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# Heterologous expression of a plastid EF-Tu reduces protein thermal aggregation and enhances $CO_2$ fixation in wheat (*Triticum aestivum*) following heat stress

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# Heterologous expression of a plastid EF-Tu reduces protein thermal aggregation and enhances $CO_2$ fixation in wheat (*Triticum aestivum*) following heat stress

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**Abstract** Heat stress is a major constraint to wheat production and negatively impacts grain quality, causing tremendous economic losses, and may become a more troublesome factor due to global warming. At the cellular level, heat stress causes denaturation and aggregation of proteins and injury to membranes leading to alterations in metabolic fluxes. Protein aggregation is irreversible, and protection of proteins from thermal aggregation is a strategy a cell uses to tolerate heat stress. Here we report on the development of transgenic wheat (*Triticum aestivum*) events, expressing a maize gene coding for plastidal protein synthesis elongation factor (EF-Tu), which, compared to non-transgenic plants, display reduced thermal aggregation of leaf proteins, reduced heat injury to photosynthetic membranes (thylakoids), and enhanced rate of $CO_2$ fixation after exposure to heat stress. The results support the concept that EF-Tu ameliorates negative effects of heat stress by acting as a molecular chaperone. This is the first demonstration of the introduction of a plastidal EF-Tu in plants that leads to protection against heat injury and enhanced photosynthesis after heat stress. This is also the first demonstration that a gene other than HSP gene can be used for improvement of heat tolerance and that the improvement is possible in a species that has a complex genome, hexaploid wheat. The results strongly suggest that heat tolerance of wheat, and possibly other crop plants, can be improved by modulating expression of plastidal EF-Tu and/or by selection of genotypes with increased endogenous levels of this protein.

**Keywords** Plastid EF-Tu · Protein aggregation · Heat injury · Transgenic wheat

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## Introduction

High temperature is a common stress for plants, restricting their growth and productivity (Boyer 1982; Lobell and Asner 2003; Peng et al. 2004) and reducing the quality of the harvested products (Stone and Nicolas 1995). Many crop plants including wheat (*Triticum aestivum*) are particularly vulnerable to heat stress (Al-Khatib and Paulsen 1989; Maestri et al. 2002). The optimum temperature for growth and yield of wheat is in the range of 18–24°C, and exposure to moderately elevated temperatures (28–32°C) even for short periods (5–6 days) reduces yield 20% or more (Stone and Nicolas 1994; Mullarkey and Jones 2000). This effect on wheat productivity is exacerbated in regions such as the North American Great Plains, where temperatures regularly exceed 30°C during the growing season (Paulsen 1994).

Heat-induced reduction in wheat yield and quality is attributed to negative effects of heat stress on cell structures and physiological and metabolic processes. High temperatures disrupt the integrity of cellular membranes and cause changes in enzyme activity that lead to alterations in the rates of biochemical reactions and ultimately an imbalance in metabolic pathways (Berry and Björkman 1980; Levitt 1980; Larkindale et al. 2005). The molecular basis of heat damage involves changes in protein conformation (Levitt 1980). The kinetic energy of polypeptide molecules increases with temperature, disrupting the forces that maintain proteins in their native state. This disruption leads to protein unfolding (denaturation) and exposure of hydrophobic residues that are normally buried in the active enzyme. Transient protein unfolding is reversible (Tanford 1968). However, during prolonged exposure to elevated temperature, the hydrophobic residues of denatured proteins interact, causing irreversible aggregation leading to loss of cell integrity and function (Levitt 1980). It is generally accepted that prevention of either protein thermal denaturation or aggregation constitutes the basis for plant thermotolerance (Levitt 1980).

Plants have evolved mechanisms to prevent or reduce protein aggregation during heat stress. One such mechanism is the synthesis of heat shock proteins (HSPs) (Vierling 1991; Schöffl et al. 1998; Feder and Hofmann, 1999; Maestri et al. 2002). Heat shock proteins act as molecular chaperones by binding and stabilizing partially unfolded or denatured proteins, protecting them from thermal aggregation and thereby facilitating their re-folding during recovery from stress (Vierling 1991; Hendrick and Hartl 1993; Feder and Hofmann 1999). Prevention of protein thermal aggregation is, thus, a critical factor contributing to plant heat tolerance.

Enhanced tolerance towards heat stress is a target for improving crop productivity. Efforts to improve the heat tolerance of wheat plants have historically relied on classical breeding techniques, utilizing both exotic and cultivated germplasm. Biotechnological approaches to enhance thermotolerance in plants have not been reported in wheat, although studies in several other plant species including *Arabidopsis*, ectopically expressing a cytosolic HSP101 (Queitsch et al. 2000), *Oryza sativa* expressing a *Arabidopsis* cytosolic HSP101 (Katiyar-Agarwal et al. 2003), *Daucus carota* carrying an extra copy of a cytosolic HSP 17.7-CI (Malik et al. 1999), and *Nicotiana tabacum* transformed with a *Lycopersicon esculentum* mitochondrial HSP 23.8 (Sanmiya et al. 2004) have demonstrated that the tools of biotechnology can be employed to improve upon a plant's response to this abiotic stress.

Earlier studies have suggested that plastid protein synthesis elongation factor, EF-Tu, may be a significant determinant of heat tolerance in plants (Bhadula et al. 2001; Rao et al. 2004; Ristic et al. 2004). Chloroplast EF-Tu (45–46 kD) is a member of a highly conserved nuclear-encoded multi-gene family (Baldauf et al. 1990; Sugita et al. 1994; Maurer et al. 1996), and plays a role in protein synthesis by promoting the GTP-dependent binding of aminoacyl-tRNA to the A site of the ribosome (Riis et al. 1990). A recent study has revealed that in wheat, this protein is upregulated under heat stress conditions and those wheat genotypes that accumulate more EF-Tu display better tolerance to heat stress (Ristic et al. 2008). Previous studies on EF-Tu in maize have revealed (a) positive correlation between the heat-induced accumulation of EF-Tu and plant ability to tolerate heat stress (Bhadula et al. 2001; Momcilovic and Ristic 2007), (b) increased thermotolerance in *Escherichia coli* expressing maize EF-Tu (Moriarty et al. 2002), (c) decreased thermotolerance in a maize mutant with reduced capacity to express EF-Tu (Ristic et al. 2004), and (d) reduced thermal aggregation of chloroplast proteins in maize with higher levels of EF-Tu (Ristic et al. 2004; Momcilovic and Ristic 2004). It has been hypothesized that EF-Tu confers heat tolerance by acting as a molecular chaperone (Rao et al. 2004). The evidence supporting this hypothesis includes the observation that the recombinant precursor of this protein (pre-EF-Tu) protects heat labile proteins, citrate synthase and malate dehydrogenase, from thermal aggregation and inactivation (Rao et al. 2004), and that the purified native pre-EF-Tu from both wheat and maize reduces thermal aggregation of photosynthetic enzyme, Rubisco activase (Ristic et al. 2007).

We hypothesized that increasing the expression of chloroplast EF-Tu may lead to protection of cellular proteins against thermal aggregation in wheat. To test this hypothesis, we introduced into wheat the maize gene coding for plastidal EF-Tu. Here we report on the expression of the transgene, and its effect on thermal aggregation of leaf proteins in transgenic plants. In addition, we also report on the heat stability of photosynthetic membranes (thylakoids) and rate of $CO_2$ fixation in young transgenic plants following exposure to heat stress.

## Materials and methods

### Transformation vector construction

A cDNA (*Zmeftu1*, 1457 bp) for maize (*Zea mays*) chloroplast protein synthesis elongation factor, EF-Tu, was previously isolated from maize line B73 (Bhadula et al. 2001). The *Zmeftul* cDNA was subcloned downstream of the maize ubiquitin 1 promoter coupled with its first intron and terminated *Agrobacterium tumefaciens* nopaline synthase polyadenylation signal. The assembled plant

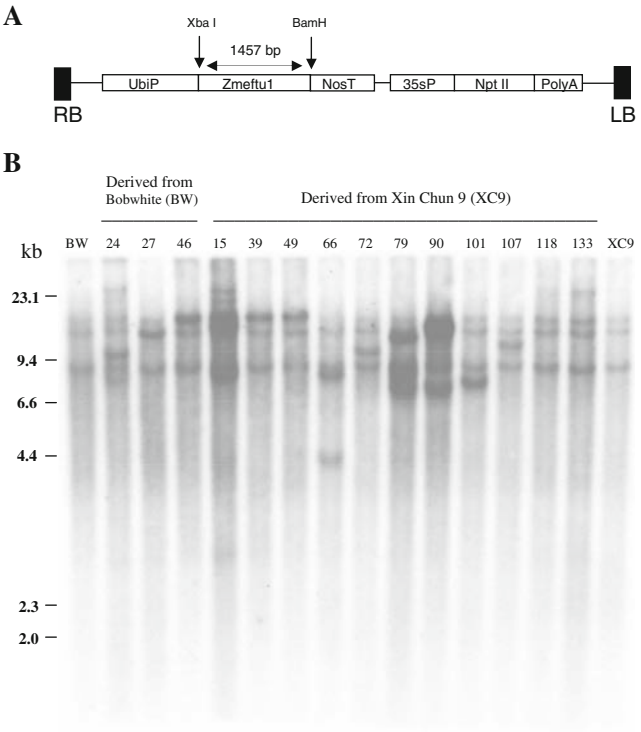



**Fig. 1** (**a**) Schematic representation of the engineered binary expression vector (*pPTN453*) used for *Agrobacterium*-mediated transformation of wheat. The vector is a derivative of *pPZP211* (Hajdukiewicz et al. 1994). *UbiP*, maize ubiquitin promoter (Christensen et al. 1992); *Zmeftu1*, cDNA for maize chloroplast protein synthesis elongation factor (EF-Tu) (Bhadula et al. 2001); *NosT*, nopaline synthase gene terminator; *35sP*, cauliflower mosaic virus 35S promoter; *NptII*, G418 resistance gene; *PolyA*, polyadenylation signal sequence; RB, right border; LB, left border. (**b**) Blot analysis of genomic DNA from transgenic wheat. Genomic DNA was extracted from PCR-positive $T_1$ plants of each putative event. DNA was digested with *Hind III*, fractionated on a 0.8% agarose gel, transferred to nylon membranes, and hybridized with the $^{32}$P-labelled *Zmeftu1* cDNA (Bhadula et al. 2001). BW, non-transgenic wheat cultivar Bobwhite; XC9, non-transgenic wheat cultivar Xin Chun 9. Numbers above the blot indicate $T_1$ generation transgenic plants from independent events. $T_1$ plant 24 (cell event 372-7-1-1), 27 (cell event 372-4-1-1), and 46 (cell event 372-6-4-2) were derived from BW; $T_1$ plant 15 (cell event 371-6-2-2), 39 (cell event 371-6-4-1), 49 (cell event 375-3-1-1), 66 (cell event 375-3-2-1), 72 (cell event 382-5-1-1), 79 (cell event 382-5-2-1), 90 (cell event 382-6-1-2), 101 (cell event 382-7-1-1), 107 (cell event 382-7-3-1), 118 (cell event 382-8-1-3), and 133 (cell event 382-8-2-4) were derived from XC9 (see Supplemental Table 1 online)

expression cassette was cloned into the binary plasmid pPZP211 (Hajdukiewicz et al. 1994) which harbors an npt II cassette for plant selection. The derived binary plasmid is referred to as *pPTN453* (Fig. 1a).

## Plant transformation

The binary vector pPTN453 was mobilized into *A. tumefaciens* strain C58C1/pMP90 (Koncz and Schell 1986) via tri-parental mating. A selected transconjugant was used for transformation. Wheat (*Triticum aestivum*) transformations were conducted as described by Cheng et al. (1997). Briefly, immature embryos, approximately 14 days post-anthesis, isolated from stock plants cultured under growth chamber conditions under a 16 h light regime, where daylight temperature was held at 20°C and a dark temperature of 12.8°C, were cultured on a MS-based medium supplemented with 0.5 mg/l 2,4-D, 2.2 mg/l picloram, 4% maltose in the dark at 24°C for four days. Following the four day preculture period embryos were inoculated for 30 min with the *A. tumefaciens* transconjugant and co-cultured for 2 day in the dark. Transgenic embryogenic tissue was selected on medium supplemented with 15 mg/l G418 for 2 weeks, followed by a step-up selection to 25 mg/l G418 during the regeneration steps. *Agrobacterium tumefaciens* counter selection was carried out with antibiotic supplements to the callus initiation and regeneration media formulations, while the antibiotic supplements used to counter select the bacterium were removed during the rooting phase. Putative primary transformants were grown to maturity under growth chamber conditions used for maintenance of stock plants.

## Transgenic plant materials and growth conditions

$T_1$ and $T_2$ generation plants were used for analysis. Seeds of both transgenic and non-transgenic [cultivars Bobwhite (BW) and Xin Chun 9 (XC9)] plants were sown in pots containing Metro Mix 200 potting soil (Hummert Intl. Topeka, KS). Plants were grown in a growth chamber (day/night temperature of 21/17°C, photoperiod 16/8 h, relative humidity 70–80%, *PPF* 280 µmol $m^{-2}$ $s^{-1}$ [Sylvania cool white fluorescent lamps]) and watered daily and fertilized weekly with "Miracle Gro" fertilizer (Stern's Miracle-Gro products, Inc. Port Washington, NY) according to manufacturer instructions. Plants of both generations, $T_1$ and $T_2$, were grown to harvest maturity. For PCR and genomic DNA blot analysis, leaf tissue was collected from 3-week-old plants; $T_1$ generation was used for genomic DNA blotting, and $T_1$ and $T_2$ generations were used for PCR. For EF-Tu protein analysis, leaf tissue was collected from flag leaves of mature (at flowering stage) $T_2$ generation plants. Collected leaves were frozen in liquid $N_2$ and stored at −80°C until used.

## Extraction of genomic DNA and PCR analysis

Genomic DNA was extracted from the leaf tissue using a published procedure (Dellaporta et al. 1983) and used as a template for PCR. PCR analysis was conducted to distinguish plants that carry *Zmeftu1* (maize gene for EF-Tu).

Maize ubiquitin promoter specific forward primer UBI2 (5′-AGCCCTGCCTTCATACGCTATTTATTTGC) was coupled with the *Zmeftu1* gene specific reverse primer EFTU2 (5′-CGATATCGACTGTGTCACCAATCTTGAC). The expected size of the PCR product was 1,066 bp. PCR reactions contained 0.4 μM of each primer, 0.4 mM dNTPs, 500 ng of genomic DNA, and 1.25 U of JumpstartREDTaq DNA polymerase (Sigma). PCR program was conducted with initial denaturation at 94°C for 3.5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 47 s, and extension at 72°C for 1.5 min. PCR products were resolved on 1% agarose gels.

## Genomic DNA blotting

*Zmeftu1* cDNA sequence (1.4 kb bp) was excised from plasmid *pPTN453* using restriction enzymes *BamH I* and *Xba I*, purified from an agarose gel using QIAGEN Gel Purification kit, and labeled with ($\alpha$-$^{32}$P)dCTP using Megapriming DNA Labeling System (Amersham, UK). The labeled cDNA was used as a probe for genomic DNA blotting; prior to genomic DNA blotting we conducted DNA blot analysis using the plasmid (*pPTN453*) as a template and maize EF-Tu cDNA as a probe, and the blot showed a very strong hybridization signal (not shown). Genomic DNA was extracted from $T_1$ plants (Dellaporta et al. 1983) and digested with restriction enzyme *Hind III*. This enzyme does not cut *Zmeftu1*. Fragments of digested DNA were separated on 0.8% agarose gels, transferred to hybridization membrane (Hybond-XL, Amersham, UK), and hybridized according to a standard protocol (Sambrook et al. 1989).

## EF-Tu protein analysis

Chloroplast EF-Tu was analyzed using 1-D SDS-PAGE and 1-D immunoblotting. Total soluble leaf proteins were extracted from five PCR-positive $T_2$ plants from each event and proteins from each $T_2$ plant were analyzed separately. Proteins were extracted in a buffer containing 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 10% glycerol, 10 mM $\beta$-mercaptoethanol, and 1% protease inhibitor cocktail (v/v, Sigma). Protein concentration was determined using Quick Start Bradford Protein Assay Kit (Bio-Rad). Proteins were separated on 10% SDS polyacrylamide gels. Equal amounts of protein (15 μg per well) were loaded on the gels. For 1-D immunoblotting, proteins separated on SDS-PAGE were transferred to PVDF membranes (Bio-Rad), and then probed for chloroplast EF-Tu using an antibody raised against maize chloroplast EF-Tu (Bhadula et al. 2001). Previous studies have shown that this antibody also cross-reacts with wheat chloroplast EF-Tu (Ristic et al. 2007, 2008). A protein sample prepared from leaf tissue of maize line B73 was also included, as a control. For checking on equal loading, the same PVDF membranes that were probed for chloroplast EF-Tu were stained with Ponceau S (Fisher Scientific).

## Assessment of protein thermal aggregation

Total soluble proteins were extracted from flag leaves of 7-week-old plants. Leaves were frozen and ground in liquid $N_2$. Proteins were extracted in a protein extraction buffer [50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 10% glycerol, and 1% protease inhibitor cocktail (v/v, Sigma)], and protein concentration was determined as described above. Aliquots (200 μl; protein concentration 300 μg ml$^{-1}$) were incubated at 53°C for 45 min in a temperature-controlled micro-multi cell spectrophotometer (Shimadzu, Japan), and the thermal aggregation of proteins was assessed by monitoring light scattering at 320 nm during incubation (Rao et al. 2004).

## Assessment of heat injury to thylakoid membranes

Heat injury to thylakoid membranes was assessed in non-transgenic plants (XC9) and in $T_2$ generation transgenic plants of events 101 and 15. Events 101 and 15 were chosen because 101 showed the highest and 15 the lowest expression of chloroplast EF-Tu (Fig. 2a). Plants of each genotype (XC9, 15, and 101) were grown in 10 pots (5 plants per pot; pot diameter at the top and the bottom was 21 and 16 cm, respectively; pot height, 20 cm) in a growth chamber. The growth conditions were as described above. Seven-week-old plants of each genotype were divided into two groups: 1) control (5 pots) and 2) heat stress (HS-18, 5 pots). The control group was maintained under growth conditions, and the heat stress group was exposed to 45°C (RH, 90–100%; *PPF* 280 μmol m$^{-2}$ s$^{-1}$) for 18 h in a growth chamber. The temperature was gradually increased from 21 to 45°C over 1 h, and exposure time for heat treatment started when temperature reached 45°C. During heat stress treatment, air temperature and relative humidity were continuously monitored. To minimize/avoid possible dehydration of the leaf tissue, pots of the HS−18 and control group were kept in trays containing ~1 cm deep water. After heat treatment, plants were allowed to recover under growth conditions in a growth chamber for 6 days. Damage to thylakoid membranes was assessed by measuring chlorophyll *a* fluorescence in intact 1-h dark-adapted leaves immediately before heat stress (0 h at 45°C), immediately after heat stress (18 h at 45°C), and after 3 and 6 days of recovery (Momcilovic and Ristic 2007). For fluorescence measurements, leaf samples were obtained from the youngest, fully expanded leaves from all plants from each of 5 pots from both control and HS-18

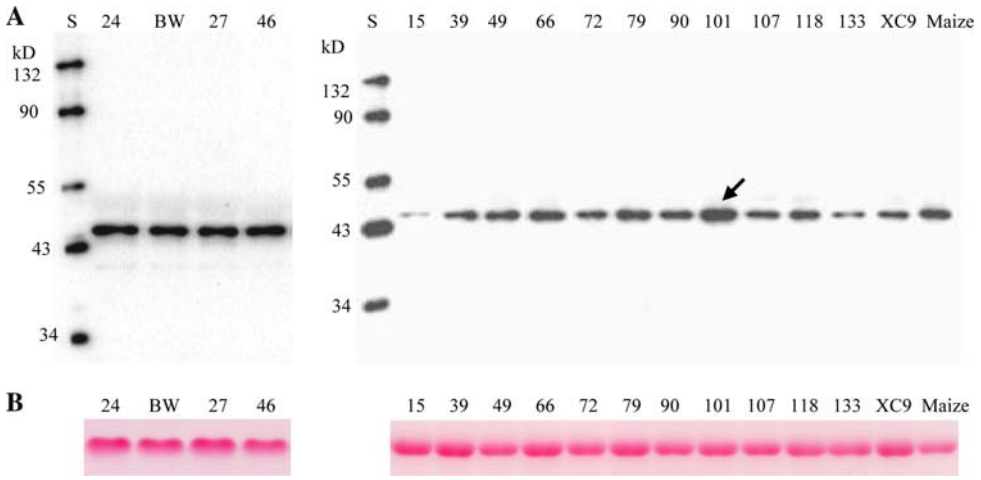



**Fig. 2** Protein analysis in $T_2$ generation plants of transgenic wheat. (**a**) One-dimensional immunoblots showing relative levels of chloroplast EF-Tu in 14 transgenic events. Total proteins were extracted from the leaf tissue of $T_2$ plants derived from 14 $T_1$ plants representing 14 independent events. Proteins were separated on 10% SDS PAGE gels and then transferred to PVDF membranes. The blots were probed with the polyclonal antibody raised against maize chloroplast EF-Tu (Bhadula et al. 2001). Equal amount of protein (15 µg) was loaded in each lane. Numbers above the immunoblots indicate plants derived from independent events (each plant was derived from one independent event); BW, non-transgenic wheat cultivar Bobwhite; XC9, non-transgenic wheat cultivar Xin Chun 9; transgenic plants 24, 27, and 46 are derived from BW; transgenic plants 15, 39, 49, 66, 72, 79, 90, 101, 107, 118, and 133 are derived from XC9; Maize, protein sample from maize line B73 (included as a control); S, Protein standards (Santa Cruz Biotechnology, CA). Note: increased relative amount of EF-Tu protein in transgenic plants/events 101, 66, and 79; decreased relative amount of EF-Tu in transgenic plants/events 15 and 133. Similar results on the relative levels of EF-Tu in transgenic and non-transgenic plants were obtained in a duplicate immunoblot. Arrow indicates transgenic event/plant that showed the highest relative level of EF-Tu. (**b**) The immunoblots shown in panel A were stained with Ponceau S to check on equal loading. The stained blots show Rubisco large subunit. Numbers above the blots indicate plants derived from independent events; these are the same plants shown on immunoblots in panel A. Maize, protein sample from maize line B73 (included as a control)

group. The ratio of constant fluorescence (O) and the peak of variable fluorescence (P) (O/P) was used for assessment of the damage to thylakoids (Krause and Weis 1984; Ristic et al. 2008). Prior to heat treatment, all transgenic plants were PCR tested (as described above), and only fluorescence data obtained from the PCR positive plants were used for assessment of thylakoid damage.

### Measurements of $CO_2$ fixation

Measurements of $CO_2$ fixation were conducted under growth conditions on the same leaves that were used for measurements of chlorophyll *a* fluorescence. Measurements were taken at the same time when fluorescence was measured using CI-340 Photosynthesis System (Camas, WA, USA) according to the manufacturer instructions.

### Statistical analysis

Analysis of variance and student *t*-test (two sample assuming unequal variances) were employed to test the difference in heat-injury of the thylakoid membranes and rate of $CO_2$ fixation between non-transgenic and transgenic genotypes (SAS 2003).

## Results

### Generation of transgenic plants and confirmation of heritable insertions

The open reading frame of the maize chloroplast EF-Tu cDNA *Zmeftu1*, isolated from line B73 (Bhadula et al. 2001), was fused to a maize ubiquitin promoter, coupled with its first intron (Christensen et al. 1992, Fig. 1a) and introduced into wheat cultivars Bobwhite (BW) and Xin Chun 9 (XC9) via *Agrobacterium*-mediated transformation. A total of 24 primary transformants ($T_0$) were established in the greenhouse. $T_0$ plants from 23 events produced $T_1$ seeds. Segregation analysis was conducted on progeny derived from 14 events via PCR and genomic DNA blot analysis using *Zmeftu1* cDNA as a probe (Bhadula et al. 2001). Inheritance of the transgenic allele was confirmed across the progeny derived from the 14 independent events, three of which were BW genetic background, while the remaining eleven events were XC9 genetic background (Supplemental Table 1 and Supplemental Fig. 1; Fig. 1b). A genomic DNA blot analysis on progeny derived from the respective events is shown in Fig. 1b. The three hybridization signals observed in the control BW and XC9 lanes most likely reflect the endogenous EF-Tu alleles in wheat,

while the unique banding patterns across the transgenic events indicate the number of transgenic loci present per independent event.

### Expression analysis of *Zmeftu1*

Relative levels of EF-Tu protein accumulation in transgenic and non-transgenic plants were monitored using one-dimensional (1-D) immunoblotting (Bhadula et al. 2001). This analysis also included leaf protein extracts from maize line B73, as a control to compare the molecular mass of wheat and maize EF-Tu. The 1-D immunoblot analysis of protein extracts from transgenic and non-transgenic plants revealed a single band across the wheat samples co-migrating with that of maize EF-Tu (Fig. 2a) suggesting that the transit peptide of the maize EF-Tu is properly processed in wheat following the import of the EF-Tu protein into the chloroplast. Importantly, this analysis also revealed that several transgenic events had modulated levels of EF-Tu. Events 101, 66, and 79 had increased plastidal EF-Tu, while events 15 and 133 displayed reduced accumulation of this protein (Fig. 2a). Of all events that displayed enhanced accumulation of EF-Tu, event 101 had the highest level of this protein. Compared to non-transformed XC9, 101 showed over three fold increase in the level of chloroplast EF-Tu (Fig. 2a, indicated by arrow).

### Expression of *Zmeftu1* does not affect general morphology and time to flowering of transgenic wheat

To investigate potential impacts on plant development due to the modulation of EF-Tu, we visually examined general morphology and time to flowering of transgenic ($T_2$ generation) and non-transgenic plants during their growth to harvest under greenhouse conditions. Increasing levels of plastidal EF-Tu did not appear to influence plant growth and development, as general morphology (appearance) (Fig. 3) and time to flowering (54–55 days) of transgenic plants were similar to those of non-transgenic controls.

### Transgenic plants expressing *Zmeftu1* display reduced thermal aggregation of leaf proteins

After confirming the expression of maize EF-Tu in transgenic wheat, we investigated thermal aggregation of total leaf proteins in 7-week-old plants from $T_2$ individuals derived from transgenic events 101 and 66 (enhanced EF-Tu events, Fig. 2a), and non-transgenic XC9. Three PCR-positive individuals (containing *Zmeftu1*) and one PCR-negative ($T_2$ null individual) plant were randomly chosen from each event for the thermal aggregation experiment (Fig. 4). Total proteins were extracted from flag leaves, incubated at high temperature (45 min at 53°C), and protein aggregation was assessed by monitoring light scattering during incubation (Rao et al. 2004). The results showed that protein extracts from all three PCR-positive plants of events 101 and 66 had a lower level of thermal aggregation, indicated by lower relative light scattering, than protein extracts from non-transgenic XC9 (Fig. 5 a–c, e–g). Protein extracts from the PCR-negative plants, on the other hand, had the level of thermal aggregation similar to that of XC9 (Fig. 5d, h).

We also investigated thermal aggregation of leaf proteins in 7-week-old plants of event 15, an event that showed reduced accumulation of EF-Tu (Fig. 2a). From this event, 150 $T_2$ plants were PCR-tested for the presence of *Zmeftu1* gene, and all of them tested positive. Protein extracts from three randomly chosen PCR-positive plants (Fig. 4) showed a pattern of protein aggregation similar to that of non-transgenic XC9 (Fig. 6 a–c).

### Transgenic plants expressing *Zmeftu1* show reduced injury to thylakoid membranes and increased rate of $CO_2$ fixation after exposure to heat stress

We examined heat injury of thylakoid membranes and measured $CO_2$ fixation in transgenic progeny derived from events 101 (enhanced EF-Tu expression) and 15 (reduced EF-Tu expression) and non-transgenic (XC9) plants following exposure to heat stress. Immediately after heat treatment, all the plants showed damage to their thylakoids as indicated by increases in the O/P ratios (Krause and Weis 1984; Momcilovic and Ristic 2007; Ristic et al. 2008), but the O/P increase in event 101, the event with higher levels of EF-Tu, was significantly less than in non-transgenic XC9 and event 15 (Fig. 7a). The differences in O/P between 101 and XC9/15 were even greater and became highly significant after 3 and 6-d recovery (Fig. 7a).

Transgenic plants with increased levels of plastidal EF-Tu (event 101) also displayed greater photosynthetic capability (greater $CO_2$ fixation) than non-transgenic plants following exposure to heat stress. $CO_2$ fixation rates were only about half after heat treatment for all three genotypes (XC9, events 101 and 15) and there was no significant difference among the genotypes right after the treatment (Fig. 7b). However, event 101 had significantly higher $CO_2$ fixation rate than non-transgenic XC9 3 and 6 d after recovery (Fig. 7b). Also, transgenic plants of event 101 appeared to have fewer visible signs of injury after 6 days of recovery from heat stress compared to plants of XC9 and event 15 (Fig. 7c).

## Discussion

We introduced a maize gene coding for plastid targeted protein synthesis elongation factor, EF-Tu (*Zmeftu1*), into

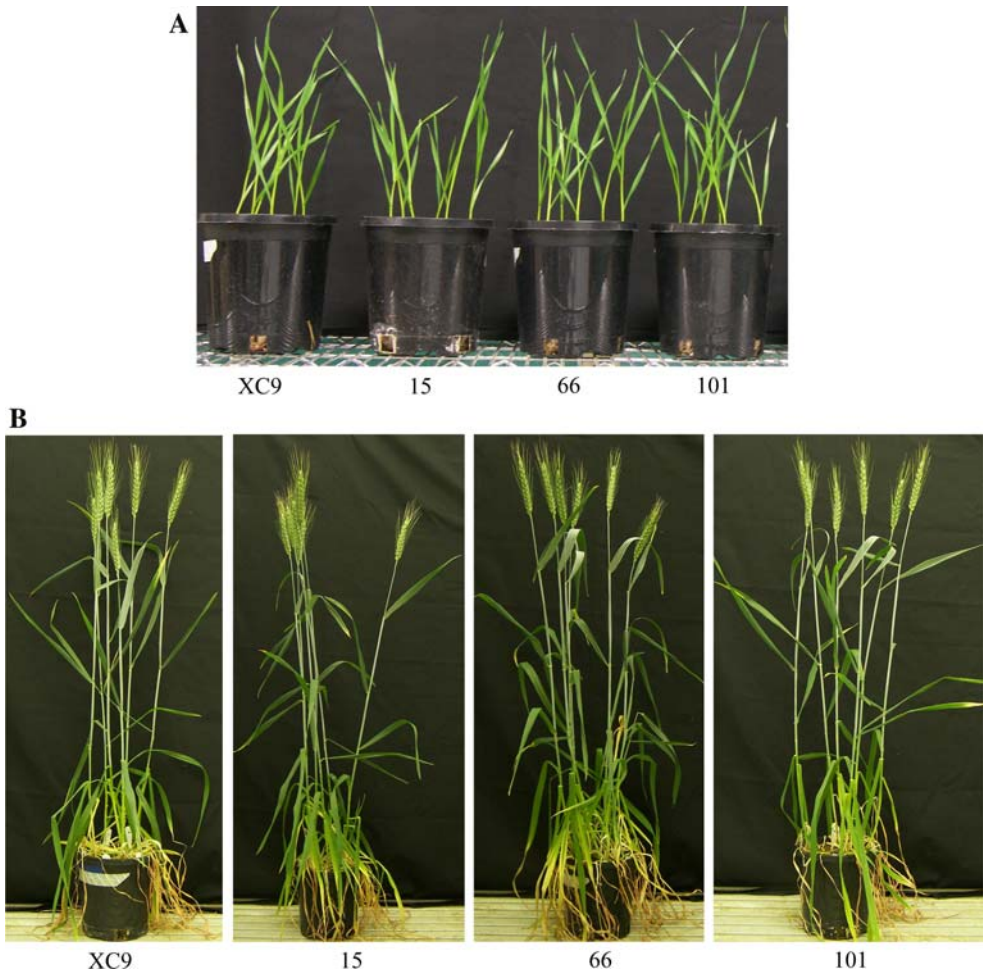



**Fig. 3** Relative levels of chloroplast EF-Tu protein do not affect general morphology and appearance of transgenic wheat plants. (**a**) 15-day-old plants. (**b**) 10-week-old plants (at flowering stage). XC9, non-transgenic wheat cultivar Xin Chun 9; 15, plant/event that underexpresses chloroplast EF-Tu (see Fig. 2a); 66 and 101, plants/events that overexpress chloroplast EF-Tu (see Fig. 2a)

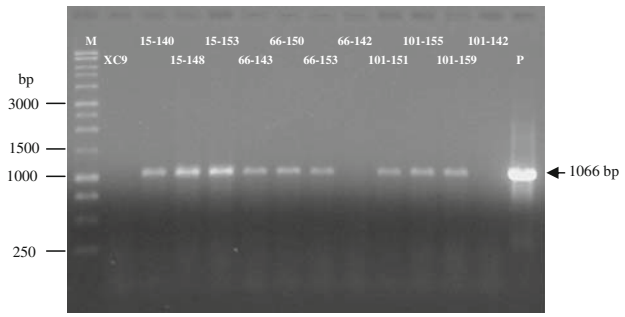
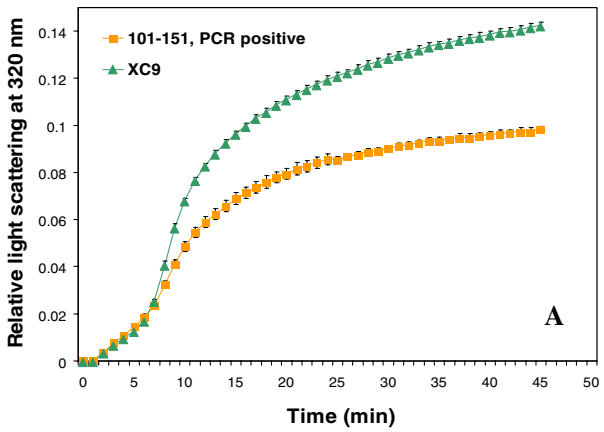
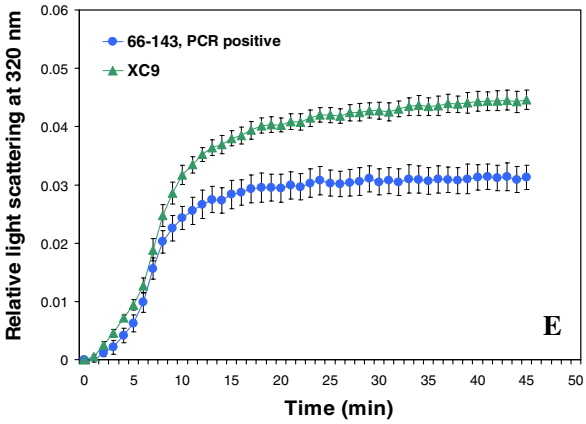


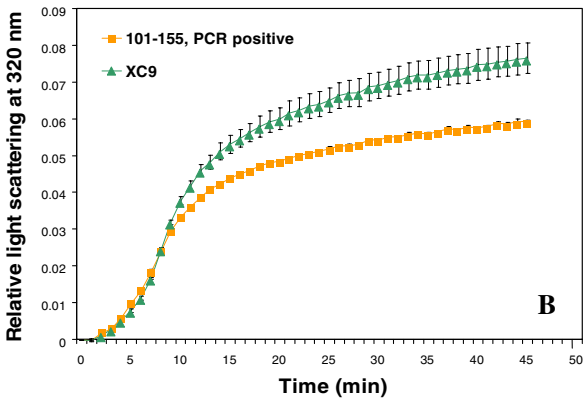

**Fig. 4** PCR products of plants for thermal aggregation assays (shown in Figs. 5 and 6). A maize ubiquitin promoter-specific forward primer and a *Zmeftu1* gene-specific reverse primer were used. $T_2$ plants 101-151, 101–155, 101–159 and 101–142 were derived from event 101 (cell line 382–7–1–1). $T_2$ plants 66–143, 66–150, 66–153 and 66–142 were derived from event 66 (cell line 375-3-2-1). $T_2$ plants 15-140, 15–148 and 15–153 were derived from event 15 (cell line 371-6-2-2) (see Supplemental Table 1 online). XC9, non-transgenic control. P, PCR products from plasmid *pPTN453* template. The 10 µl out of 25 µl PCR reaction volume was loaded on a 1% agarose gel

two cultivars of hexaploid wheat (BW and XC9). Molecular analysis (PCR and genomic DNA blotting) of transgenic plants confirmed the insertion of the transgene into the wheat genome. Moreover, molecular analysis revealed possible existence of three copies of endogenous EF-Tu gene in wheat as genomic DNA blots of two non-transgenic controls (BW and XC9) probed with maize EF-Tu cDNA showed three hybridization bands (Fig. 1b). These hybridization bands also indicate a high similarity between maize and wheat EF-Tu gene sequences. Indeed, alignment of a wheat plastidal cDNA sequence (TC264979) from wheat cDNA (EST) database (http://compbio.dfci.harvard.edu/tgi/) and the maize EF-Tu cDNA probe sequence shows 88% identity. At the protein level, these two plastidal EF-Tu sequences show 88% identity and 93% similarity (BLAST 2 Sequences Program; www.ncbi.nlm.nih.gov).

Several transgenic events showed modulated expression of chloroplast EF-Tu. Three events (101, 66, and 79)


101-151, PCR positive
XC9
Relative light scattering at 320 nm
Time (min)
A


66-143, PCR positive
XC9
Relative light scattering at 320 nm
Time (min)
E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Time (min)
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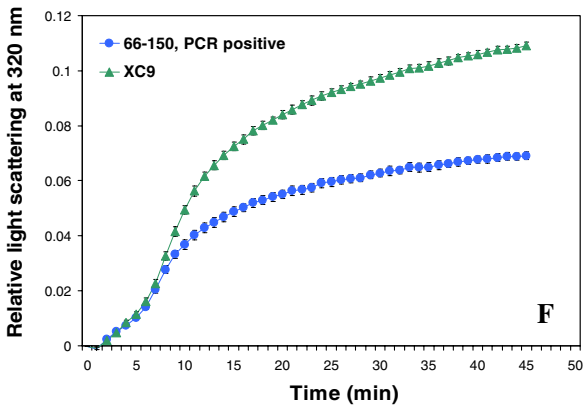
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XC9
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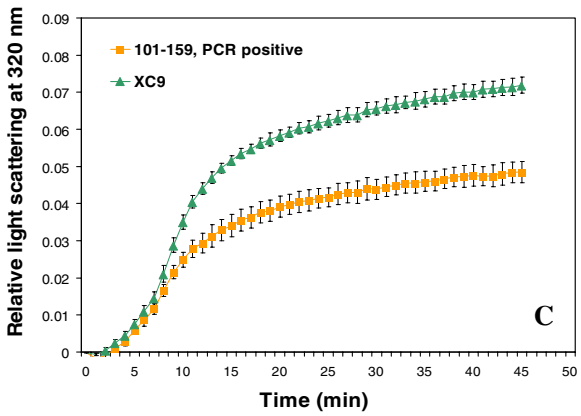
Time (min)
F


101-159, PCR positive
XC9
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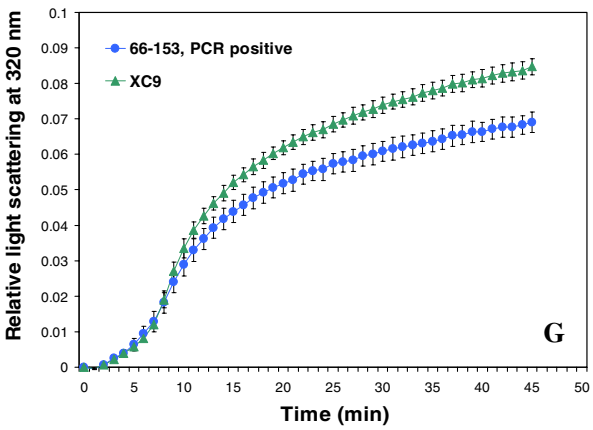
Time (min)
C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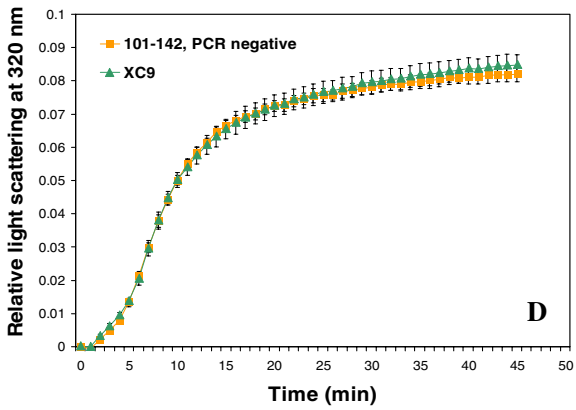
XC9
Relative light scattering at 320 nm
Time (min)
G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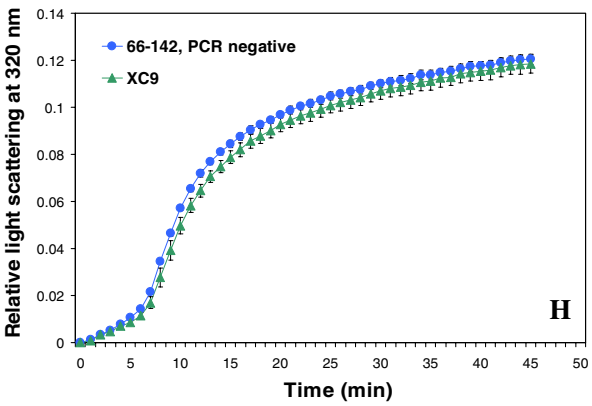
XC9
Relative light scattering at 320 nm
Time (min)
D


66-142, PCR negative
XC9
Relative light scattering at 320 nm
Time (min)
H

◀**Fig. 5** Overexpression of maize chloroplast protein synthesis elongation factor, EF-Tu, leads to reduction of thermal aggregation of leaf proteins in transgenic wheat. (**a**–**h**), Relative light scattering of leaf protein extracts from transgenic plants of events 101 (**a**–**d**), and 66 (**e**–**h**). Proteins were extracted from flag leaves of 50-days old PCR-positive (containing *Zmeftu1* gene) and PCR–negative (without *Zmeftu1* gene) transgenic plants from the segregated $T_2$ population of events 101, 66 (showed increased accumulation of EF-Tu; Fig. 2a). Total soluble proteins were also extracted from flag leaves of non-transgenic (XC9) 50-d-old plants and used as a control. Protein concentration was adjusted to 300 µg $ml^{-1}$. Protein samples (200 µl) were incubated at 53°C for 45 min in a temperature-controlled micro-multi cell spectrophotometer (Shimadzu, Japan), and protein aggregation was assessed by monitoring light scattering at 320 nm during incubation (Rao et al. 2004). In each panel, data represent averages of three independent measurements. Bars indicate standard errors. Increase in light scattering indicates protein aggregation (Rao et al. 2004). Note that compared to non-transgenic wheat XC9, transgenic wheat overexpressing EF-Tu (events 101 and 66) showed reduced thermal aggregation of leaf proteins

showed increased and two (15 and 133) decreased levels of this protein. The increased level of EF-Tu in transgenic 101, 66, and 79 was likely due to the contribution of *Zmeftu1*. On the other hand, the reduction in the level of EF-Tu in transgenic events 15 and 133 was possibly caused by down-regulation of transcript accumulation induced by post-transcriptional gene silencing (Matzke and Matzke 1995) due to the high degree of homology between the endogenous wheat EF-Tu alleles and the introduced transgenic allele, *Zmeftu1* (Fig. 1b). Importantly, the increased expression of EF-Tu in transgenic events did not seem to have any detrimental effect on plant growth and development as the general morphology and time to flowering of transgenic plants were comparable to those of non-transgenic control. This suggests that transformation of wheat with a maize gene for EF-Tu may not compromise agronomic performance in wheat under non-stress conditions, however, to ascertain impact on productivity requires extensive field evaluations.

We tested the hypothesis that increased expression of chloroplast EF-Tu may lead to protection of cellular proteins against thermal aggregation. This hypothesis was tested by investigating thermal aggregation of total leaf proteins in $T_2$ plants of transgenic events 101 and 66 (enhanced EF-Tu events) and non-transgenic XC9. The results supported the above hypothesis. They showed that transgenic events with increased expression of chloroplast EF-Tu display reduced thermal aggregation of leaf proteins (Fig. 5). This observation is in concurrence with a study of Momcilovic and Ristic (2004) who examined thermal aggregation of chloroplast proteins from two lines of maize with contrasting levels of EF-Tu. They observed that thermal aggregation of chloroplast proteins (55°C) was less extensive in a line of maize with higher levels of EF-Tu relative to a line with lower levels of EF-Tu (Momcilovic and Ristic 2004).

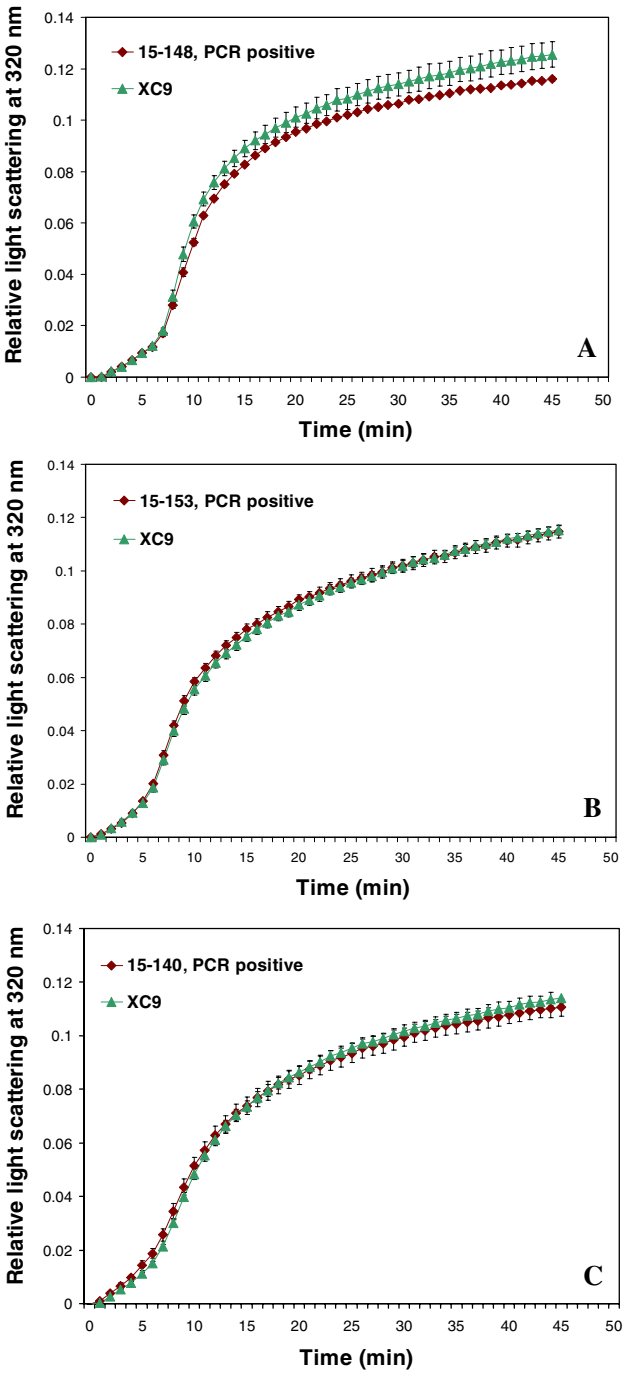



**Fig. 6** Relative light scattering of leaf protein extracts from non-transgenic, XC9, and transgenic plants of event 15 (showed under-expression of chloroplast EF-Tu; Fig. 2a). Total soluble proteins were extracted from flag leaves of 50-days old plants. Protein concentration was adjusted to 300 µg $ml^{-1}$. Protein samples (200 µl) were incubated at 53°C for 45 min in a temperature-controlled micro-multi cell spectrophotometer (Shimadzu, Japan), and protein aggregation was assessed by monitoring light scattering at 320 nm during incubation (Rao et al. 2004). In each panel, data represent averages of three independent measurements. Bars indicate standard errors. Increase in light scattering indicates protein aggregation (Rao et al. 2004)

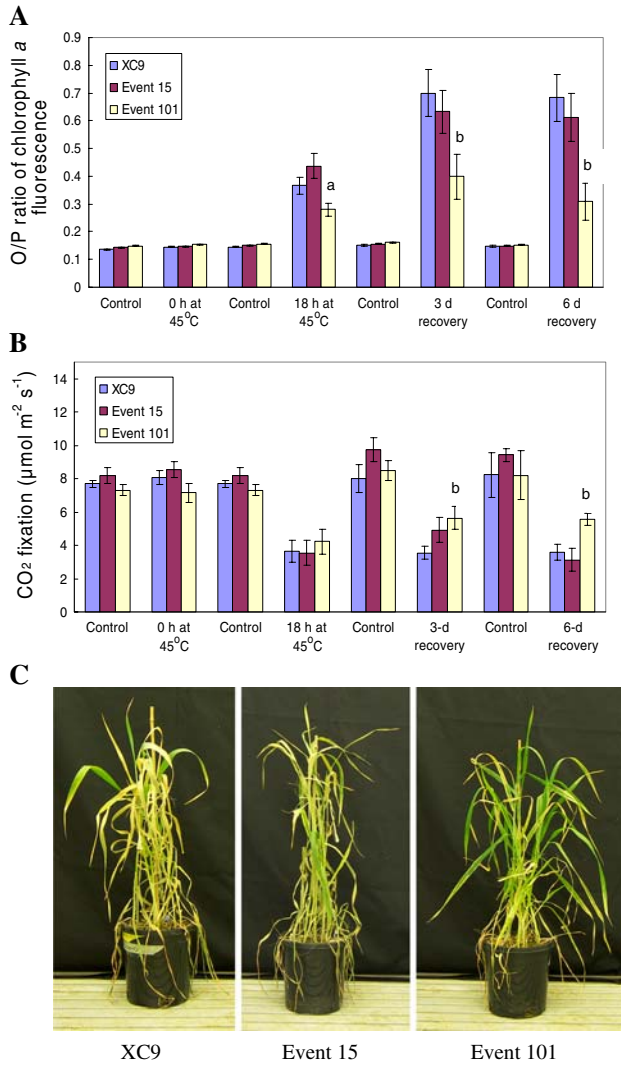



**Fig. 7** Transgenic plants showing increased expression of chloroplast EF-Tu display reduced injury to thylakoid membranes (**a**), enhanced rate of $CO_2$ fixation (**b**), and reduced visible signs of injury (**c**) following heat stress. Seven-week-old plants were exposed to 45°C for 18 h in a growth chamber. Following heat stress plants were allowed to recover for 6 days. Damage to thylakoid membranes was assessed by measuring chlorophyll *a* fluorescence in 1-h dark-adapted leaves (Ristic et al. 2008). For fluorescence measurements, leaf samples were obtained from the youngest, fully expanded leaves. The ratio of constant fluorescence (O) and the peak of variable fluorescence (P) (O/P) was used for assessment of the damage to thylakoid membranes (Ristic et al. 2008; Krause and Weis 1984). Increases in O/P indicate damage to thylakoids (Ristic et al. 2008; Krause and Weis 1984). Measurements of chlorophyll *a* fluorescence and $CO_2$ fixation were taken on the same leaves at indicated times. Bars indicate standard errors. Letters "a" and "b" above the bars indicate significant difference in O/P ratio or rate of $CO_2$ fixation between non-transgenic (XC9) and transgenic plants. The "*n*" values were: XC9-control, $n = 19$; Event 15-control, $n = 18$; Event 101-control, $n = 19$; XC9-heat stress, $n = 19$; Event 15-heat stress, $n = 19$; Event 101-heat stress, $n = 15$. a, $P = 0.032$; b, $P \leq 0.01$

We also investigated thermal aggregation of leaf proteins in plants of event 15, an event that showed reduced levels of EF-Tu, but a pattern of protein aggregation was similar to that of non-transgenic XC9. This may be viewed as somewhat unexpected since event 15 had a lower level of EF-Tu than XC9. It is possible that the pre-existing amount of EF-Tu in wheat leaf cells is insufficient to provide any appreciable protection to cellular proteins against thermal aggregation and that a further decrease in the level of this protein will have no detectible effect on protein aggregation. Alternatively, a decrease in the amount of EF-Tu below a certain level may be compensated by the production of molecular chaperones and/or activation of other protective mechanisms. Further studies are needed to test these possibilities.

Our study with transgenic wheat demonstrates that heterologous expression of the maize plastidal EF-Tu (increase in endogenous levels of EF-Tu) leads to enhanced protection of cellular proteins against thermal aggregation. The results support the concept that EF-Tu reduces protein aggregation by acting as a molecular chaperone. This idea is consistent with the results communicated by Ristic et al. (2007) who observed reduced thermal aggregation of heat-labile photosynthetic enzyme Rubisco activase (Salvucci et al. 2001) in the presence of purified native precursor of EF-Tu from both wheat and maize. The above concept is also in accord with studies on recombinant maize (Rao et al. 2004) and prokaryotic (Caldas et al. 1998) EF-Tu, which showed that this protein displays chaperone activity and protects heat-labile proteins from thermal aggregation.

It is tempting to hypothesize that expression of maize plastidal EF-Tu will be beneficial to wheat's response to heat stress. The beneficial effect of EF-Tu enhanced expression will be manifested by protection of cellular proteins against thermal aggregation, as demonstrated in our study (Fig. 5). Moreover, the beneficial effect of EF-Tu expression may also be manifested through the EF-Tu's well-characterized function in protein synthesis (Riis et al. 1990; Nissen et al. 1995; Willson and Noller 1998). Increase in the endogenous level of EF-Tu may enhance the overall efficiency of protein synthesis and this, in turn, may improve cell ability to alleviate the negative effects of heat stress. Furthermore, EF-Tu may be contributing to heat tolerance through some other mechanisms. This protein is involved in multiple cellular activities, such as the formation of RNA replicase (Blumenthal et al. 1972), interaction with adenylate cyclase (Reddy et al. 1986), the formation of cytoskeleton-like filament bundles (Beck 1979), catalysis of protein disulfide formation and isomerization (refolding) (Richarme 1998), and regulation of RNA synthesis (transcriptional activation) (Young and Bernlohr 1991). It has been suggested that EF-Tu may be contributing to heat tolerance through its activities in the formation of cytoskeleton-like bundles (Takai et al. 1998), protein isomerization (Richarme 1998), and transcriptional activation (Young and Bernlohr 1991). More studies,

however, are needed to investigate the mechanisms by which EF-Tu may confer heat tolerance.

Although all heat-tolerance related activities of EF-Tu are not fully understood, the results of our experiments on thermal aggregation of leaf proteins from transgenic 101 and 66 and non-transgenic XC9 suggest that enhanced expression of this protein contributes to heat tolerance. If EF-Tu contributes to heat tolerance, it is reasonable to expect that transgenic plants with increased constitutive expression of this protein may suffer less injury during exposure to heat stress and/or display better recovery following exposure to stress. To this end, we tested this possibility by examining the response of whole transgenic plants of events 101 (enhanced EF-Tu expression), 15 (reduced EF-Tu expression), and non-transgenic XC9 to a brief heat stress under laboratory conditions and the results supported our hypothesis. Transgenic plants of event 101, the event with increased levels of plastidal EF-Tu, displayed reduced injury to photosynthetic membranes (thylakoids), enhanced rate of $CO_2$ fixation, and fewer visible signs of heat injury following exposure to heat stress (Fig. 7). The reduced injury and enhanced $CO_2$ fixation in transgenic 101 is probably due to the protection of photosynthetic membranes and photosynthetic-related enzymes under heat stress as we previously reported that both recombinant maize pre-EF-Tu purified from *E. coli* and native pre-EF-Tu purified from maize and wheat leaf tissues protect the photosynthetic enzyme Rubisco activase from thermal aggregation (Ristic et al. 2007). The results of the whole plant response to heat stress, thus, support the above hypothesis that increased constitutive expression of EF-Tu is beneficial to plant tolerance to heat stress. The reduced injury and enhanced photosynthesis capability in heat-stressed transgenic plants of event 101 suggests that increases in endogenous levels of chloroplast EF-Tu could lead to better yield in heat environments. Field testing in adverse environments, however, is needed to test this hypothesis.

Several studies have previously shown that manipulation of expression of specific genes can enhance plant tolerance to heat stress (Malik et al. 1999; Queitsch et al. 2000; Katiyar-Agarwal et al. 2003; Sanmiya et al. 2004). These studies have used genes coding for HSPs to enhance heat tolerance in several diploid species. Although they showed that over-expression of specific HSPs enhances thermotolerance in transgenic plants, the mechanism by which the transgenes confer heat tolerance is unclear. Our study, in contrast, provides evidence on a possible mechanism by which the transgene (EF-Tu) helps plant cells ameliorate the negative effects of heat stress. It demonstrates that an increase in the constitutive expression of a plastidal EF-Tu has a positive effect on the basis of plant thermotolerance, which is prevention of protein thermal aggregation. Moreover, our study also demonstrates that a gene other than a HSP can be used for improvement of heat tolerance and that the improvement is possible in a species that has a complex genome, hexaploid wheat.

In summary, we transformed wheat using a maize gene for plastid targeted protein synthesis elongation factor, EF-Tu. The enhanced expression of chloroplast EF-Tu in transgenic wheat led to improved protection of leaf proteins against thermal aggregation, reduced damage to thylakoid membranes and enhanced photosynthetic capability following exposure to heat stress. The results strongly suggest that heat tolerance of wheat, and possibly other crop plants, can be improved by modulating expression levels of plastidal EF-Tu.

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**Supplemental Table S1.** Number of $T_1$ seeds harvested from $T_0$ plants of transgenic wheat, and summary of PCR analysis on $T_1$ generation plants

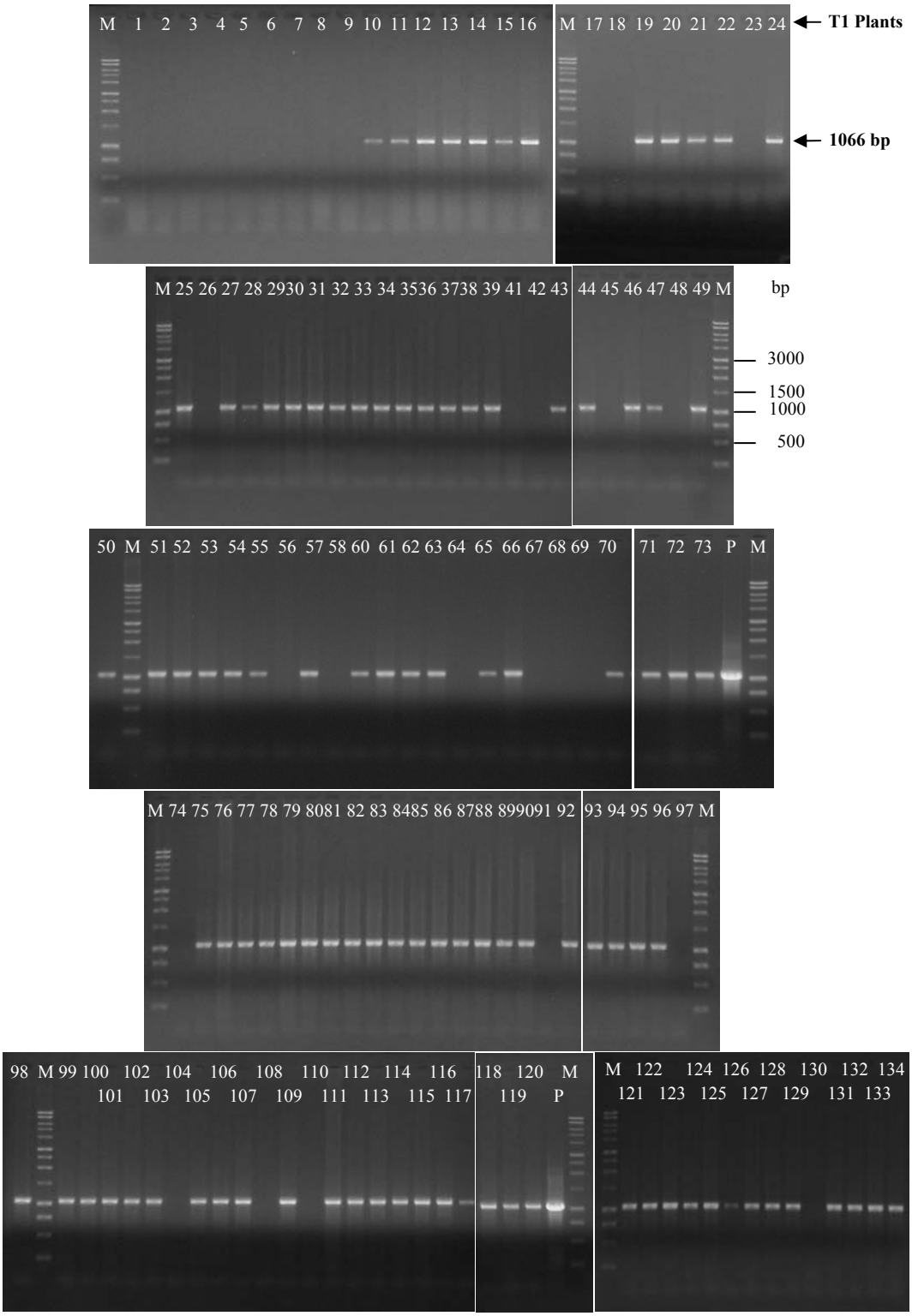
| Cell event (Background) | T1 seeds harvested | T1 seeds germinated/sown | PCR-positive plants/ plants tested | PCR-tested T1 plant No. | T1 plant ID* |
|---|---|---|---|---|---|
| 372-7-1-1 (BW) | 92 | 8/8 | 5/8 | 17 to 24 | 24 |
| 372-4-1-1 (BW) | 73 | 10/10 | 9/10 | 25 to 34 | 27 |
| 372-6-4-2 (BW) | 26 | 8/8 | 4/8 | 41 to 48 | 46 |
| 371-6-2-2 (XC9) | 131 | 8/8 | 7/8 | 9 to 16 | 15** |
| 371-6-4-1 (XC9) | 18 | 5/6 | 5/5 | 35 to 39 | 39 |
| 375-3-1-1 (XC9) | 51 | 10/10 | 8/10 | 49 to 58 | 49 |
| 375-3-2-1 (XC9) | 38 | 10/10 | 6/10 | 60 to 68 | 66** |
| 382-5-1-1 (XC9) | 40 | 10/10 | 8/10 | 69 to 78 | 72 |
| 382-5-2-1 (XC9) | 38 | 10/10 | 10/10 | 79 to 88 | 79 |
| 382-6-1-2 (XC9) | 31 | 8/8 | 7/8 | 89 to 96 | 90 |
| 382-7-1-1 (XC9) | 69 | 10/10 | 8/10 | 97 to 106 | 101** |
| 382-7-3-1 (XC9) | 89 | 10/10 | 8/10 | 107 to 116 | 107 |
| 382-8-1-3 (XC9) | 84 | 10/10 | 10/10 | 117 to 126 | 118 |
| 382-8-2-4 (XC9) | 44 | 8/10 | 7/8 | 127 to 134 | 133 |

From 14 events above $T_1$ seeds were planted and PCR-tested, $T_2$ seeds were collected and $T_2$ plants were analyzed.

| | | |
|---|---|---|
| 406-2-1-1 (BW) | 0 | These events were not analyzed. |
| 372-4-1-2 (BW) | 37 | |
| 371-6-2-1 (XC9) | 86 | |
| 375-3-2-2 (XC9) | 35 | |
| 382-5-1-2 (XC9) | 15 | |
| 382-5-2-2 (XC9) | 33 | |
| 382-7-1-2 (XC9) | 36 | |
| 382-8-1-2 (XC9) | 26 | |
| 382-8-2-1 (XC9) | 22 | |
| 382-8-2-5 (XC9) | 22 | |

A total of 24 events regenerated. Twenty-three events produced T1 seeds. Fourteen events have been analyzed. BW: wheat cv. Bobwhite. XC9: wheat genotype Xin Chun 9. * T1 plant used for Southern and immunoblot analyses.
** T1 plant from which T2 generation plants were raised for further analysis

## Supplemental Figure S1



**Supplemental Figure S1**. PCR analysis in $T_1$ generation transgenic wheat plants. Genomic DNA was extracted from leaf tissues of 3 week-old plants, and used as a template for PCR. Maize ubiquitin promoter specific forward primer UBI2 (5'-AGCCCTGCCTTCATACGCTATTTATTTGC)] was coupled with the *Zmeftu1* gene specific reverse primer EFTU2 (5'- CGATATCGACTGTGTCACCAATCTTGAC). The expected PCR product is 1,066 bp. Plants No. 1 to 4 and 5 to 8 are from non-transgenic wheat cultivars Bobwhite (BW) and Xin Chun 9 (XC9), respectively. $T_1$ plants No. 9 to 16 are from event 371-6-2-2, 17 to 24 from 372-7-1-1, 25 to 34 from 372-4-1-1, 35 to 39 from 371-6-4-1, 41 to 48 from 372-6-4-2, 49 to 58 from 375-3-1-1, 60 to 68 from 375-3-2-1, 69 to 78 from 382-5-1-1, 79 to 88 from 382-5-2-1, 89 to 96 from 382-6-1-2, 97 to 106 from 382-7-1-1, 107 to 116 from 382-7-3-1, 117 to 126 from 382-8-1-3 and 127 to 134 from 382-8-2-4 (see Supplemental Table 1 online). Seeds (plants) No. 40 and 59 did not germinate. M: DNA markers; P: PCR products from plasmid *pPTN453* template (positive control).