

Expression of ethylene-forming enzyme (EFE) of *Pseudomonas syringae* pv. *glycinea* in *Trichoderma viride*

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Abstract The *efe* gene encoding an ethylene-forming enzyme from *Pseudomonas syringae* pv. *glycinea* has been expressed for the first time under the control of *Trichoderma reesei cbh1* promoter in *Trichoderma viride*. Reverse transcription polymerase chain reaction analysis showed that transformant Y2 produced mRNA of the *efe* gene. Southern blot analysis showed that there was one copy of *efe* gene which was integrated into the chromosomal DNA of *T. viride*. Ethylene production by transformant Y2 was efficiently induced by cellulose, while very low level of ethylene was produced when sodium carboxymethyl cellulose or lactose was used as carbon source. Peptone exerted a much greater stimulatory effect on ethylene production. A high level of ethylene was produced when transformant Y2 was cultured in solid fermentation medium containing wheat straw, indicating that plant wastes could be directly converted to ethylene by the recombinant filamentous fungus.

Keywords Ethylene · Ethylene-forming enzyme (EFE) · *cbh1* promoter · *Trichoderma viride* · *Pseudomonas syringae*

Introduction

Ethylene is the most important starting material in petroleum chemistry and it is also a gaseous plant hormone involved in the regulation of numerous physiological processes such as fruit ripening and senescence. It is well-known that ethylene is not only produced by plants, but also by microorganisms (Chalutz and Lieberman 1977; Nagahama et al. 1992; Sato et al. 1997; Chague et al. 2002). However, for large-scale industrial application, ethylene is made from hydrocarbon feedstocks such as natural gas liquids or crude oil. As the global fossil fuel reserves decline, it is desirable to develop a process for the microbial production of ethylene from various renewable resources.

Microorganisms produce ethylene via two different biochemical pathways. Both pathways are distinct from that of higher plants, which use only one pathway from methionine via 1-aminocyclopropane-1-carboxylic acid (Jiao et al. 1986; Hall and Smith 1995; Weingart et al. 1999). Most microorganisms produce only trace amounts of ethylene from methionine via 2-keto-4-methyl-thiobutyric acid by an NADH:Fe(III)EDTA oxidoreductase (Weingart et al. 1999). Four pathovars of *Pseudomonas syringae* and a few species of fungi such as *Penicillium digitatum* produce efficiently ethylene from 2-oxoglutarate by an ethylene-forming enzyme (EFE) (Nagahama et al. 1991; Völksh and Weingart 1997). The *efe* gene from *P. syringae* pv. *phaseolicola* PK2 has been cloned and expressed in *Escherichia coli* at high level (Ishihara et al. 1995). In order to make the biological conversion of carbon dioxide into ethylene, the *efe* gene from *P. syringae* pv. *phaseolicola* PK2 has been introduced into cyanobacterium *Synechococcus* sp. PCC 7942 and the recombinant cyanobacterial strains produce ethylene at a low level (Sakai et al. 1997; Takahama et al. 2003).

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The filamentous *Trichoderma* species, such as *Trichoderma reesei* and *Trichoderma viride*, are known to be good cellulase producers and they produce a complete set of cellulases consisted of three general classes of enzymes: cellulohydrolases (CBHI and CBHII), endoglucanases (EGI, EGII, and EGIII), and β -glucosidase, which act synergistically to hydrolyze crystalline cellulose to glucose (Henrique-Silva et al. 1996; Ilmen et al. 1997; Sun et al. 1997; Van Wyk and Mohulatsi 2003). Homologous and heterologous genes under the strong main cellobiohydrolase (*cbh1*) promoter of *T. reesei* have been successfully over-expressed in *T. reesei* and the related species *Trichoderma harzianum* (Joutsjoki et al. 1993; Miettinen-Oinonen et al. 1997; Margolles-Clark et al. 1996a, b). Extensive studies showed that a high level of cellulase gene expression of *T. reesei* was induced by insoluble polymer such as cellulose and plant material (Ilmen et al. 1997). Thus it will be a very important potential value to introduce *efe* gene into *Trichoderma* species and to enable the recombinant *Trichoderma* species to produce ethylene from agricultural wastes.

In this study, *efe* gene encoding EFE was cloned and sequenced from *P. syringae* pv. *glycinea* ICMP2189 and was then successfully expressed under the control of *T. reesei* *cbh1* promoter in *T. viride*. We further characterized the effect of different carbon sources or nitrogen source peptone on *efe* gene expression.

Materials and methods

Microorganisms and plasmids

P. syringae pv. *glycinea* ICMP2189 was obtained from Plant Protection Institute of Chinese Academy of Agricultural Science. *T. viride* TL124, which has a strong cellulolytic activity, was isolated by our lab. Transformant Y2 carrying *efe* gene from *P. syringae* pv. *glycinea* ICMP2189 was developed in this study. *E. coli* DH5 α was used as a host for cloning and sequencing. Plasmid pMD18-T was used as a cloning vector and pTRIL, which carries *T. reesei* *cbh1* promoter and terminator, was used as an expression vector in *T. viride* (Wang et al. 2004).

Cloning of *efe* gene and construction of recombinant plasmid pTRIL-efe

A 1,071-bp fragment including the *efe* coding region, was amplified with primers P1 (5'-ccg**tcgacat**gacaaactacagactt-3' [a *Sall* site italicized and the *efe* start codon indicated by bold letters]) and P2 (5'-tatggatcca**actcat**gagcctgtcgcg-3' [a *Bam*HI site italicized and the *efe* stop codon indicated by bold letters]) from *P. syringae* pv.

glycinea ICMP2189. The polymerase chain reaction (PCR) product was ligated to vector pMD18-T and then sequenced. At the same time, the amplified fragment was digested with *Sall* and *Bam*HI and ligated to vector pTRIL digested with the same enzymes, yielding recombinant plasmid pTRIL-efe (Fig. 1).

Protoplast preparation and transformation of *T. viride* TL124

Protoplasts of *T. viride* TL124 were prepared as described by Penttila et al. (1987). The recombinant plasmid pTRIL-efe was cotransformed with plasmid pAN7-1 carrying a hygromycin resistance cassette into the protoplasts of *T. viride* TL124 as described by Wang et al. (2004). The hygromycin-resistant transformants were selected on minimal medium containing 250 $\mu\text{g ml}^{-1}$ hygromycin. The positive transformant Y2 was obtained and identified by PCR amplification with the above-mentioned primers P1 and P2 for *efe* gene and by Southern blot analysis.

Southern blot analysis

The entire *efe* gene fragment was recovered from the recombinant plasmid pTRIL-efe and then was labeled using DIG-DNA labeling and detection kit purchased from Roche (Germany). The chromosomal DNA of transformant Y2 was isolated by using a modified CTAB procedure as described by Tel-Zur et al. (1999). Southern blot analysis was carried out using the labeled *efe* gene as a probe

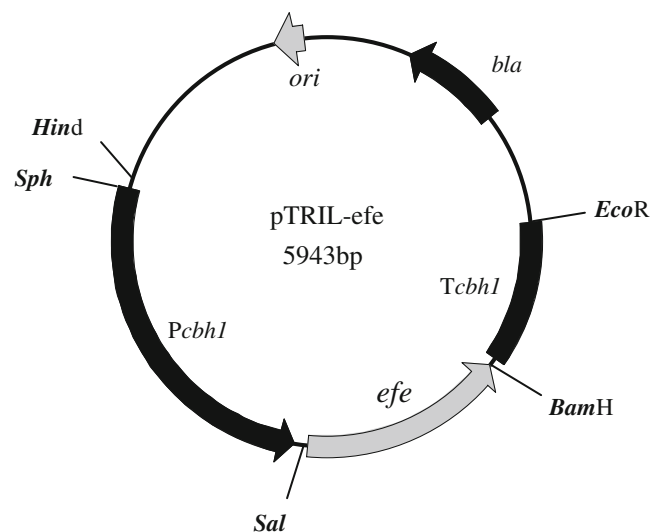


Fig. 1 The expression plasmid pTRIL-efe was constructed by cloning the *P. syringae* *efe* gene into vector pTRIL which carries *T. reesei* *cbh1* promoter and terminator

according to the instructions of the DIG-DNA labeling and detection kit.

RT-PCR amplification

Total RNAs were isolated from the fresh mycelia of transformant Y2 using the Trizol reagent (Invitrogen) according to the manufacturer's protocol, and then reverse-transcribed to cDNA by using oligo-dT primers in the reverse transcription (RT)-PCR with the instructions of AMV reverse transcriptase (Calo et al. 2006). The resulting cDNA was amplified with primers P1 and P2 for *efe* gene under the above-mentioned PCR conditions.

Ethylene production of transformant Y2 on cellulose-induction medium

T. viride strains were maintained on potato dextrose agar. For the inducible production of ethylene under the control of *T. reesei cbh1* promoter, cellulose-inducing (CI) medium was used, which was *Trichoderma* minimal medium (Ilmen et al. 1997), in which glucose was substituted with 0.5% cellulose (Penttila et al. 1987).

To determine ethylene production of transformant Y2 on CI medium, experiments were carried out as follows: 0.5 ml of 10^7 ml⁻¹ spore suspension, collected from cultures grown on potato dextrose agar, was inoculated into 50 ml of the minimal medium in a 250-ml flask and grown at 30 °C with shaking at 200 rpm. At 48 h after incubation, mycelia were collected, washed, and transferred into 2 ml cellulose-induction medium in 12×120 mm test tube covered with a rubber stopper and grown with shaking at 200 rpm at 30 °C. After 36 h of incubation, gas samples were then removed from test-tube using a syringe and the dry weight of mycelia was determined following drying for 48 h at 70 °C. Ethylene levels were determined by injecting 0.1 ml gas samples into a HP6890 gas chromatograph as described by Chalutz and Lieberman (1977). Ethylene production rates were calculated as nanoliter ethylene produced in 1 h per gram of dry weight of mycelium (nl h⁻¹ g⁻¹ dry wt) (Chalutz and Lieberman 1977; Chague et al. 2002).

Effects of carbon sources and peptone on ethylene production

To determine the effect of different carbon sources and peptone on ethylene production, *Trichoderma* minimal medium was used, in which 2% glucose was substituted with different carbon sources such as 0.5% cellulose, 2% cellulose, 0.5% sodium carboxymethyl cellulose, or 3% lactose. Nitrogen source such as 0.2% peptone was added to the medium containing different carbon sources when indicated.

Ethylene production cultured in solid state fermentation

To evaluate the effect of plant material as substrate on ethylene production, the solid fermentation medium containing wheat straw is here developed. The wheat straw used here was washed, chopped, dried (70 °C to constant weight), and ground to 40-mesh size. The solid fermentation medium in 25-ml serum bottle contains pretreated wheat straw, 0.72 g; bran, 0.08 g; (NH₄)₂SO₄, 0.02 g; and water, 2.0 ml, being autoclaved at 121 °C for 30 min. Then 0.08 ml of 10^7 ml⁻¹ spore suspension was inoculated into 25-ml serum-bottle containing the above-described fermentation medium and grown at 30 °C under stationary conditions. At 48 h after incubation, cotton plugs covered in serum bottle was replaced with rubber stoppers and the cultures were further grown for 48 h and then the total amount of ethylene that accumulated in the flask was determined. The total amount of ethylene per flask was calculated by multiplying the free volume in the flask (25-ml) by the amount of ethylene in 1 ml gas sample as determined by gas chromatographic analysis (Chague et al. 2002).

During the determination of ethylene production, all treatments were in three replicates and all the experiments were repeated three times.

Results

Cloning and sequencing of *efe* gene

A 1,071-bp fragment including the complete *efe* coding region was PCR-amplified from *P. syringae* pv. *glycinea* ICMP2189 using primers designed according to the known *efe* genes of *P. syringae*. The GenBank accession number for the *efe* gene isolated in this study is EF17587. Sequence alignments showed that the sequence of the open reading frame from *P. syringae* pv. *glycinea* strain ICMP2189 was completely identical to those derived from *P. syringae* pvs. *glycinea* 7a/90, *cannabina* GSPB2553 and *sesami* 962 and differed in only two single-base pair substitutions from the *efe* nucleotide sequence of *P. syringae* pv. *phaseolicola* PK2. Our data were in agreement with Weingart et al's report (1999, 2001) that a high conservation of the *efe* gene among *P. syringae* pvs. *phaseolicola*, *glycinea*, *cannabina* and *sesami*, all of which were efficient ethylene-producers (Sato et al. 1997; Weingart and Völksch 1997).

Transformation of *T. viride* with expression plasmid carrying *efe* gene

For overexpression of the *efe* gene in *T. viride*, the powerful promoter of the *T. reesei cbh1* gene was used. The plasmid

pTRIL-efe containing the entire coding region of the *P. syringae* pv. *glycinea* *efe* gene linked to the *chb1* promoter and terminator was constructed. The constructed plasmids pTRIL-efe was cotransformed into *T. viride* TL124 with plasmid pAN7-1, which contains a hygromycin resistance cassette. Transformants were selected on plates containing hygromycin. About 200 hygromycin-resistant transformants obtained here were further identified by PCR analysis using P1 and P2 primers for *efe* gene. The PCR product of plasmid DNA (pTRIL-efe) was used as a positive control. PCR analysis showed that five potential transformants contained *efe* gene, while no corresponding amplified fragment could be detected in the wild-type *T. viride*.

Southern blot analysis

Southern blot analysis using the labeled *efe* gene as a probe was further performed with genomic DNA isolated from the wild-type *T. viride* and from the potential transformants identified by PCR analysis. The results showed that *efe* gene in transformant Y2 was integrated into the genome of the recipient, whereas no hybridization signals were observed in the other four transformants or wild-type *T. viride* in this study. Figure 2 showed that *Hind*III- and *Sal*I-digested genomic DNA isolated from transformant Y2 gave a different single positive signal band at the positions of 5 kb and 1.2 kb, respectively. Since the length of the coding region of the *P. syringae* pv. *glycinea* *efe* gene is 1.053 kb, thus we deduce from the current results that there is one

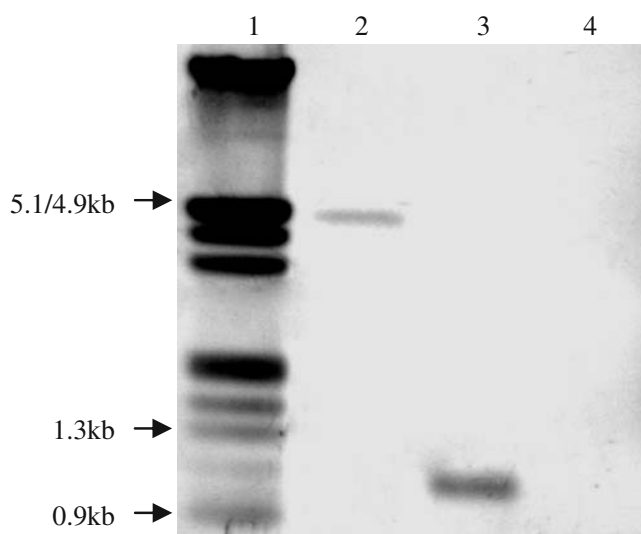


Fig. 2 Southern blot analysis of genomic DNA isolated from transformant Y2. Lane 1 DNA molecular weight marker (λ DNA/*Hind*III+*Eco*RI); Lanes 2 and 3 genomic DNA of transformant Y2 digested respectively with *Hind*III and *Sal*I; Lane 4 genomic DNA of non-transformed *T. viride* digested with *Hind*III. The whole coding sequence of *efe* gene (1,053 bp) was used as a hybridizing probe

copy of *efe* gene which was integrated into the chromosomal DNA of transformant Y2.

RT-PCR analysis

RT-PCR analysis was performed with total RNA isolated from transformant Y2 that were confirmed by Southern blot analysis. The total RNA of the wild-type *T. viride* was analyzed as a parallel control. The data showed that accumulation of mRNA of the *efe* gene could only be detected in transformant Y2 (Fig. 3). No detectable level of transcript, however, was observed in the recipient *T. viride*.

Ethylene production of transformant Y2

Previous reports have demonstrated that cellulose induces the expression of *T. reesei* *chb1* promoter in *T. reesei* or *T. harzianum* (Margolles-Clark et al. 1996a, b). To determine if ethylene was produced by transformant Y2, CI medium which contains 0.5% cellulose as the sole carbon source was used. Before ethylene assay, it has been determined that there were no differences in growth rate or morphology between transformant Y2 and the wild-type *T. viride*, indicating that integration of *efe* gene into the genome of *T. viride* does not seem to be harmful to the host. Gas chromatograph assay showed that transformant Y2 produced ethylene efficiently (the ethylene production rate was 1,059.7 nl h⁻¹ g⁻¹ dry wt), while no production of ethylene was observed in the wild-type *T. viride* (Table 1). The ethylene level produced by transformant Y2 was similar to that produced by *P. digitatum*, which is the most efficient ethylene-producer among fungi (Spalding and Lieberman 1964). The results indicate that the *efe* gene from *P. syringae* pv. *glycinea* was successfully expressed in *T. viride*. This is the first time to express the *efe* gene of *P. syringae* under the control of *T. reesei* *chb1* promoter in *Trichoderma* species.

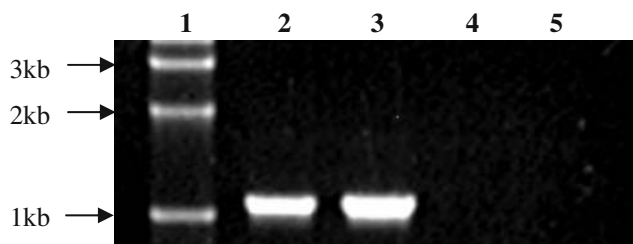


Fig. 3 RT-PCR analysis of EFE mRNA in transformant Y2. Lane 1 1 kb DNA ladder; Lane 2 RT-PCR amplification of first-strand cDNA from transformant Y2; Lane 3 PCR product from plasmid pTRIL-efe as a positive control; Lane 4 PCR amplification of total RNA treated with DNaseI as a negative control. Lack of a signal in this lane indicates that PCR product in lane 2 are due to the amplification of cDNA, rather than low levels of genomic DNA; Lane 5 RT-PCR amplification of the wild-type *T. viride* as a negative control

Table 1 Primary determination of ethylene production of transformant Y2 on CI-medium

	Carbon source (% cellulose)	Time (h)	Ethylene production rate of three replicates (nl h ⁻¹ g ⁻¹ dry wt)			The average ethylene production rate (nl h ⁻¹ g ⁻¹ dry wt)
			1	2	3	
Wild type (control)	0.5	36	0	0	0	0
Transformant Y2	0.5	36	935.73	1,068.10	1,175.27	1,059.7±119.99

Effects of carbon sources and peptone on ethylene production

As different carbon sources and peptone affect the expression of *cbh1* promoter, we here determined the ethylene production rates in media containing different carbon sources or peptone as described in “Materials and methods”. Our results, presented in Fig. 4, revealed that 2% cellulose induced ethylene production more efficiently than 0.5% cellulose did. Very low level of ethylene was produced when sodium carboxymethyl cellulose or lactose was used as carbon source. The results are a little different from the previous report that lactose induced a moderate expression of *cbh1* in *T. reesei* (Ilmen et al. 1997). Nitrogen source peptone exerts a much greater stimulatory effect on ethylene production when added to minimal medium supplemented with different carbon sources, especially in which containing 2% cellulose, in agreement with the report that peptone provoked production of cellulases in *Chaetomium globosum* (Umikalsom et al. 1997).

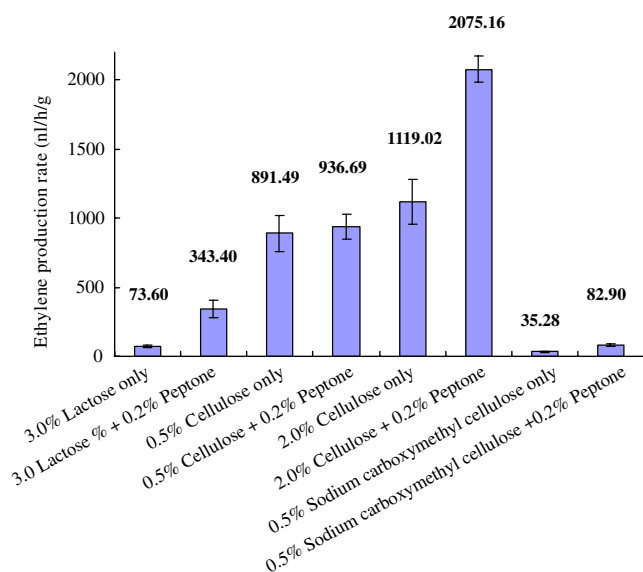


Fig. 4 Effects of carbon sources and peptone on ethylene production by transformant Y2. The values are averages of triplicates and standard deviations are shown

Ethylene production cultured in solid state fermentation

To determine if transformant Y2 could utilize plant wastes to produce ethylene, here we developed a solid fermentation medium which contains wheat straw as the main carbon source. Transformant Y2 was found to produce ethylene at the amount of 2,280 nl when cultured in this solid fermentation medium for 48 h at 30 °C. The result suggests that plant wastes could be directly converted to ethylene by the recombinant filamentous fungus.

Discussion

In this study, we have established a high expression system of *P. syringae* pv. *glycinea efe* gene using *T. viride* as a host. We further analyzed the effects of different carbon and nitrogen sources on ethylene production by transformant Y2.

So far, overexpression of *P. syringae* pv. *phaseolicola efe* gene has been performed by using *E. coli* and *Synechococcus elongates* as hosts (Ishihara et al. 1995; Sakai et al. 1997; Takahama et al. 2003). However, expression of *P. syringae efe* gene has not been carried out in filamentous fungi. In this study, the bacterial *efe* gene was linked to the *T. reesei cbh1* promoter and then transformed to *T. viride*. The positive transformant Y2 carrying *P. syringae* pv. *phaseolicola efe* gene was obtained from about 200 hygromycin-resistant transformants and identified by PCR amplification and by Southern blot analysis. Initially, five potential transformants containing *efe* gene were identified by PCR amplification. However, Southern blot analysis using the labeled *efe* gene as a probe showed that only transformant Y2 gave a positive signal while the other four potential transformants did not. The data suggested that *efe* gene was integrated into the genome in transformant Y2 while *efe* gene was not in the other four hygromycin-resistant transformants. Thus we chose transformant Y2 for further experiments. Since Southern blot analysis showed that *HindIII*- and *SalI*-digested genomic DNA gave a different single positive signal band at the positions of 5 kb and 1.2 kb, we deduce that there is one copy of *efe* gene integrated into the chromosomal DNA of transformant

Y2. RT-PCR and ethylene assay demonstrated that the *efe* gene encoding an EFE has been successfully expressed in transformant Y2. The current results that cellulose and peptone induced a high level of ethylene production indicated that expression of the bacterial *efe* gene in transformant Y2 was strictly under the control of *T. reesei cbh1* promoter. The data are consistent with the report that cellulose or plant materials induces expression of the *T. reesei cbh1* promoter in *T. reesei* or in *T. harzianum* (Margolles-Clark et al. 1996a, b; Ilmen et al. 1997). Our results also showed that transformant Y2 produced efficiently ethylene on the solid fermentation medium which contains wheat straw as a main carbon source. Since *T. viride* has a very strong cellulolytic activity, using *T. viride* as a host to produce ethylene seems to be attractive in industrial use.

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