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Chloroplast elongation factor BcEF-Tu responds to turnip mosaic virus infection and heat stress in non-heading Chinese cabbage

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Abstract

Eukaryotic elongation factor Tu has been implicated in responses to heat stress and viral infection. In this study, the turnip mosaic virus (TuMV)-response gene *BcLRK01*, which encodes a leucine-rich repeat receptor-like kinase, was probed using the cDNA library of TuMV-infected leaves of non-heading Chinese cabbage (*Brassica campestris* ssp. *chinensis*). The *BcEF-Tu* gene, which encodes chloroplast elongation factor Tu, was obtained and verified by a yeast two-hybrid system to interact with the *BcLRK01* gene. TuMV infection depressed the expression of this gene, whereas a heat stress induced its expression. Overexpression of *BcEF-Tu* enhanced the viability of *Escherichia coli* transformants under the heat stress. These results demonstrate that elongation factor BcEF-Tu responded to the TuMV infection and heat stress. This is the first report on chloroplast EF-Tu in non-heading Chinese cabbage which provides a theoretical basis for the functional research of EF-Tu.

Additional key words: *Brassica campestris,* chlorophyll fluorescence, heterologous expression, high temperature, real-time PCR, yeast two-hybrid system.

Introduction

A chloroplast elongation factor in turnip (EF-Tu) plays important roles in polypeptide elongation during the translation (Bhadula *et al.* 2001). It is conserved in higher plants and its sequence is highly similar to prokaryotic elongation factors (EF). Recent studies have shown that chloroplast EF-Tu plays an important role in heat tolerance in maize. Heat stress could induce the synthesis and accumulation of chloroplast EF-Tu in the heat-tolerant maize line ZPBL 1304 (Ristic *et al.* 1996, Bhadula *et al.* 2001) but not in the heat-sensitive line ZPL 389 (Ristic *et al.* 1998). Overexpression of maize EF-Tu can enhance the viability of *Escherichia coli* transformants under heat stress (Moriarty *et al.* 2002).

Abundant data have demonstrated that viral replication was involved in the host translation machinery. It seems thereare two interaction mechanisms between EFs and viruses. One case is that host EFs directly interact with viral RNA. For example, eukaryotic EFs interact with viruses of plants and animals and are involved in viral replication or transcription (Blackwell and Brinton 1997, De Nova-Ocampo *et al.* 2002, Zeenko *et al.* 2002). The other case is that eukaryotic EFs bind directly to the RNA-dependent RNA polymerase which catalyzes the synthesis of viral RNA from both negative- and positive-strand RNA templates. A classic example is that the bacteriophage Qβ replicase complex consists of EFs whose removal results in the loss of Qβ replicase activity (Blumenthal and Carmichael 1979). The interactions of EFs with viruses were also demonstrated for a vesicular stomatitis virus (Das *et al.* 1998), tobacco mosaic virus (Yamaji *et al.* 2006), and turnip mosaic virus (TuMV) (Thivierge *et al.* 2008). TuMV is a positive-strand virus with a ~10-kb RNA genome in length. In the process of duplication, it can be a template for both translation and replication, leading to the participation of multiple host factors. A recent study shows that TuMV 6K2-induced vesicles in chloroplast contain a viral protein (VPg), eukaryotic initiation factor

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Abbreviations: EF - elongation factor; F_v/F_m - variable to maximum chlorophyll fluorescence ratio; IPTG - isopropyl-b-D-thiogalactopyranoside; TuMV - turnip mosaic virus.

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(iso) 4E (eIF[iso]4E), translation factor poly(A)-binding protein (PABP), eukaryotic elongation factor 1A (eEF1A), and heat shock protein 70-3 (Thivierge *et al.* 2008). These findings may indicate that chloroplast EF-Tu isinvolved in viral infection and heat stress.

In our earlier study, we cloned the TuMV-response gene *BcLRK01* from non-heading Chinese cabbage by the cDNA-amplified fragment length polymorphism technology (Peng *et al.* 2012a). In this study, the *BcLRK01* gene was probed using a cDNA library that was constructed from TuMV-inoculated leaves of non-heading Chinese cabbage. The *BcEF-Tu* gene-encoding chloroplast elongation factor was obtained from the library. The interaction of the *BcEF-Tu* gene with *BcLRK01* and possible responses to TuMV infection and heat stress were studied.

Materials and methods

Yeast two-hybrid system: Non-heading Chinese cabbage (*Brassica campestris* ssp. *chinensis* Makino) cv. Taisankang was grown in a growth chamber under day/night temperatures of 25/18 °C, relative humidities of 85/65 %, a 16-h photoperiod, and an irradiance of 280 μmol m^{-2} s^{-1}. Four-week-old plants were TuMV-inoculated and sampled at 0, 6, 12, and 24 h, and 7 and 30 d post-inoculation. RNA was isolated and equally pooled for reverse transcription. A cDNA library was constructed according to the protocols of the *Make Your Own* "*Mate & Plate*™" library system (*Clontech*, Palo Alto, USA). In brief, cDNA was generated from pooled RNA using the *SMART* technology. The cDNA and a linearized pGADT7-Rec vector were co-trans-formed into yeast strain Y187 and plated on a synthetically defined medium without leucine (SD/-Leu) agar medium for growth. After 4 - 5 d, colonies were harvested and pooled in a rich broth medium. The medium (1 cm^3) was used for library screening. The *BcLRK01* gene was probed using the cDNA library according to the protocols of the *Matchmaker Gold* yeast two-hybrid system (*Clontech*). In brief, a BcLRK01/pGBKT7 recombinant was constructed and tested for its autoactivation and toxicity. Yeast two-hybrid screening was carried out by yeast mating. Positive interactions between prey and bait proteins were verified according to the protocols of the *Yeast Transformation System 2* (*Clontech*).

Gene expression by real-time polymerase chain reaction (PCR): Non-heading Chinese cabbage cv. Taisankang (resistant to TuMV) and cv. Xiangqingcai (susceptible to TuMV) were used. The expression analyses of the *BcEF-Tu* gene under a TuMV infection and heat stress werre carried out according to our earlier methods (Peng *et al.* 2012a). In brief, RNA was extracted using an RNA isolation kit (*Tiangen*, Peking, China) following the manufacturer's protocols. Single-strand cDNA was synthesized on 2 μg of RNA using *AMV* reverse transcriptase (*TaKaRa*, Tokyo, Japan) according to the manufacturer's instructions. The quantitative analysis of the genes was performed using the *Rotor Gene* system (*Corbett*, Sydney, Australia). PCR reaction mixtures had a volume of 0.025 cm^3 and contained 0.0125 cm^3 of a *SYBR Green* PCR mix (*TaKaRa*), 0.002 cm^3 of a template (5× diluted cDNA), 10 pmol of each primer, and 0.0085 cm^3 of sterile water. Denaturation at 95 °C for 2 min was followed by 40 cycles at 90 °C for 20 s, at 56 °C for 15 s, and at 72 °C for 20 s. Subsequently, a melting curve at 65 - 95 °C was performed to detect primer dimerization or other amplification artefacts. The primer forward sequence was 5′-GTGAGCAAGGGATGAGGTTT-3′ and the reverse sequence, 5′-TGCGAATCAAGAGTGAAACAA-3′. Results were standardized by comparing the data with the glyceradehyde-3-phosphate dehydrogenase gene (DDBJ, accession No. AB303568). Relative gene quantification was calculated using the comparative ΔC_T method (Livak and Schmittgen 2001). The statistical analysis was carried out by the Duncan's new multiple range method using the *SAS* (Chicago, USA) software.

Assessment of heat tolerance: Plants were grown in the growth chamber under the above mentioned conditions. Heat tolerance was assessed in 4-week-old seedlings cvs. Taisankang and Xiangqingcai exposed to 45 °C and 100 % relative humidity for 3 h. The temperature was increased from 25 °C to 45 °C for about 20 min. Treatment time started when the temperature reached 45 °C. After the heat stress, the plants were watered immediately and transferred to 25 °C for recovery. A control group was maintained under the normal growth conditions. After 3-d recovery, leaves were sampled from plants adapted in dark for 30 min. Chlorophyll *a* fluorescence parameters were detected using an imaging *PAM* fluorometer (*Walz*, Effeltrich, Germany) and the ratio of variable to maximal fluorescence (F_v/F_m) was calculated.

Based on the database of non-heading Chinese cabbage (http://nhccbase.njau. edu.cn), we cloned the full length *BcEF-Tu* gene (the forward primer, 5′-ATG GCGATATCGTCTCCCGCCTATTC-3′; the reverse primer, 5′-CTCGATGATAGACTGAATAACTCCAG-3′) and trans-formed it into an expression vector pGEX-4T-1 to form the BcEF-Tu/pGEX-4T-1 recombinant. The recombinant and the pGEX-4T-1 vector were transformed into the competent *Escherichia coli* strain BL21 (DE3). Heterologous gene expression was detected after 0.4 mM isopropyl-b-D-thiogalacto-pyranoside (IPTG) induction by sodium dodecyl sulfate polyacryl-amide gel electrophoresis. The transformants were then grown in lysogeny broth (LB) media at 37 °C with shaking to an absorbance of A_{600} = 0.8. Three equivalent aliquots of cultures were induced by IPTG at 51, 52, and 53 °C, respectively. Two hours after induction, the

cultures were diluted 100× times with phosphate-buffered saline (PBS) and incubated on LB agar plates at 37 °C. Cell viability was determined by counting colony-forming units.

Results

In our earlier research, we cloned the TuMV-response gene *BcLRK01* (Peng *et al.* 2012a,b) and probed it using a cDNA library of TuMV-infected leaves of non-heading Chinese cabbage. One fragment encoding chloroplast EF-Tu was obtained. We cloned the complete sequence of this gene, named *BcEF-Tu* (acc. No. KF006995), which is 1 413 bp long. A yeast two-hybrid system was used to verify a positive interaction. Co-transformation of BcLRK01/pGBKT7 and BcEF-Tu/pGADT7 was able to activate the reporter in SD/-Leu/-Trp (DDO) and SD/-Leu/-Trp/-His/-Ura (QDO) media. Co-transformation of pGBKT7 and BcEF-Tu/pGADT7 was able to activate the reporter in the DDO but not in QDO medium (Fig. 1). These findings demonstrate that *BcEF-Tu* was the interactive gene of *BcLRK01*.

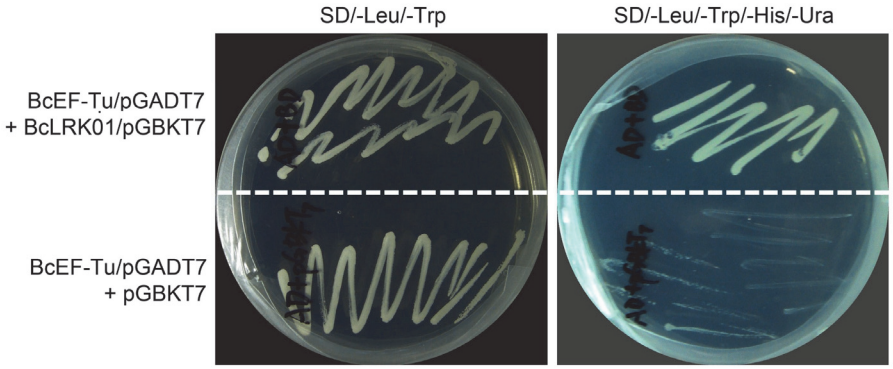

Fig. 1. Verification of an interaction between BcEF-Tu and BcLRK01 using a yeast two-hybrid system. Recombinants of BcEF-Tu/pGADT7 and BcLRK01/pGBKT7 as well as BcEF-Tu/pGADT7 and pGBKT7 were co-transformed into competent AH109 yeast cells. The transformants were grown on SD/-Leu-Trp and SD/-Leu/-Trp/-His/-Ura agar plates at 30 °C for 3 d.

Table 1. The relative expression of the *BcEF-Tu* gene under mock and TuMV inoculations. Means ± SD, $n = 3$. Values followed by different letters in the column are significantly different at $P < 0.05$.

Treatment	0 h	2 h	6 h	12 h	24 h	7 d
Mock	0.18 ± 0.01[a]	0.07 ± 0.01[a]	0.12 ± 0.03[a]	0.17 ± 0.02[a]	0.24 ± 0.06[a]	0.07 ± 0.02[a]
TuMV	0.18 ± 0.04[a]	0.08 ± 0.01[a]	0.09 ± 0.02[a]	0.14 ± 0.05[a]	0.14 ± 0.02[b]	0.07 ± 0.02[a]

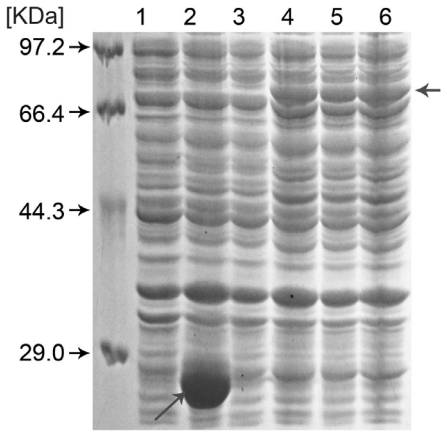

Fig. 2. The heterologous expression of the BcEF-Tu/pGEX-4T-1 recombinant. *Line* 1 - pGEX-4T-1 vector, *line* 2 - IPTG-induced pGEX-4T-1 vector; *line* 3- BcEF-Tu/pGEX-4T-1 recombinant, *lines* 4 to 6 - IPTG-induced BcEF-Tu/pGEX-4T-1 recombinant. The *arrows* point to the induced bands.

Since *BcEF-Tu* interacted with the TuMV-response gene *BcLRK01*, we wondered whether it also responds to TuMV infection. To address this question, we quantified the expression of the *BcEF-Tu* gene. The results show that TuMV obviously depressed its expression at 24 h post-inoculation (hpi). Seven days post-inoculation (dpi), the depression disappeared completely (Table 1). Since EF-Tu has been suggested to be involved in heat tolerance in maize, we wondered whether *BcEF-Tu* also responds to heat stress. To address this question, we quantified the expression of the *BcEF-Tu* gene under the heat stress in non-heading Chinese cabbage cvs. Taisankang and Xiangqingcai (Table 2). The *BcEF-Tu* gene was obviously induced by the heat stress in TuMV sensitive Xiangqingcai but not in resistant Taisankang.

To verify heat tolerance of these two cultivars, the F_v/F_m ratio, which characterizes a maximum photo-

chemical efficiency, was detected on four blades (three spots per each blade) from different plants of both the cultivars. No obvious difference in F_v/F_m before and after the heat stress was detected in cv. Taisankang. However, compared to the control, the heat stress sharply decreased F_v/F_m in cv. Xiangqingcai (Table 2).

The *BcEF-Tu* gene was transformed into the expression vector pGEX-4T-1. The heterologous expression of the transformant was induced by IPTG (Fig. 2). The vector-only form was induced with an increased expression of a GST tag (26 kDa). The IPTG-induced recombinant showed a molecular mass of approximately 78 kDa in accordance with our prediction in the *DNAman* software. Compared to the controls, the proteins did not show any increased expression without the IPTG induction. To evaluate the effect of the recombinant BcEF-Tu/pGEX-4T-1 on *E. coli* survival, *E. coli* cells transformed with BcEF-Tu/pGEX-4T-1 and pGEX-4T-1 were both subjected to a 51 - 53 °C heat stress. The viability of *E. coli* cells transformed with pGEX-4T-1 decreased significantly with the increasing temperature. There was no obvious difference in the viability of *E. coli* cells with or without the IPTG induction (Fig. 3*A*). However, survival rates were obviously greater for cells overexpressing the recombinant BcEF-Tu polypeptide (Fig. 3*B*).

Table 2. The relative expression of the *BcEF-Tu* gene and the variable to maximum chlorophyll fluorescence ratio (F_v/F_m) under a heat stress (45 °C for 3 h) in 4-week-old plants of cvs. Taisankang and Xiangqingcai. Controls were maintained under normal growth conditions and sampled at the same time as the heat stress-induced plants. The gene expression was measured immediately after the heat stress, but F_v/F_m after 3-d recovery in the normal growth conditions. Means ± SD, $n = 3$ for the gene expression, and $n = 12$ for F_v/F_m. Values followed by different letters in the column are significantly different at $P < 0.05$.

Treatment	*BcEF-Tu* expression		F_v/F_m	
	Taisankang	Xiangqingcai	Taisankang	Xiangqingcai
Control	0.24 ± 0.07[a]	0.23 ± 0.04[a]	0.78 ± 0.01[a]	0.78 ± 0.01[a]
Heat stress	0.32 ± 0.13[a]	0.67 ± 0.16[b]	0.74 ± 0.03[a]	0.63 ± 0.02[b]

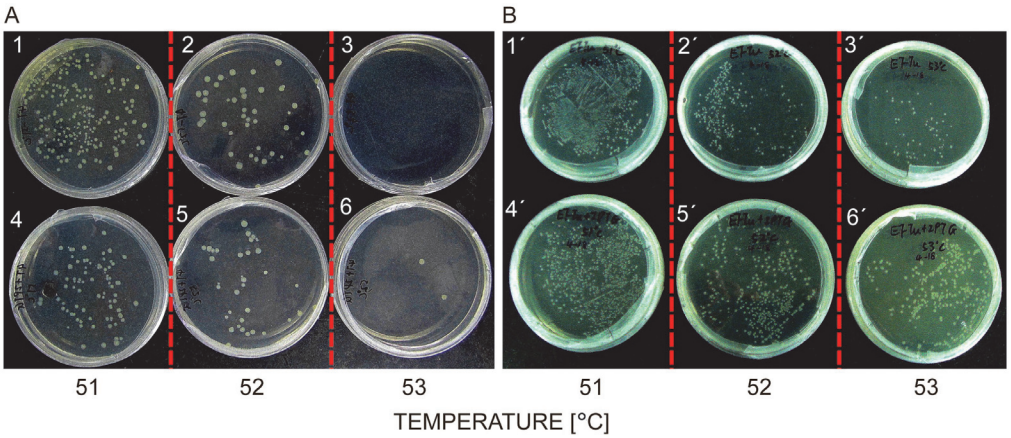

Fig. 3. Lysogeny broth agar plates inoculated with *E. coli* cultures of transformants pGEX-4T-1 (*A*) and BcEF-Tu/pGEX-4T-1 (*B*) under a heat stress. Cultures were treated at 45 °C for 2 h, diluted 1:100 (v/v) with phosphate-buffered saline, and then spread on each plate (0.05 cm^3). The plates were then incubated overnight at 37 °C. 1 to 3 - transformant pGEX-4T-1 without isopropyl-b-D-thiogalactopyranoside (IPTG) induction, 4 to 6 - transformant pGEX-4T-1 with IPTG induction; 1′ to 3′- transformant BcEF-Tu/pGEX-4T-1 without IPTG induction, 4′ to 6′ - transformant BcEF-Tu/pGEX-4T-1 with IPTG induction.

Discussion

In this study, we sampled the TuMV-inoculated leaves of non-heading Chinese cabbage at 0, 6, 12, and 24 hpi, and 7 and 30 dpi, isolated their RNAs individually, and then pooled them. The mixed RNAs were used to construct a cDNA library. Thus, the genes involved in the hyper-sensitive response and systemic acquired resistance were all included in the library.

Once attacked by pathogens, host receptors recognize the virulence and pass the signals to other parts of the organ or even to the whole plant. Leucine-rich repeat receptor-like kinases (LRR-RLKs) play important roles in signal transduction, disease defence, and environmental stresses (Torii 2004). In our earlier study, we cloned the *BcLRK01* gene which encodes a LRR-RLK protein and responds to TuMV infection. This gene was probed using the cDNA library. Twenty-eight genes were obtained, among which one gene encoding chloroplast EF-Tu was of interest. Some eukaryotic EFs have been verified to

interact with viral components and respond to heat stress (Bhadula *et al.* 2001, Zeenko *et al.* 2002, Yamaji *et al.* 2006, Thivierge *et al.* 2008). Recent research has shown that TuMV 6K-induced vesicles predominantly target chloroplasts, where they amalgamate and induce chloroplast membrane invaginations. Viral RNA, double-stranded RNA, and viral replicase components are concentrated at the 6K vesicles that are associated with chloroplasts in infected cells suggesting that chloroplast-bound 6K vesicles are the sites for potyvirus replication (Wei *et al.* 2010). Thus, we hypothesized that *BcEF-Tu* was involved in TuMV infection.

To verify the positive interaction, the BcLRK01/ pGBKT7 and BcEF-Tu/pGADT7 recombinants were co-transformed into AH109 yeast cells. Co-transformed cultures were able to grow on DDO and QDO LB agar plates, whereas co-transformants of BcLRK01/pGBKT7 and pGADT7 did not grow on QDO plates (Fig. 1). These findings indicate that the *BcEF-Tu* gene interacted with *BcLRK01*.

To determine whether the *BcEF-Tu* gene responds to TuMV infection, its expression was quantified in the mock- and TuMV-inoculated leaves (Table 1). The results show that the TuMV infection obviously depressed the expression of the *BcEF-Tu* gene at 24 hpi. The depression peaked at 24 hpi and disappeared until 7 dpi. Because the chloroplast is the site of TuMV aggregation and multiplication in plants, some host factors are involved in the process of viral infection (Thivierge *et al.* 2008). We infer that the accumulation of TuMV in chloroplasts restrained the chloroplast protein synthesis. With the infection, the virus increasingly reached an equilibration in the chloroplasts and activated systemic-acquired plant resistance. Thus, the TuMV-induced depression of the *BcEF-Tu* gene weakened gradually and then disappeared.

Chloroplast fluorescence parameters, such as F_v/F_m, are considered as markers of plant stress responses. The value of F_v/F_m is generally 0.75 - 0.85 and declines remarkably under stress. Li *et al.* (2011) used F_v/F_m to evaluate heat tolerance of *Lonicera japonica*. In this study, the heat stress significantly decreased the F_v/F_m in cv. Xiangqingcai, but not in cv. Taisankang (Table 2). This finding suggests that Taisankang is heat tolerant, whereas Xiangqingcai is heat sensitive. We detected the relative expression of the *BcEF-Tu* gene in both the cultivars under the heat stress. Compared with the controls, the heat stress induced the gene expression in cv. Xiangqingcai, but not in cv. Taisankang (Table 2). A recent study also showed that heat tolerant and heat sensitive maize lines differ in the expression of *EF-Tu* under heat stress (Momcilovic and Ristic 2007). The results suggest that the expression regulation of *EF-Tu* may differ among heat tolerant and heat sensitive cultivars under heat stress conditions and indicates that the *BcEF-Tu* gene responds to heat stress.

The *E. coli* cells transformed with BcEF-Tu/ pGEX-4T-1 and pGEX-4T-1 were subjected to the heat stress (51 - 53 °C). This temperature course was determined in our preliminary experiment, in which 54 °C was sufficient to cause death of transformants (data not shown). In our study, the heat stress obviously decreased the cell survival of the vector-only transformants regardless of the IPTG induction status (Fig. 3*A*). With increasing temperature, the *E. coli* cells overexpressing the polypeptide encoded by the *BcEF-Tu* cDNA survived at a significantly higher rate than their non-induced counterparts or the control transformants (Fig. 3*B*). These results demonstrate that the protective effect against the heat stress in the IPTG-induced transformants was a consequence of the recombinant BcEF-Tu polypeptide. Thus, the BcEF-Tu protein played a role in the development of heat tolerance in non-heading Chinese cabbage.

Since BcEF-Tu responds to TuMV infection and heat stress, we infer that it may function as molecular chaperone in plant development. This is the first report on chloroplast EF-Tu in non-heading Chinese cabbage which provides a theoretical basis for the functional research of EF-Tu.

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