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Identification of the *TaBTF3* gene in wheat (*Triticum aestivum* L.) and the effect of its silencing on wheat chloroplast, mitochondria and mesophyll cell development

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ABSTRACT

The full-length cDNA (882 bp) and DNA (1742 bp) sequences encoding a basic transcription factor 3, designated as *TaBTF3*, were first isolated from common wheat (*Triticum aestivum* L.). Subcellular localization studies revealed that the TaBTF3 protein was mainly located in the cytoplasm and nucleus. In *TaBTF3*-silenced transgenic wheat seedlings obtained using the Virus-induced gene silencing (VIGS) method, the chlorophyll pigment content was markedly reduced. However, the malonaldehyde (MDA) and H_2O_2 contents were enhanced, and the structure of the wheat mesophyll cell was seriously damaged. Furthermore, transcripts of the chloroplast- and mitochondrial-encoded genes were significantly reduced in *TaBTF3*-silenced transgenic wheat plants. These results suggest that the *TaBTF3* gene might function in the development of the wheat chloroplast, mitochondria and mesophyll cell. This paper is the first report to describe the involvement of *TaBTF3* in maintaining the normal plant mesophyll cell structure.

1. Introduction

The nascent polypeptide-associated complex (NAC) is a heterodimeric complex composed of α and β subunits and is an abundant and conserved protein complex that exists in mammalian, yeast, and plants cells [1]. Evidence shows that NAC directly interacts with the signal recognition particle (SRP) to ensure the proper folding and targeting of synthesized proteins, thereby inhibiting the targeting of nonsecretory proteins to the endoplasmic reticulum [2,3]. BTF3 (basic transcription factor 3), the β-subunit of NAC, had originally been identified as a basic transcription factor that is both involved in the transcriptional initiation of RNA polymerase II and associated with apoptosis in mammalian cells [4,5]. Two BTF3 homologs in *Saccharomyces cerevisiae* exert negative effects on the expression of several genes that are transcribed by RNA polymerase II [6]. The *Caenorhabditis elegans ICD-1* gene encodes the β-NAC protein, which functions in preventing apoptosis [7]. The absence of NAC also leads to the embryonic lethal phenotype in both mice and *Drosophila*, indicating that NAC plays a crucial biological role in vivo [8,9]. Moreover, it has been shown that BTF3 acts as a transcriptional regulator in PDAC (pancreatic ductal adenocarcinoma) tissues [10].

Although NAC is highly conserved and present in eukaryotic cells, the functions of BTF3 in higher plants still remain unclear. Arabidopsis *BTF3* can interact with the translation initiation factor (iso) 4E (eIFiso4E) and might be involved in the regulation of translation initiation [11]. A *Capsicum annuum BTF3* gene was found to involve in hypersensitive response (HR) cell death and could function as a transcription factor in the nucleus through the transcriptional regulation of HR-related gene expression [12]. However, the function of the *TaBTF3* gene in maintaining the normal plant mesophyll cell structure has not yet been reported. In this study, the cDNA and DNA sequences of the *TaBTF3* gene were first isolated from common wheat, its subcellular localization was observed using GFP fluorescence, and its cellular function in wheat was further explored by using BSMV-VIGS.

2. Materials and methods

2.1. Plant materials and growth conditions

The common wheat (*Triticum aestivum* L.) cultivar Yumai 34, a resistant wheat cultivar, was used in the current study. Seeds were grown in an illumination incubator under a photoperiod of 16 h light/8 h dark, with 23 °C/18 °C day/night temperatures and a relative humidity of 60%/75% (day/night). Uniform seedlings at the 2–3 leaf stage were used for BSMV-VIGS inoculation.

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2.2. Cloning the full-length cDNA and DNA sequences of TaBTF3 gene from wheat

The full length cDNA sequence of the *TaBTF3* gene was obtained by using the RACE method (BD SMARTTM RACE cDNA Amplification Kit, Invitrogen, Carlsbad, CA, USA). To confirm its authenticity, a pair of primers (its sense primer was located before the start codon ATG, and its reverse primer was located after the stop codon TAA) was designed to clone the ORF (open reading frame) sequence of the *TaBTF3* gene. The DNA sequence of the *TaBTF3* gene was also cloned by using this same pair of primers, and the PCR products were cloned and sequenced. Sequence data were analyzed by using BLAST, the CAP3 Sequence Assembly Program (http://pbil.univ-lyon1.fr/cap3.php), ORF Finder (http://www.ncbi.nih.gov/gorf/gorf.html), InterProScan (http://www.ebi.ac.uk/InterProScan/) and PROSITE (http://au.expasy.org/prosite/) network services. The alignment of the deduced protein sequences and phylogenetic tree were computed using DNAMAN (version5.2.2, Lynnon Biosoft) and the ClustalX version1.81 program employing standard parameters, respectively.

2.3. Subcellular localization of TaBTF3

The ORF sequence of *TaBTF3* (the termination codon was removed) was amplified using a pair of primers (BTF-SL1 and BTF-SL2). The obtained ORF sequence was digested by *BamH* I and *Hind* III and further cloned into the pJIT163-hGFP plasmid, which was digested with the corresponding enzymes, to generate the pJIT163-*TaBTF3*-hGFP recombinant plasmid. For transient expression in Arabidopsis protoplasts, 10–15 μg of the purified pJIT163-*TaBTF3*-hGFP plasmid were used to transform Arabidopsis protoplasts by PEG transformation according to a described method [13]. The GFP fluorescence in Arabidopsis protoplasts was detected using a Confocal Laser Scanning Microscope (Olympus, FV1000, Japan). The GFP fluorescence was observed at an excitation wavelength of 488 nm and an emission wavelength of 506–538 nm, and the autofluorescence of the chloroplasts was observed at an excitation wavelength of 488 nm and an emission wavelength of 664–696 nm.

2.4. BSMV vector construction and inoculation for TaBTF3 silencing

A conserved fragment (275 bp) of the *TaBTF3* gene was cloned into the BSMV-γ plasmid by using an *Nhe*I site. The schematic construction of the recombinant BSMV-*TaBTF3* virus vector is described in Supplementary Fig. 1 (S-Fig. 1). Capped transcripts were prepared in vitro by using the RiboMAXTM Large Scale RNA Production System-T7 (Promega). The details of the BSMV inoculation and wheat plant growth conditions were as described by Liang et al. [14].

2.5. Molecular identification of the BSMV-VIGS plants

Total RNA was extracted from the leaves of the BSMV-*TaBTF3*- and BSMV-GFP-inoculated wheat plants using the TRIZOL reagent (Takara), and the first strand of cDNA was synthesized using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) after DNaseI (Fermentas) treatment. Real time RT-PCR analysis (qRT-PCR) was used to examine the reduction in the expression levels of the *TaBTF3* gene in both the BSMV-*TaBTF3*- and BSMV-GFP-inoculated wheat plants [15]. RT-PCR was performed to test the GFP control plants with the specific GFP primers (GFP-RT-S/AS), and the fluorescence of the GFP protein was observed under confocal microscopy (Olympus, FV1000, Japan) in both leaves and roots.

2.6. Physiological parameters test of the BSMV-VIGS plants

Symptomatic leaves of the *TaBTF3*-silenced and GFP control wheat plants were selected for measuring the chlorophyll, MDA and H_2O_2 content. The MDA content was determined using the TBA (Thiobarbituric acid) test method [16]. A Minolta Chlorophyll Meter (model:SPAD-502, Japan) was used to measure the total chlorophyll content, and six SPAD readings that randomly measured from base to tip were taken and averaged on each selected leaf to represent the individual plant value. To measure the in vivo H_2O_2 concentration in the epidermal cells, the fluorescence intensity of H_2O_2 in the epidermal cells was visualized using a fluorescent dye molecular probe, H_2DCFDA (2′, 7′-dichlorodihydrofluorescein diacetate), as described by Pei et al. [17]. The experiments described above were repeated at least three times.

2.7. Preparation of paraffin slices

The wheat leaf paraffin slices were prepared and observed as described by Naija et al. [18], with minor modification. For the paraffin sections, the leave segments were embedded in paraffin, after infusion, the samples were divided into 8 μm sections by using the Leica slicer (Leica, Inc., Germany). Staining was carried out with Hematoxylin–Eosin, and the samples were photographed using a BX60 light microscope (Olympus).

2.8. Primers

All primers used in this study are listed in Supplemental Table 1 (S-1).

3. Results

3.1. Isolation and characterization of the TaBTF3 gene

The results indicated that the full-length cDNA sequence of the *TaBTF3* gene is 882 bp, and contained the ORF of 534 bp, which is flanked by 5′- and 3′-untranslated regions of 113 and 235 bp, respectively (GenBank accession no: JX508709). The predicted TaBTF3 protein has 177 amino acids. Scansite analysis (http://scansite3.mit.edu) indicated that TaBTF3 is a hydrophilic protein, characterized by the N-terminal and C-terminal regions. The N-terminal region contains a nuclear localization signal (NLS) sequence 'RRKKK' between amino acid residues 22–26 and a NAC domain (amino acid residues 36–92), and functions as a DNA-binding domain, which is highly conserved across different species (Fig. 1A and D). Corresponding to the ORF region of its cDNA sequence, the *TaBTF3* genomic sequence is 1742 bp, containing five exons and four introns (Fig. 1B). Phylogenetic analysis was performed between the TaBTF3 and BTF3-like members from other species. The deduced amino acid sequence of TaBTF3 showed high similarities (85.8–94.4%) to other monocot BTF3 family members, but lower similarities (25.4–80.2%) to the BTF3 family members from dicots, yeast and humans (Fig. 1C and D).

3.2. Subcellular localization of TaBTF3

Plasmid DNA encoding either TaBTF3-GFP or GFP alone under the control of the CaMV35S promoter was introduced into Arabidopsis protoplasts, respectively. The cells that were transformed with the GFP control plasmid showed GFP signals in the entire cytoplasm (Fig. 2). However, the TaBTF3-GFP signal was detected in both the cytoplasm and the nucleus (Fig. 2). These results suggest that TaBTF3 might be predominantly localized in the nucleus and cytoplasm.

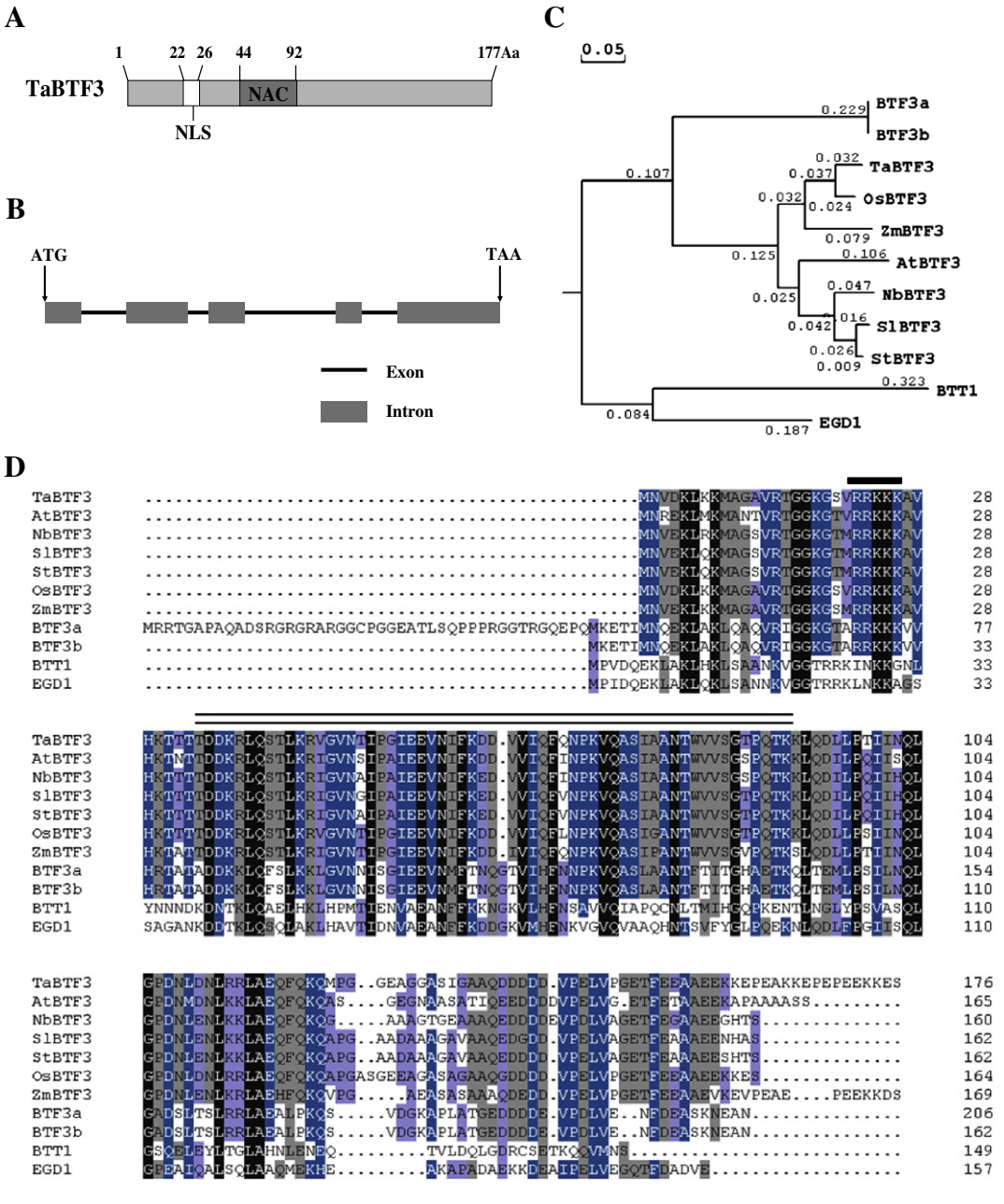

Fig. 1. Genomic sequence and protein structure analyses, multiple alignment and phylogenetic tree. (A) Structure of the TaBTF3 protein. (B) The exon/intron organization of the *TaBTF3* genomic sequence. (C) The phylogenetic tree. (D) Multiple alignment of TaBTF3 with the BTF3 proteins from other species. The *overline* indicates a putative NLS sequence (RRKKK). The *double overline* indicates the conserved NAC domain. Genbank accession number for the proteins in the alignment are as follows: AtBTF3 (*Arabidopsis thaliana*) NM_101651.3; BTF3a and BTF3b (human), NP_001032726.1 and NP_001198.2; BTT1 and EGD1 (*S. cerevisiae*) CAA55370.1 and CAA55371.1; NbBTF3 (*Nicotiana benthamiana*) ABE01085.1; OsBTF3 (*Oryza sativa*) NM_197052.1; SlBTF3 (*Solanum lycopersicum*) ABC24973.1; StBTF3 (*Solanum tuberosum*) ABB87130.1; ZmBTF3 (*Zea mays*) ACG38424.1. The cDNA sequence and deduced amino acid sequence of the *TaBTF3* gene were analyzed using the DNAMAN software.

3.3. Molecular identification of the BSMV-VIGS plants

The expression levels of the *TaBTF3* gene in the VIGS plants were analyzed using the qRT-PCR method, with β-actin being used as internal control, at 10, 15 and 20 dpi (Fig. 3B), respectively. The expression level of *GFP* was examined by RT-PCR (S-Fig. 2B), and the GFP fluorescence was detected in both the leaves and the roots in both the BSMV-GFP-infected wheat plants and the control plants (uninoculated) (S-Fig. 2C and D).

The results indicated that the expression level of the *TaBTF3* gene in the *TaBTF3*-silenced plants was significantly reduced compared to the BSMV-GFP plants; similar results were obtained when compared with the BSMV-MCS plants (Fig. 3B and S-Fig. 2A). RT-PCR analyses of the BSMV-GFP plants revealed that the GFP gene only existed and expressed stably in GFP plants (S-Fig. 2B). Furthermore, strong GFP fluorescence was observed in both the leaves and roots of the BSMV-GFP-infected wheat plants (S-Fig. 2C and D). These results showed that the BSMV RNAs moved systemically from the leaf to the root in inoculated wheat plants and suggested that the BSMV-based VIGS system used in this study was efficient and successful in Yumai 34 wheat plants.

3.4. Morphological and physiological changes of the TaBTF3-silenced wheat plants

Silencing of the *TaBTF3* gene caused slight leaf yellowing and abnormal leaf morphology, but almost normal wheat growth was maintained compared to the GFP control plants (Fig. 3A and

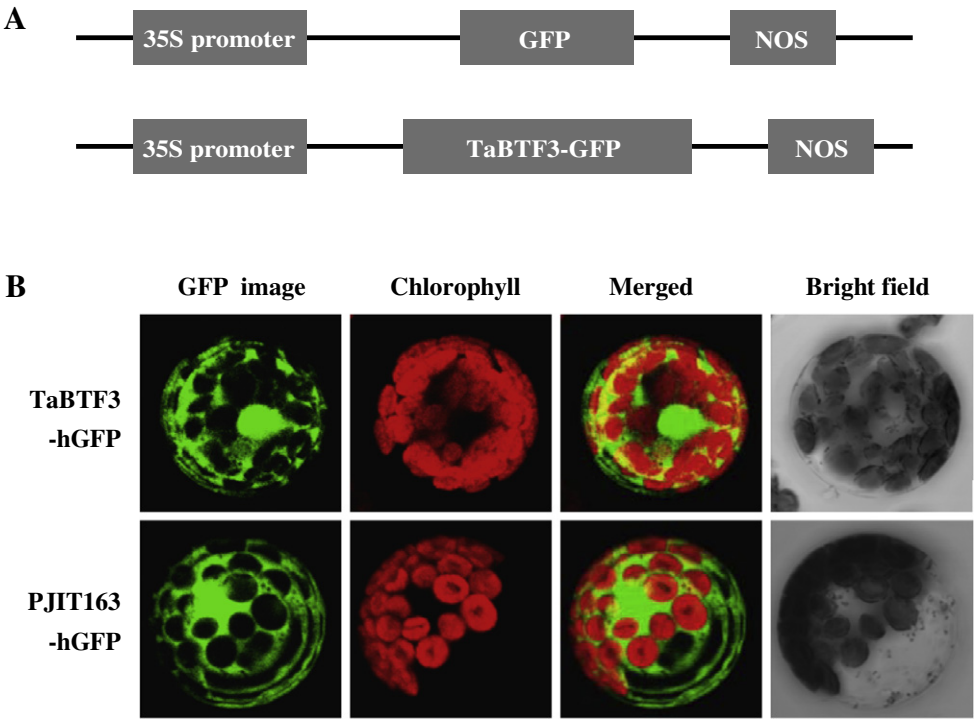

Fig. 2. Subcellular localization of *TaBTF3* in Arabidopsis protoplasts. (A) Schematic representation of the 35S::*TaBTF3*-GFP fusion construct and the control 35S::GFP construct. (B) The 35S::*TaBTF3*-GFP fusion construct and the control 35S::GFP construct were transformed and transiently expressed in Arabidopsis protoplasts. All images were obtained using a confocal Laser Scanning Microscope (Olympus, FV1000, Japan). GFP, chlorophyll autofluorescence, merged and bright-field images are shown.

S-Fig. 3). A significant decrease in the chlorophyll content was detected in the *TaBTF3*-silenced plants (Fig. 3C), but the MDA and H_2O_2 contents increased (Fig. 3D and E). The chlorophyll content in the *TaBTF3*-silenced plants was only 76.36% of that found in GFP control plants. However, the MDA content in the *TaBTF3*-silenced plants reached 8.4 nmol/g, which was markedly higher than that of the GFP control plants (6.26 nmol/g). The accumulation rates of fluorescent H_2DCFDA in the epidermal cells of the *TaBTF3*-silenced plants were significantly higher than those of the GFP control plants, and the relative fluorescence intensity in epidermal cells from the *TaBTF3*-silenced VIGS plants was approximately 4-fold than that in the GFP control plants.

3.5. Transcript levels of the chloroplast- and mitochondrial-encoded genes were reduced in the TaBTF3-silenced plants

The formation of excess H_2O_2 and MDA in a cell is the consequence of organelle dysfunction when plants are undergoing or during exposure to stresses [19,20]. To further evaluate the effect of silencing the *TaBTF3* gene on the integrity of the chloroplasts and mitochondria, we examined the transcript levels of the chloroplast- and mitochondrial-encoded genes, which can be transcribed by plastid-encoded RNA polymerases (PEP), by nucleus-encoded phage-type RNA polymerases (NEP), by both PEP and NEP, or by undefined promoters [21]. The expression levels of the PEP-dependent photosynthesis genes, including *psbB*, *rbcL*, *psbA*, *ndhG* and *psaA*, decreased dramatically in the *TaBTF3*-silenced wheat plants (Fig. 4A). In addition, the expression levels of two mitochondrial-encoded genes, *atp1* (undefined promoter) and *cox3* (NEP-dependent), were also reduced greatly (Fig. 4B). These results indicated that silencing of the *TaBTF3* gene suppressed the transcription of both PEP- and NEP-dependent genes in the chloroplasts and mitochondria and that the *TaBTF3* gene could be required for the biogenesis of the chloroplasts and mitochondria in wheat plants.

3.6. Silencing of the TaBTF3 gene damaged the wheat mesophyll cell structure

Normal plant cells contain a profusion of mitochondria and chloroplasts, which are essential for optimized and proper plant development, but down-regulation of the chloroplast- and mitochondrial-encoded genes can lead to an abnormal shape and a reduction in the number of these organelles [22]. To further examine the structural changes of the wheat mesophyll cells, paraffin slices were prepared and observed under light microscopy. Interestingly, significant differences in the mesophyll cell structure were observed. Under normal growth conditions, the morphology of mesophyll cells in the GFP control plants were intact, closely arranged and filled the whole transverse section; fewer intercellular spaces can be observed (Fig. 4C). However, significant changes in the structure and morphology of the mesophyll cells appeared in the *TaBTF3*-silenced wheat plants; the cells were smaller in size, irregularly shaped, disorderly and loosely arranged, and had enlarged intercellular spaces (Fig. 4D).

4. Discussion

In the present study, we first cloned the cDNA and DNA sequences of the *TaBTF3* gene from wheat, which encodes a protein with a conserved NAC domain and shows high similarity to other BTF3s (Fig. 1A, C and D). The DNA sequence of the *TaBTF3* gene was also cloned, and the exon and intron structures of the *TaBTF3* gene were similar to those of the *SmBTF3* gene in *Salvia miltiorrhiza* [23], except for differences in the lengths of the exons and introns. A nuclear localization signal 'RRKKK' is putatively located between amino acid residues 22–26 in the N-terminus (Fig. 1A and D), indicating that the TaBTF3 protein might be located in the nucleus. The subcellular localization of TaBTF3 further verified this predication (Fig. 2). Furthermore, our results also showed that TaBTF3 also

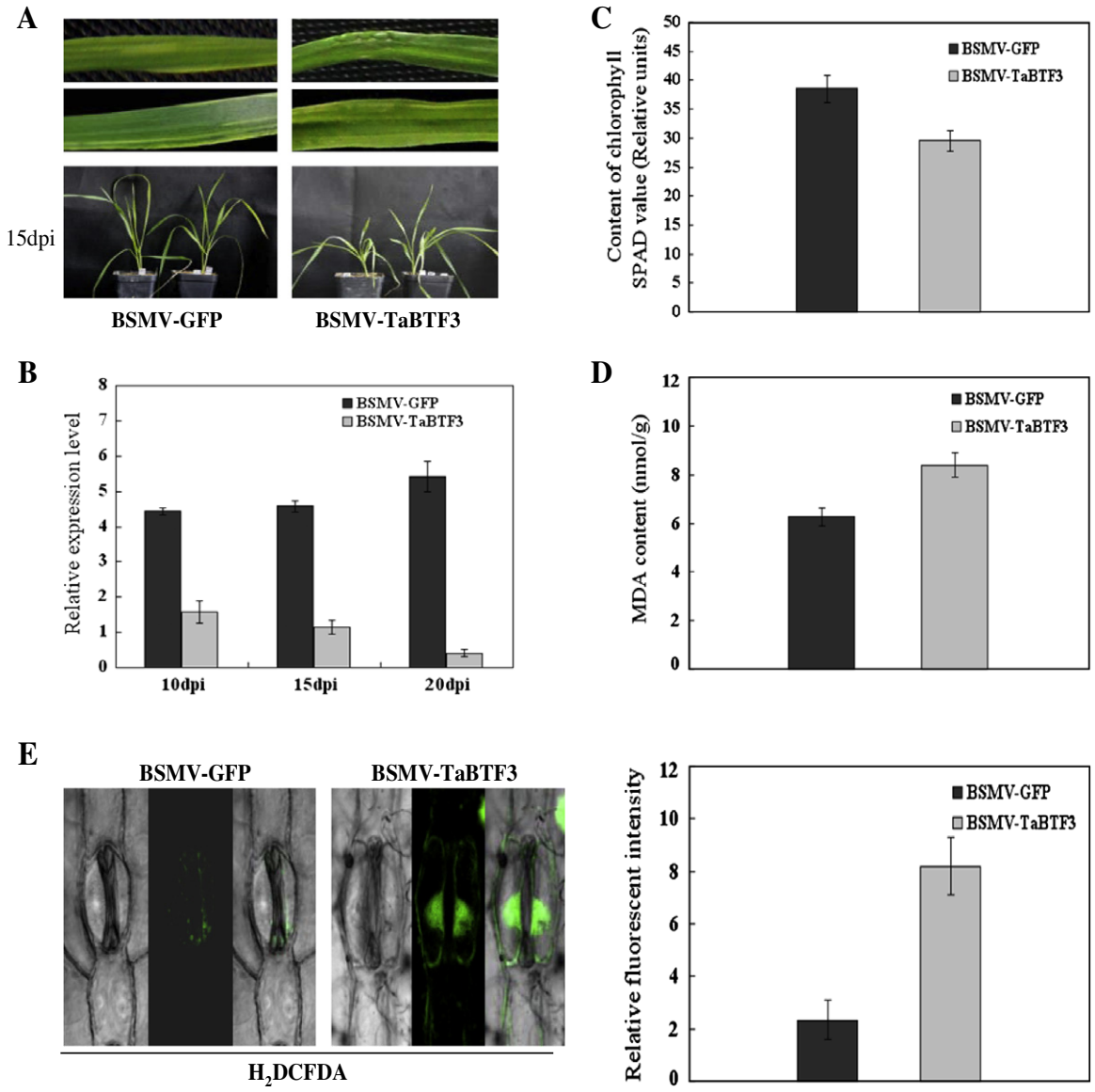

Fig. 3. Identification of the BSMV-VIGS wheat plants and examination the H_2O_2, MDA and chlorophyll content. (A) The VIGS phenotypes of the leaves and the whole plants were taken at 15 dpi. (B) Relative expression level of the *TaBTF3* gene in the BSMV-*TaBTF3*- and the BSMV-GFP-inoculated plants. (C and D) The chlorophyll and MDA content in the *TaBTF3*-silenced and the GFP plants at 15 dpi. (E) Detection of the H_2O_2 accumulation in the *TaBTF3*-silenced plants and the GFP plants at 15 dpi using confocal microscopy. The means were generated from three independent measurements; bars indicate standard errors.

located in cytoplasm, implying a diverse localization of TaBTF3 that is similar to the Homo sapiens BTF3 [24]. The subcellular localization provides the first indication of function, and the diverse subcellular localization of a gene indicates that it may play distinct functional roles in the cell. Thus, the diverse localization may help to enhance the role of *TaBTF3* in the nucleus and its functions in the cytoplasm.

The qRT-PCR analysis showed that the transcript levels of both PEP- and NEP-dependent genes in the chloroplast and mitochondria were inhibited in *TaBTF3*-silenced plants (Fig. 4A and B). Although both the chloroplasts and mitochondria possess their own genomes and transcription machineries, the majority of the proteins and the specialized components for gene expression are encoded or controlled by the nucleus before being imported into these organelles [25]. It has been reported that the PEP and NEP promoters are tightly controlled or encoded by the nucleus in Arabidopsis [26,27]. The transcription levels of both PEP- and NEP-dependent genes in the chloroplasts and mitochondria were inhibited regardless of their promoter difference. Moreover, given that NAC has been reported to function in both the cytoplasm and the nucleus in mammals and yeast [1], we predicted that the suppression of gene expression is due to the hampered synthesis and import of PEP and NEP promoter caused by the down-regulation of the *TaBTF3* gene, which further affects the development of these organelles. The altered organelle biogenesis in turn disturbed the physiological metabolism, including the significant reduction in chlorophyll content and the excessive accumulation of H_2O_2 and MDA in the *TaBTF3*-silenced wheat plants (Fig. 3C–E).

In eukaryotic cells, newly synthesized polypeptide chains must first enter the endoplasmic reticulum (ER) for folding and maturation to be transported to their final destinations. Disruption of this process leads to the accumulation of numerous unfolded aberrant proteins in the ER, a condition called 'ER stress' [28]. The deletion of NAC in budding yeast results in the induction of the ER stress response pathway [29], and the suppression or depletion of BTF3 also results in an abnormal, defective, or lethal phenotype in plants,

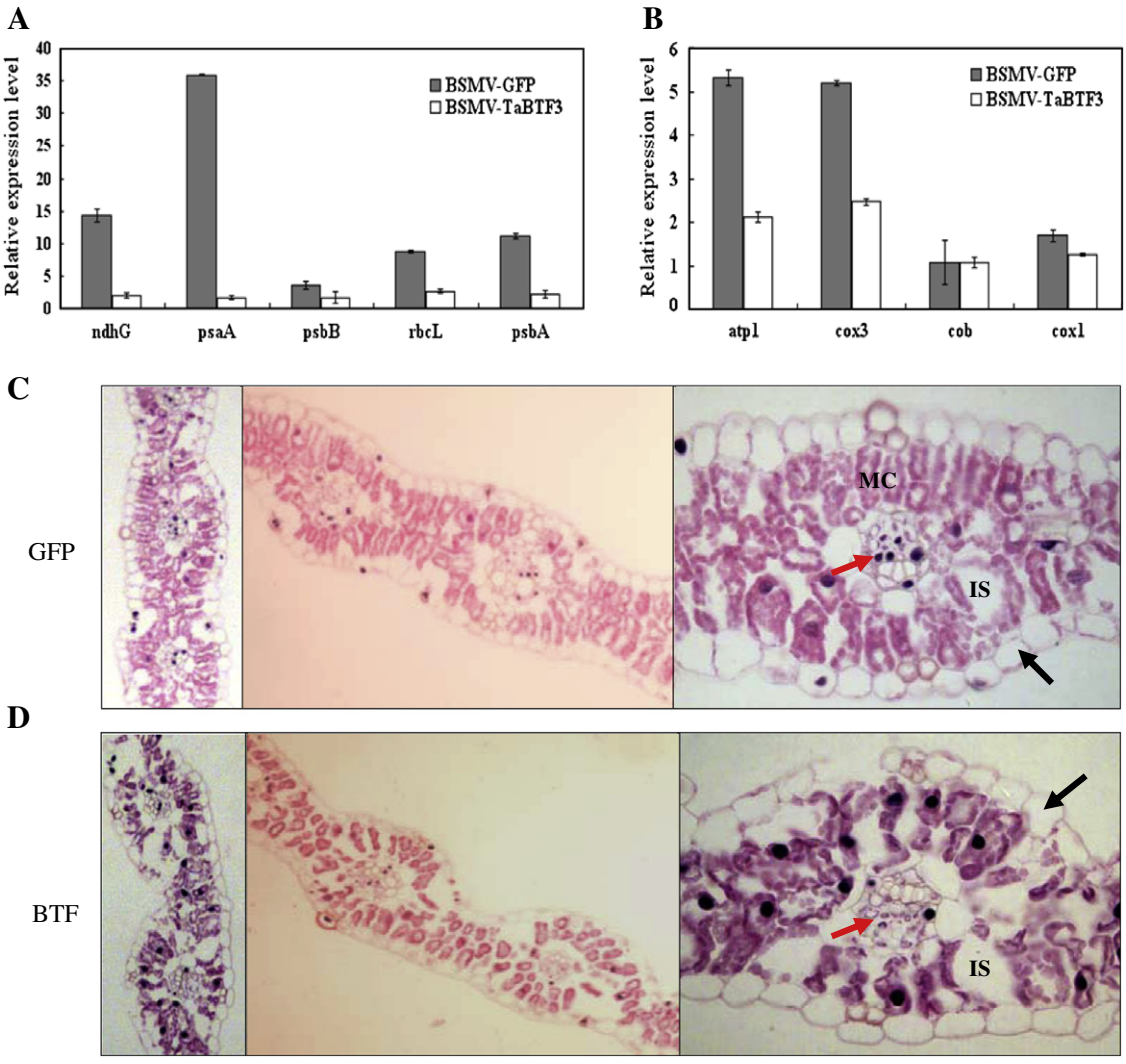

Fig. 4. The transcription levels of the chloroplast- and mitochondrial-encoded genes, and the leaf anatomical (transverse section) characteristics of BSMV-GFP- and BSMV-*TaBTF3*-inoculated wheat plants. (A) Expression of the chloroplast-encoded genes. (B) Expression of the mitochondrial-encoded genes. (C) The mesophyll cell structure of the GFP and BTF wheat leaves. The leaf vein is indicated by a red arrowhead; the epidermal cell (black arrows), intercellular spaces (IS) and mesophyll cell (MC) are clearly shown. BTF denotes *TaBTF3*-silenced plants; GFP denotes BSMV-GFP-infected wheat plants. The data for all chloroplast- and mitochondrial-encoded genes selected for qRT-PCR are listed in S-Table 1.

mice and *C. elegans* [3,7,8,30]. In the present study, we found that silencing of the *TaBTF3* gene resulted in an abnormal mesophyll cell structure, causing reduced size and irregular shape, disorderly and loose arrangement, and enlarged intercellular space (Fig. 4D). We speculated that silencing of the *TaBTF3* gene might induce 'ER stress' and disrupt correct protein folding and targeting, thus further affecting the mesophyll cell development and arrangement.

These findings further highlight the crucial roles of *BTF3* in the process of plant growth and development. Wheat is an important crop for the world population; thus, further research is needed to determine the functions of the *TaBTF3* gene and the related molecular mechanisms that are involved in wheat mesophyll cell development.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.08.137.

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