGGAGCAG	TCAGGAACTT	GAGATAATTA	450
AGGTGGTTGC	TGACCAGGTG	GCTGTGGCCT	TATCCCATGC	TGCAATCCTT	500
GAAGAGTCCC	AACTTATGCG	GGAACAATTG	GCTGAGCAAA	ACCGGGCCTT	550
GCAACAGGCG	AAAATGAATG	CCATGATGGC	AAGCCATGCA	AGAAACTCAT	600
TCCAAAAGGT	GATGAGTGAT	GGGATGAGAA	GGCCAATGCA	TTCAGTATTG	650
GGGCTGCTTT	CCATGATGCA	GGATGAGAGT	TTGAATAATG	ATCAGCGAGT	700
TATTGTTGAT	GCAATGGTAA	GGACAAGCAA	TGTCTTATCG	ACGTTGATAA	750
ATGATGCTAT	GGATAATCCA	GCCAAGGATA	GTGGAAGATT	TCCTTTGGAG	800
ATGAGGCCTT	TCCGGTTACA	ACCAATGATA	AAAGAAGCAG	CTTGCCTTGC	850
CAAATGCTTG	TGTGTGTATA	GGGGGTTTGG	TTTTGCAATT	GAGGTTGACA	900
AGTCCATAGC	TGATCATGTA	ATTGGAGATG	AAAGAAGGGT	TTTTCAGGTG	950
ATTTTGCATA	TGGTTGGTAG	CCTGTTGAAT	GGAAACCAGG	GCGGAGGGTT	1000
GGTCGTATTT	CGGGTTTCTT	CAGAGAATGG	AAGTCAGGGG	AGGAATGAT	1049

Fig. 3 The nucleotide sequence of all star strawberry
Note: Underlined sequences were the primer sequences.

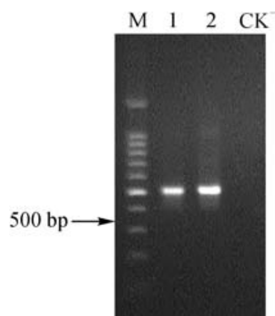

Fig. 4 Agrose gel electrophoretogram of PCR products
Note: M: 100 bp ladder marker; 1, 2: PCR products of antisense; CK^{-}: negative comparison of PCR products.

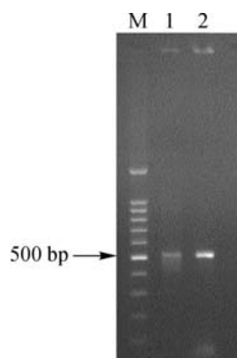

Fig. 5 Agrose gel electrophoretogram of antisense PCR digested by enzymatic
Note: M: 100 bp ladder marker; 1: antisense PCR digested by *Xba* I + *Bam*H I ; 2: PCR products of antisense.

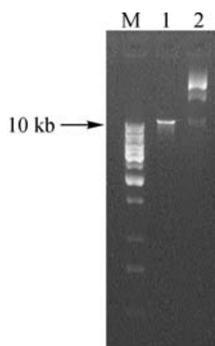

Fig. 6 Agrose gel electrophoretogram of pBI121 plasmid digested by *Xba* I and *Bam*H I
Note: M: 1 kb ladder marker; 1: pBI121 plasmid digested by *Xba* I and *Bam*H I ; 2: pBI121 plasmid.

3.3.2 Identification of antisense expression vector

If the antisense expression vector was digested with *Xba* I and *Eco*R I, the small fragment should be about 2.6 kb. If it was digested with *Xba* I and *Bam*H I the small fragments of the electrophoresis band should be about 500 bp. After digestion with *Hin*d III and *Xba* I or *Hin*d III and *Bam*HI the small fragments should be about 0.9 kb and 1.3 kb, respectively. Electrophoresis results (Fig. 7) were in line with expectations.

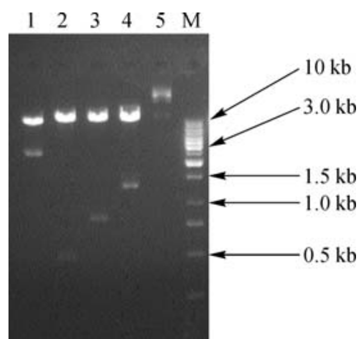

Fig. 7 Detection of the antisense expression vector by enzymatic digestion
Note: 1: *Xba* I /*Eco*R I ; 2: *Xba* I/*Bam*H I ; 3: *Hin*d III/*Xba* I ; 4: *Hin*d III/*Bam* H I ; 5: pBI -*anti-Etr2*; M: 1 kb ladder marker.

3.4 Identification of expression vector in *Agrobacterium* by bacterium liquid PCR

The pBI121-anti-*Etr2* expression vector plasmid was transformed into *Agrobacterium fumefeciens* LBA4404 competent cells. Positive colonies were screened on solid YEB medium containing Kan ($100\,\mu g \cdot mL^{-1}$) and rif ($50\,\mu g \cdot mL^{-1}$). A single colony was chosen and shaken in the YEB liquid medium containing the same antibiotic. Using the pBI121-anti-*Etr2* plasmid (positive control), non-transformed *Agrobacterium* liquid (negative control) and transformed *Agrobacterium* liquid as the template, transformation was verified by PCR amplification with specific primers. If the pBI121-anti-*Etr2* expression vector was transformed into *Agrobacterium*, the PCR product should be about 500 bp. The PCR product electrophoresis (Fig. 8) was in line with expectations. That is to say, the expression vector was transformed into *Agrobacterium fumefeciens* LBA4404.

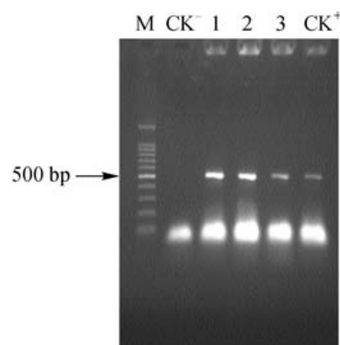

Fig. 8 Agrose gel electrophoretogram of antisense expression vector transformed *Agrobacterium fumefeciens* LBA4404
Note: M: 100 bp ladder marker; CK^{-}: comparison of negative control; 1–3: PCR products of bacterium liquid; CK^{+}: PCR product of the plasmid.

4 Discussion

Since the 1980s, antisense RNA technology has become a powerful tool for regulation and control of plant gene expression. Antisense RNA technology can delay or suppress fruit ripening through the suppression of the fruit mature gene expression (Luo et al., 1995; Waterhouse et al., 1998). Strawberry is a non-climacteric fruit, but it can also produce a small amount of ethylene. Only when ethylene binds with the receptor can it be effective in the signal transduction process (Klee, 2002).

In contrast to the great deal of information regarding the regulation of ripening in climacteric fruits (Giovanoni, 2001; Giovanoni, 2004), much less is known about non-climacteric ones. Since non-climacteric fruits are also able to synthesize ethylene, and in some cases it has been seen that ethylene can hasten post-harvest deterioration, the possible involvement of this hormone in the ripening of non-climacteric fruits has been studied in different laboratories (Ferrarese et al., 1995; Harpster et al., 1997; Harpster et al., 1998; El-Kereamy et al., 2003; Katz et al., 2004; Tesniere et al., 2004). However, no results have been obtained that can demonstrate a clear relation between ethylene and the ripening of these fruits.

In our study, an antisense expression vector of *FaEtr2* was constructed for further research on gene function to improve the varieties of strawberry through transgenic technology. Transgenic experiments with antisense plasmids are in progress. However, attempting to construct the antisense expression vector is difficult due to the fact that the two fragments to be connected vary widely. Connection is not successfully done in accordance with the recommended mole concentration ratio (Sambrook et al., 2001). In this study mole concentration ratio was optimized, so this issue was resolved fundamentally.

Acknowledgements This research was supported by National Natural Science Foundation of China (Grant No. 30500349) and Beijing Natural Science Foundation, China (No. 6062020)

References

Cancel J D, Larsen P B (2002). Loss-of-function mutations in the ethylene receptor ETR1 cause enhanced sensitivity and exaggerated response to ethylene in *Arabidopsis*. Plant Physiology, 129: 1557–1567

El-Kereamy A, Chervin C, Roustan J P, Cheynier V, Souquet J M, Moutounet M, Raynal J Ford C, Latche A, Pech J C, Bouzayen M (2003). Exogenous ethylene stimulates the long-term expression of genes related to anthocyanin biosynthesis in grape berries. Physiologia Plantarum, 119: 175–182

Ferrarese L, Trainotti L, Moretto P, Polverinode L P, Rascio N, Casadoro G (1995). Differential ethylene-inducible expression of cellulase in pepper plants. Plant Molecular Biology, 29: 735–747

Giovannoni J (2001). Molecular biology of fruit maturation and ripening. Annual Review of Plant Molecular Biology, 52: 725–749

Giovannoni J J (2004). Genetic regulation of fruit development and ripening. The Plant Cell, 16: S170–S180

Harpster M H, Brummell D A, Dunsmuir P (1998). Expression analysis of a ripening-specific, auxin-repressed endo–1,4–beta-glucanase gene in strawberry. Plant Physiol, 118: 1307–1316

Harpster M H, Lee K Y, Dunsmuir P (1997). Isolation and characterization of a gene encoding endo-β-1,4 –glucanase from pepper (*Capsicum annuum* L.). Plant Molecular Biology, 33: 47–59

Jimenez-Bermudez S, Redondo-Nevado J, Munoz-Blanco J, Caballero J L, Lopez-Aranda J M, Valpuesta V, Pliego-Alfaro F, Quesada M A, Mercado J A (2002). Manipulation of strawberry fruit softening by antisense expression of pectate lyase gene. Plant Physiol, 128: 751–759

Katz E, Lagunes P M, Riov J, Weiss D, Goldschmidt E E (2004). Molecular and physiological evidence suggests the existence of a system II-like pathway of ethylene production in non-climacteric *Citrus* fruit. Planta, 219: 243–252

Klee H J (2002). Control of ethylene–mediated processes in tomato at level of receptors. J Exp Bot, 53: 2057–2063

Knee M, Sargent J A, Osborne D J (1977). Cell wall metabolism in developing strawberry fruits. J Exp Bot, 28: 377–396

Luo Y B, Sheng J P, Shen L (1995). Control of tomato ethylene biosynthesis by the use of antisense gene. Journal of Agricultural Biotechnology, 3(2): 37–44 (in Chinese)

Marty I, Douat C, Tichit L, Jungsup K, Leustek T, Albagnac G (2000). The cystathionine-gamma-synthase involved in methionine biosynthesis is highly expressed and auxin-repressed during wild strawberry (*Fragaria vesca* L.) fruit ripening. Theor Appl Genet, 100: 1129–1136

Medina-Escobar N, Cardenas J, Munoz-Blanco J, Caballero J L (1998). Cloning and molecular characterization of a strawberry fruit ripening-related cDNA corresponding a mRNA for low-molecular-weight heat-shock protein. Plant Mol Biol, 36: 33–42

Sambrook J, Russsell D W, Huang P T (2001). Molecular Cloning: A Laboratory Manual. 3rd ed (in Chinese, trans. Peitang HUNG). Beijing: Academic Press, 2002, 68–70

Tesniere C, Pradal M, El-Kereamy A, Torregrosa L, Chatelet P, Roustan J P, Chervin C (2004). Involvement of ethylene signaling in a non-climacteric fruit: new elements regarding the regulation of ADH expression in grapevine. Journal of Experimental Botany, 55: 2235–2240

Trainotti L, Pavanello A, Casadoro G (2005). Different ethylene receptors show an increased expression during the ripening of strawberries: Does such an increment imply a role for ethylene in the ripening of these non-climacteric fruits. Journal of Experimental Botany, 56(418): 2037–2046

Trainotti L, Spolaore S, Pavanello A, Baldan B, Casadoro G (1999). A novel E-type endo-beta-1,4–glucanase with a putative cellulose-binding domain is highly expressed in ripening strawberry fruits. Plant Mol Biol, 40: 323–332

Waterhouse P M, Graham M W, Wang M B (1998). Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. Proc Natl Acad Sci, 95: 13959–13964

Wooly L C, James D J, Manning K (2001). Purification and properties of an endo-beta–1,4–glucanase from strawberry and down-regulation of the corresponding gene. Cell Planta, 214: 11–21