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RESEARCH ARTICLE

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Strawberry *FaEtr2* gene RNAi expression vector construction and genetic transformation

Abstract The short hairpin RNA (shRNA) expression vector of the *FaEtr2* gene was constructed by inserting the sense fragment into the constructed antisense vector of *FaEtr2* (pBI121-Anti-*Etr2*) in sense orientation. The constructed RNA interference (RNAi) expression vector was transformed into *Agrobacterium fumefeciens* LBA4404 and used to infect strawberry leaves. Using *in vitro* plantlet leaves as explants, the transformation conditions of All-Star strawberry were studied systemically. The results showed that infecting the leaves with *A. fumefeciens* resuspension liquid of OD_{600} = 1.0 by preculturing for 3 d, co-culturing for 3 d, infecting for 10 min, and adding acetosyringone (AS) 50–100 $\mu mol \cdot L^{-1}$ was suitable for genetic transformation of All-Star strawberry. Seven lines of transgenic plants were preliminarily identified by PCR and β-glucurondiase (GUS) histochemical staining.

Keywords strawberry, *FaEtr2*, RNA interference (RNAi), genetic transformation

1 Introduction

Ethylene is a critical component of diverse developmental processes, such as seed germination, fruit ripening, abscission, and senescence. Strawberry is a kind of non-climacteric fruit, but it can also produce a small amount of ethylene. Only when ethylene is bound with receptors can it be effective in the signal transduction process (Klee, 2002). The possible involvement of this hormone in the ripening of the climacteric and non-climacteric has been studied (Ferrarese et al., 1995; Harpster et al., 1997, 1998; Giovannoni, 2001, 2004; Katz et al., 2004; Tesniere et al., 2004), but no results can demonstrate a clear relation between ethylene and the ripening of non-climacteric fruits.

Efforts have long been made to control the softening of strawberry fruits, yet none has an effect (Harpster et al., 1998; Woolley et al., 2001; Jimenez-Bermudez et al., 2002). *FaEtr2* is mostly expressed in ripening strawberries (Trainotti et al., 2005). It may be closely related to the maturing of strawberry fruits. Further research on gene function of *FaEtr2* may demonstrate a relation between ethylene and the ripening of non-climacteric fruits. Intron-containing constructs encoding self-complementary 'hairpin' RNA (ihpRNA) have the potential to efficiently silence specific genes and the silencing effect stably inherited over many generations (Fire et al., 1998; Smith et al., 2000; Hoffmann et al., 2006). The specificity of double-stranded RNA (dsRNA) silencing enables researchers to generate custom knockout plants and analyze the related gene function (Chen et al., 2007). Compared with co-operation and anti-sense technology, RNA interference (RNAi) has a higher efficiency of gene silencing.

Genetic transformation has been described in different cultivars of strawberry (Zhang and Wu, 1998; Zhang et al., 2001; Zhu et al., 2008). As the regeneration and transformation conditions have cultivar difference, so the genetic transformation conditions of All-Star strawberry were studied in our work. To elucidate the function of *FaEtr2*, *Agrobacterium fumefeciens* cells harboring an intron-hairpin construct of a partial *FaEtr2* were used to infect All-Star strawberry leaves. RNAi gene was successfully transformed into All-Star strawberry. Further research on gene function is under way.

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2 Materials and methods

2.1 Materials

All-Star strawberry *in vitro* plantlet leaf disks were selected for transformation. The *A. tumefaciens* strain LBA4404 carrying the vector plasmid pBI121-*FaEtr2*-RNAi that contains kanamycin (kanamycin, Kan)-resistant npt II (neomycin phosphotransferase), and uidA (β-D-glucuronidase) genes were used for the genetic transformation of strawberry.

Murashige and Skoog basal medium with sucrose 30 g·L^{-1}, Thidiazuron (TDZ) 1 mg·L^{-1}, and NAA 0.05 mg·L^{-1} was used for leaf regeneration (MS_1). Cefotaxime (Cef) 200 mg·L^{-1} and Kan 20 mg·L^{-1} were added into the MS_1 medium for resistant buds screening. Beef-Yeast Extract (YEB) medium for *A. fumefeciens* culture contained peptone 10 g·L^{-1}, yeast extract 1 g·L^{-1}, sucrose 5 g·L^{-1}, and $MgSO_4 \cdot 7H_2O$ 5 g·L^{-1}, at pH 7.0.

2.2 Methods

2.2.1 Amplification of sense fragments

Sense fragment oligos were commercially synthesized with the sense strand sequence: 5′-GGGATCCA-CATTCTTCGGCCAG-3′ and antisense strand sequence: 5′-TATCCCGGGCTGGATTATCCAT-3′ (underlined sequences were *Bam*HI and *Sma*I sites, respectively). The PCR amplification reaction conditions were predegeneration at 94°C for 3 min, followed by 30 cycles of degeneration at 94°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 90 s, and the final extension at 72°C for 10 min.

2.2.2 Construction of pBI121-*Etr2*-RNAi expression vector

The PCR product of sense fragments were subcloned into the *Bam*HI-*Sma*I site of vector pBI121-Anti-*Etr2*, resulting in plasmid pBI121-*Etr2*-RNAi. The plasmid was used for transformation with *E. coli* strain *DH5α* competent cells. Positive plasmids were verified with the restriction endonucleases. Finally, the vector was transferred into the *A. fumefeciens* strain LBA4404 by the freezing-thaw method. The RNAi vector map is shown in Fig. 1.

2.2.3 Optimization of pre-culture time, infection time, acetosyringone concentration, and *A. fumefeciens* concentration

A. fumefeciens liquid of OD_{600} = 0.5 was centrifuged and resuspensed to different concentrations with MS liquid medium. Leaves were pre-cultured for 1, 2, 3, 4, and 5 d respectively to optimize pre-culture time. Different concentrations (OD_{600} = 0.5, 1.0, 1.5, 2.0) of *A. fumefeciens* resuspension liquid were used to infect leaves. Four levels (0 μmol·L^{-1}, 50 μmol·L^{-1}, 100 μmol·L^{-1}, and 150 μmol·L^{-1}) were established to optimize acetosyringone concentration. For infection time, four levels (5, 10, 15, and 20 min) were designed. Each treatment had three replicates, with 150 pieces of leaf explants per replicate and all the trials were conduced twice.

2.2.4 Genetic transformation

Pre-cultured explants were immersed in *A. fumefeciens* resuspension solution for 10 min. Then the redundant solution on the explant surfaces was filtrated with sterile filter paper. Co-cultured on the antibiotic-free regeneration medium for three days, the explants were transferred to MS_1 medium with Cef 200 mg·L^{-1} and cultured for three days. The regeneration rates of resistant adventitious buds were assayed 35 days after selective culture on MS_1 medium with Cef 200 mg·L^{-1} and Kan 20 mg·L^{-1}.

2.2.5 Statistical analyses

The data were subjected to analysis of variance (ANOVA). Multiple range tests were used to compare different treatments.

Regeneration rate of resistant adventitious buds (%) = (number of the differentiated leaves/number of the leaves inoculated) × 100 %.

2.2.6 PCR analysis

Genomic DNA was extracted and tested from freezing young leaves of transformed plants with a CTAB protocol. The nontransformed plants were considered as control. The specific primers (5′-TCAGAAAGAATGCTAACC-CACAG-3′/5′-GAGGAAGGGTCTTGCGAAGG ATA-3′)

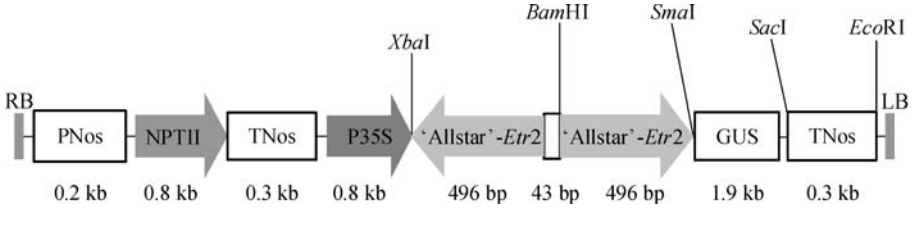

Fig. 1 Map of All-Star *FaEtr2* gene RNAi expression vector

were designed based on the coding site sequence of CaMV 35 s promotor. PCR was carried out in a total volume of 20 μL under the following condition: the template was denatured at 94°C for 3 min, followed by 30 cycles of amplification (94°C for 30 s, 62.4°C for 30 s, 72°C for 60 s), and the final extension at 72°C for 10 min. The PCR products were subjected to electrophoresis using 1.0% agarose gel. The amplification of a fragment about 0.78 kb was predicted.

2.2.7 GUS histochemical assay

The transformed leaves were placed in tubes with X-Gluc staining solution at 37°C overnight. The materials were transferred into 70% alcohol and kept at room temperature for 24 h. Non-transformed leaves were used as negative control. The transgenic plant leaves that expressed GUS gene should appear blue in color.

3 Results and analysis

3.1 Construction and identification of expression vector for RNAi

Sense PCR products and pBI121-Anti-*Etr2* plasmids were digested by *Bam*HI and *Sma*I at the same time. The size of the fragments did not change significantly with only a few bases cut (Fig. 2(a), 2(b)). The RNAi expression vector was verified by restriction endonucleases. When it was digested by *Xba*I and *Sma*I, *Bam*HI and *Eco*RI, *Hin*dIII and *Sma*I, *Hin*dIII and *Bam*HI, the small fragments should be about 1.0 kb, 2.6 kb, 1.9 kb, and 1.3 kb, respectively. Electrophoresis results (Fig. 2(c)) were in accordance with expectations. All kinds of digestion detection showed that the RNAi expression vector of *FaEtr2* gene was successfully constructed.

The constructed vector was transferred into the

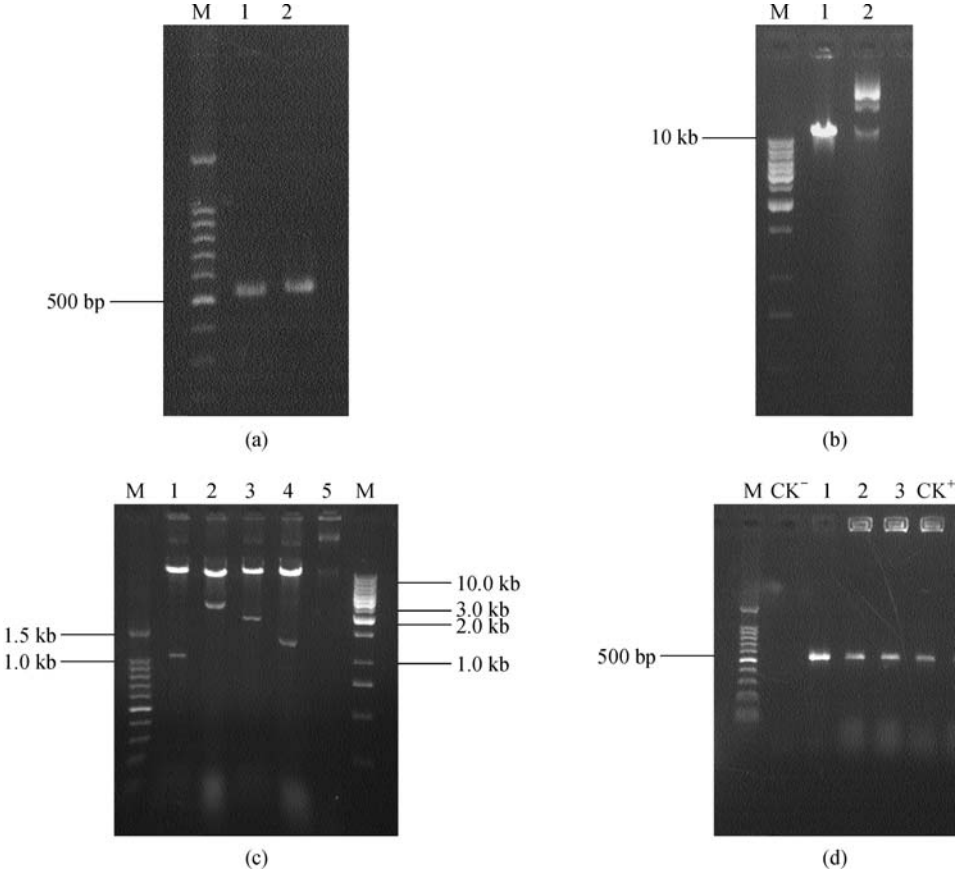

Fig. 2 construction of expression vector for RNAi

Note: (a): agrose gel electrophoretogram of sense PCR digested by enzymatic, M: 100 bp ladder marker, 1: sense PCR products digested by *Bam*HI/*Sma*I, 2: PCR products of sense; (b): agrose gel electrophoretogram of pI121-Anti-*Etr2* plasmid digested by *Bam*HI/*Sma*I, M: 1 kb ladder marker, 1: pBI121-Anti-*Etr2* plasmid digested by *Bam*HI/*Sma*I, 2: pBI121-Anti-*Etr2* plasmid; (c): detection of the RNAi experssion vector by restriction endonucleases, left M: 100 bp ladder marker, 1: plasmid digested by *Xba*I/*Sma*I, 2: plasmid digested by *Bam*HI/*Eco*RI, 3: plasmid digested by *Hin*dIII/*Sma*I, 4: plasmid digested by *Hin*dIII/*Bam*HI, 5: control plasmid, right M: 1 kb ladder marker; (d): agrose gel electrophoretogram of RNAi expression vector in *Agrobacterium fumefeciens* LBA4404, M: 100 bp ladder marker, CK^-: comparison of negative control, 1–3: PCR products of bacterium liquid, CK^+: PCR product of plasmid.

A. fumefeciens strain LBA4404 by the freezing-thaw method. Using *A. fumefeciens* liquid as template, the transformation was identified by PCR. If the pBI121-*Etr2*-RNAi expression vector was transformed into *A. fumefeciens*, the PCR products should be about 500 bp. The PCR products in electrophoresis Fig. 2(d) were in accordance with our expectations.

3.2 Genetic transformation conditions

3.2.1 Effects of dark pre-culture time and *A. fumefeciens* concentrations on the transformation rate

The effects of different dark pre-culture time on strawberry genetic transformation are shown in Table 1. After pre-culture, the rate of resistant adventitious buds improved gradually. The highest rate of resistant adventitious buds generated by a 3-d pre-culture was 18.9%, significantly higher than that of other pre-culture time. The rate declined when the pre-culture was prolonged. Few resistant adventitious buds were generated on leaves without pre-culture. Therefore, the appropriate pre-culture time was found to be 3 days.

Table 1 Effects of dark pre-culture time and *A. fumefeciens* concentration on regeneration rate of resistant adventitious buds

dark pre-culture time/d	regeneration rate of resistant adventitious buds/%	*A. fumefeciens* concentration /OD_{600}	regeneration rate of resistant adventitious buds/%
1	0E	0.5	5.09Cc
2	8.01Cc	1.0	18.55Aa
3	18.9Aa	1.5	9.63Bb
4	13.34Bb	2.0	3.79Cc
5	5.04Dd	–	–

Note: Different small and capital letters in a column represent significance at 0.05 and 0.01 probability level, respectively.

The effects of *A. fumefeciens* concentration on genetic transformation are shown in Table 1. The highest rate (18.55%) of resistant adventitious buds was achieved when the concentration of *A. fumefeciens* solution was at 1.0, significantly higher than that of other treatments. The rate declined when the concentration of *A. fumefeciens* liquid rose.

3.2.2 Effects of acetosyringone concentration and infection time on the transformation rate

The effects of acetosyringone (AS) concentration on the transformation rate are shown in Table 2. The highest rate of resistant adventitious bud was generated at the concentration of 50 $\mu mol \cdot L^{-1}$ AS, significantly different from the 0 $\mu mol \cdot L^{-1}$ AS treatment but not significantly different from the 100 $\mu mol \cdot L^{-1}$ AS treatment. Therefore, the appropriate concentration of AS was at 50–100 $\mu mol \cdot L^{-1}$ when infecting All-Star strawberry leaves.

The effects of infection time on genetic transformation of All-Star strawberry are shown in Table 2. When the conditions such as pre-culture time and concentration of *A. fumefeciens* solution were determined, the infection time had a significant influence on the conversion efficiency. The highest rate of resistant adventitious buds generated when the infection time was 10 min, and the treatment was significantly different from others. The rate declined with too long or too short of infection time, therefore, 10 min was the suitable infection time for All-Star strawberry transformation.

Table 2 Effects of acetosyringone concentration and infection time on regeneration rate of resistant adventitious buds

acetosyringone concentration /($\mu mol \cdot L^{-1}$)	regeneration rate of resistant adventitious buds/%	infection time /min	regeneration rate of resistant adventitious buds/%
0	9.53BCb	5	10.47BCc
50	15.09Aa	10	21.46Aa
100	14.55ABa	15	16.38ABb
150	8.89Cb	20	7.77Cc

Note: Different small and capital letters in a column represent significance at 0.05 and 0.01 probability levels, respectively.

3.3 Detection of transformed plants

Seven lines of putative transgenic plants (non-transformed plants listed only as a control) were detected from 180 lines of All-Star strawberries resistant plantlets by PCR (Fig. 3(a)). It was preliminarily proved that strawberry ethylene receptors *FaEtr2*-RNAi gene was transferred into All-Star strawberry plantlets. The photos of the resistant buds are shown in Fig. 3(b). In order to identify the transgenic plantlets, GUS histochemical staining was done.

When resistant buds grew to 1 to 2 cm high, the leaves of the buds were detected by dyeing in GUS Staining Solution. The leaves of control plantlets reacted negatively (Fig. 3(d)) while the transformed plantlets reacted positively with a blue color (Fig. 3(c)). Seven lines of leaves from 180 lines showed positive reaction. The positive rate of GUS histochemical staining was 3.93%. The results were consistent with those by PCR detection, which proved *FaEtr2*-RNAi gene was already transferred into All-Star strawberry plantlets.

4 Discussion

In application of RNA interference, vector optimization is critical. hpRNA constructs require two copies of the target sequence in an inverted-repeat orientation in order to produce duplex RNA (Waterhouse et al., 1998; Smith

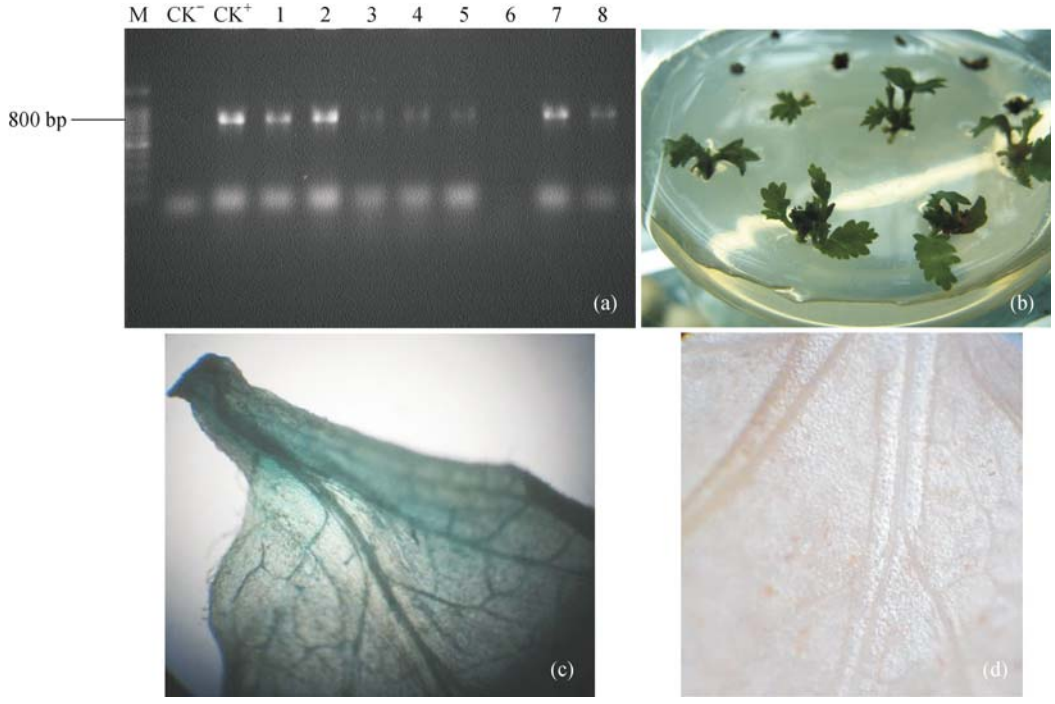

Fig. 3 Detection of transgenic plants

Note: (a): PCR amplification results of transgenic plants, M: 100 bp Ladder Marker, CK^- negative control, CK^+: PCR product of the plasmid, 1–5, 7, 8: PCR products of transgenic plants, 6: PCR product of non-transformed plant; (b): photos of resistant adventitious buds; (c): results of transgenic plant leaf by GUS histochemical staining detection; (d): results of non-transgenic plant leaf by GUS histochemical staining detection (CK^-).

et al., 2000; Wesley et al., 2003). Only dsRNA sequences from exons have an effect while the sequences from introns have no effect. Increasing the size of the dsRNA fragments from 100 to 450 nt may cause an increase in the degradation of the corresponding mRNAs. It is necessary to include a spacer region between the arms of hpRNA constructs for stability of the inverted repeat DNA (Wang and Waterhouse, 2000). We paid full attention to these key points, and successfully constructed the RNAi vector of *FaEtr2*.

Several factors and steps affect gene transformations mediated by *A. fumefeciens*. Zhang and Wang (2005) preliminarily studied the transformation system of All-Star strawberry and obtained Kanamycin-resistant shoots, but the rate was very low. In our study, the transformation conditions were optimized. AS and β-hydroxy acetosyringone (OH-AS) are usually required for Virulence (vir) gene activity (Wang et al., 2004). AS is more widely used than OH-AS in practice. In the gene transformation system of Idaho locust, the transformation ratio is more than doubled when AS is added to the *A. fumefeciens* solution (Sun et al., 2006). Most researchers suggest that 100 μmol·L^{-1} AS is suitable for the strawberry. Our study showed no significant difference between 50 μmol·L^{-1} and 100 μmol·L^{-1} AS treatments. This may due to the difference of cultivars. Pre-culture of leaves is needed in the strawberry transformation system. In our experiment, the ratio of the transformation system of All-Star strawberry was low when pre-culture time was less than or more than 3 days, which was consistent with most reports on strawberry. Many researchers also report that *A. fumefeciens* concentration of 0.5 is suitable for the strawberry. In our study, *A. fumefeciens* liquid of OD_{600} = 0.5 was centrifuged and resuspensed to OD_{600} = 1.0 with MS liquid medium to infect the leaves, which dramatically promoted the regeneration rate of resistant buds. The reason might be that resuspension by MS liquid medium can decrease the injury of the leaves from *A. fumefeciens*.

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