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Visualization of nuclear localization of transcription factors with cyan and green fluorescent proteins in the red alga *Porphyra yezoensis*

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Running title Nuclear localization of transcription factors in a red alga

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Abstract

Transcription factors play a central role in expression of genomic information in all organisms. The objective of our study is to analyze the function of transcription factors in red algae. One way to analyze transcription factors in eukaryotic cells is to study their nuclear localization, as reported for land plants and green algae using fluorescent proteins. There is, however, no report documenting subcellular localization of transcription factors from red algae. In the present study, using the marine red alga *Porphyra yezoensis*, we confirmed for the first time successful expression of humanized fluorescent proteins (ZsGFP and ZsYFP) from a reef coral *Zoanthus* sp. and land plant-adapted sGFP(S65T) in gametophytic cells comparable to expression of AmCFP. Following molecular cloning and characterization of transcription factors DP-E2F-like 1 (PyDEL1), transcription elongation factor 1 (PyElf1) and multiprotein bridging factor 1 (PyMBF1), we then demonstrated that ZsGFP and AmCFP can be used to visualize nuclear localization of PyElf1 and PyMBF1. This is the first report to perform visualization of subcellular localization of transcription factors as genome-encoded proteins in red algae.

Introduction

Eukaryotic mRNA synthesis is a complex biochemical process, which is controlled by the concerted action of multiple transcription factors that modify the activity of RNA polymerase II at both the initiation and elongation stages of transcription (Reines et al. 1996). In land plants and green algae, the important role of transcription factors during stress responses and development has been demonstrated. For example, CBF1, 2 and 3 direct cold-inducible gene expression in *Arabidopsis thaliana* (Chinnusamy et al. 2007), while Myb transcription factor LCR1 regulates the CO₂ responsive gene *Cah1* under CO₂ limiting stress in *Chlamydomonas reinhardtii* (Yoshioka et al. 2004). Moreover, in *Volvox carteri*, RegA plays a central role as a master regulatory gene in germ-soma differentiation by suppression of reproductive activities in somatic cells (Babinger et al. 2006). These findings and additionally accumulating data suggest that stress responses and development of land plants and green algae are mainly regulated by gene expression at the transcription level (Shinozaki and Yamaguchi-Shinozaki 2007; Goodenough et al. 2007).

Knowledge of subcellular localization of transcription factors contributes towards our understanding of their function in eukaryotic cells. Green fluorescent protein (GFP) is widely recognized as a powerful tool for monitoring localization of transcription factors in plants (Li et al. 2006; Saleh et al. 2006; Hu et al. 2008). Recently, the GFP variants such as yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP) were engineered to improve brightness and to provide additional emission wavelengths, resulting in facilitation of multicolor imaging in studies of gene expression and

subcellular protein localization (Shah et al. 2002; Nowak et al. 2004; Vermeer et al. 2009). To date, a number of reports have documented the nuclear localization of transcription factors using these fluorescent proteins (for example, Camuzeaux et al. 2005; Zhu et al. 2008).

The marine red alga *Porphyra yezoensis* has been proposed as a model marine plant for physiological and genetic studies in seaweed because of its biological and economical importance (Saga and Kitade 2002). Of particular importance, an analysis of expressed sequence tags (ESTs) is a powerful method to identify various genes of interest in the genome of *P. yezoensis* (Nikaido et al. 2000; Asamizu et al. 2003). However, the lack of effective systems to analyze gene function prevents progression of molecular biological studies in red algae, and foreign gene expression systems are essential for the studies of gene function. Recently, we found that optimization of codon usage in a β -glucuronidase (GUS) reporter gene is critical for achievement of high expression in *P. yezoensis* cells (Fukuda et al. 2008). Subsequently, we developed a system for the expression of a humanized cyan fluorescent protein from the sea anemone *Anemonia majano* (AmCFP). The codon usage of this gene resembles that of *P. yezoensis*, enabling us to monitor the correct plasma membrane localization of the Pleckstrin homology (PH) domain from human proteins as a fusion protein with AmCFP (Mikami et al. 2009). However, no report has successfully visualized the subcellular localization of proteins encoded in the *P. yezoensis* genome so far.

The aim of the study was to visualize subcellular localization of transcription factors in *P. yezoensis* cells. First, molecular cloning and characterization of cDNAs encoding transcription factors were carried out. Secondly, we successfully showed

expression of humanized fluorescent proteins ZsGFP and ZsYFP, whose codon usage is similar to that of genes from *P. yezoensis*, and that of plant-adapted fluorescent protein sGFP(S65T), which provides up to 100-fold brighter fluorescence signals than the original GFP from jellyfish *Aequorea victoria* in plants (Niwa et al. 1999). Thirdly, we showed successful application of the above transient gene expression system for visualization of the nuclear localization of transcription factors from *P. yezoensis* by fusion with the above fluorescent proteins.

Materials and Methods

Cultivation of P. yezoensis

The cultivation of leafy gametophytes of *P. yezoensis* strain TU-1 was performed as described by Fukuda et al. (2008), except for culture conditions at both 15°C and 25°C. Filamentous sporophytes were also cultured under the same conditions. The medium (enriched sea life; ESL) was renewed weekly in both gametophytes and sporophytes.

Plasmid construction

Complete ORFs for humanized fluorescent proteins (ZsGreen and ZsYellow) from a reef coral *Zoanthus* sp. (Clontech) and plant-adapted fluorescent protein sGFP(S65T) (Niwa et al. 1999) were amplified with the primer sets BamHI-ZsGFP-F/SacI-FP-R, BamHI-ZsYFP-F/SacI-FP-R and BamHI-sGFP-F/SacI-sGFP-R, respectively (Table 1), under the following program:

94°C for 2 min followed by 30 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 1 min, and final incubation at 72°C for 7 min with LA *Taq* polymerase plus GC buffer (TaKaRa). The coding region of AmCFP in pPyAct1-AmCFP (Mikami et al. 2009) after digestion with *Bam*HI and *Sac*I was then replaced with *Bam*HI-*Sac*I-digested PCR fragments. Resultant plasmids were designated pPyAct1-ZsGFP, pPyAct1-ZsYFP and pPyAct1-sGFP, respectively.

We used Gateway Technology (Invitrogen) to construct expression plasmids of fluorescent proteins fused with an ORF of the *PyDEL1* (EST AU195873), *PyElf1* (EST AV437360) or *PyMBF1* (EST AV439047) gene. To make entry plasmids, ORFs of *PyDEL1*, *PyElf1* and *PyMBF1* were amplified from the corresponding EST clones with the primer sets PyDEL1-F/PyDEL1-R, PyElf1-F/PyElf1-R and PyMBF1-F/PyMBF1-R, respectively (Table 1). The PCR conditions were 30 cycles of 98°C for 10 s, 60°C for 5 s and 72°C for 2 min with Prime STAR HS DNA polymerase plus GC buffer (TaKaRa). The amplified DNA fragments were then directly subcloned into pENTR/D-TOPO (Invitrogen). To produce destination vectors, pPyAct1-AmCFP and pPyAct1-ZsGFP were digested with *Bsp*EI positioned between the ORF of the fluorescent protein and a terminator of the gene for nopaline synthase (*nos*) from *Agrobacterium tumefaciens*. They were then filled with Klenow enzyme and ligated with an Rf cassette amplified using primers Rfb-F and Rfc-R (Table 1). The resultant destination plasmids were designated pPyAct1N-AmCFP-DES and pPyAct1N-ZsGFP-DES, respectively.

Next, LR recombination reactions were performed according to the manufacturer's instructions using the above entry plasmids with pPyAct1N-AmCFP-DES to produce expression plasmids pPyAct1-AmCFP-DEL1, pPyAct1-AmCFP-Elf1 and

pPyAct1-AmCFP-MBF1; with pPyAct1N-ZsGFP-DES to produce plasmids pPyAct1-ZsGFP-DEL1, pPyAct1-ZsGFP-Elf1 and pPyAct1-ZsGFP-MBF1; and with pAct1C-AmCFP-DES (Mikami et al. 2009) to produce expression plasmids pPyAct1-DEL1-AmCFP, pPyAct1-Elf1-AmCFP and pPyAct1-MBF1-AmCFP (Fig. 6A).

Transient transformation of gametophytic cells and observation of fluorescent signals

Transient transformation of gametophytic cells of *P. yezoensis* by particle bombardment was performed as described previously (Mikami et al. 2009), except for use of a fluorescence microscope (DM5000B; Leica, Germany) equipped with fluorescence filter L5 to yield GFP signals and the fluorescence filter YFP to yield YFP signals in addition to the fluorescence filter CFP to yield CFP signals. To visualize nuclear localization, transiently transformed gametophytic cells were fixed with 80% methanol for 10 s and then stained with SYBR Gold (Invitrogen) for 30 min. SYBR Gold staining was observed using the fluorescence filter YFP. Images were captured with a Leica DFC 300FX camera running on the Leica Application Suite.

Analysis of gene expression by RT-PCR

Total RNA extraction from gametophytes and sporophytes was carried out with a RNeasy plant mini kit (buffer RLC; Qiagen) and resultant total RNA was further purified with an RNase-Free DNase Set (Qiagen). Total RNA (0.5 µg per reaction) and the oligo-dT primer were used for first strand cDNA synthesis using a PrimeScript II 1st

strand cDNA Synthesis kit (TaKaRa) according to the manufacturer's instructions.

RT-PCR with LA *Taq* polymerase plus GC buffer (TaKaRa) was performed as follows:

94°C for 2 min followed by 30 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 1 min,

and final incubation at 72°C for 7 min with primer sets PyGAPDH-F/PyGAPDH-R,

PyDEL1-F/PyDEL1-R, PyElf1-F/PyElf1-R and PyMBF1-F/PyMBF1-R (Table 1) to

detect expression of *PyGAPDH*, *PyDEL1*, *PyElf1* and *PyMBF1*, respectively.

Results

Cloning and structural characterization of transcription factors from *P. yezoensis*

We surveyed EST clones encoding full-length ORFs, and found three full-length cDNAs of transcription factors as follows: DP-E2F-like 1 (PyDEL1), transcription elongation factor 1 (PyElf1) and multiprotein bridging factor 1 (PyMBF1). The nucleotide sequences of these cDNAs were deposited in GenBank under accession numbers AB480826, AB480827 and AB480828, respectively.

PyDEL1 is a homologue of DP-E2F-like (DEL)/E2F7 protein. DNA-binding domains of DEL/E2F7 homologues consist of two conserved separated subdomains named DB1 and DB2 (Fig. 1). The DB1 and DB2 domains of PyDEL1 share 55 and 56% identical amino acids with DEL1 from *A. thaliana* (AtDEL1), respectively. In addition, they possess the RXYD DNA recognition motif (Fig. 1), which is responsible for interacting with half of the palindromic promoter binding sites (CGCGCG and CGCGCG) (Vandepoele et al. 2002).

PyElf1 is a homologue of transcription elongation factor 1 (Elf1). Amino acid

sequences of Elf1 homologues are conserved among yeast, humans, *A. thaliana*, *C. reinhardtii*, the unicellular red alga *Cyanidioschyzon merolae* and *P. yezoensis*, containing a C4 type-zinc finger as the DNA-binding region (Fig. 2); this is also found in other transcriptional elongation factors including Spt4 and TFIIS (Malone et al. 1993; Qian et al. 1993).

PyMBF1 is a homologue of multiprotein bridging factor 1 (MBF1). As shown in Fig. 3, eukaryotic MBF1 homologues contain a helix-turn-helix motif. MBF1 regulates transcription by bridging a sequence-specific activator and TATA box-binding protein (TBP) (Takemaru et al. 1998; Liu et al. 2003, 2007). Although the amino acid residue required for binding plant and animal MBF1 homologues to TBP is glutamic acid residue, the corresponding residue in PyMBF1 is glutamine as seen in the fungus *Neurospora crassa* (Fig. 3).

Expression of gene for transcription factors in *P. yezoensis*

RT-PCR was carried out to examine expression of the *PyDEL1*, *PyElf1* and *PyMBF1* genes. The glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) is a known housekeeping gene in various organisms and was therefore used as a positive control. As a result, the *PyElf1* and *PyMBF1* genes were shown to be expressed in gametophytic and sporophytic cells without any changes in their quantity under both 15°C and 25°C culture conditions, although the *PyGAPDH* mRNA level varied depending on the temperature (Fig. 4). Such constitutive expression of the *PyMBF1* gene is different from that of other *MBF1* genes. For example, tissue-specific expression of the *MBF1* gene were observed in the silk gland of the silkworm *Bombyx mori* (Li et al. 2008), while

Arabidopsis thaliana *MBF1c* and *Retama raetam* *MBF1* (*ERTCA*) are up-regulated by heat stress (Tsuda et al. 2004; Pnueli et al. 2002). On the other hand, DEL transcripts are preferentially expressed in growing tissues in *A. thaliana* (Kosugi and Ohashi 2002). The highest expression of the *PyDEL1* gene was observed in sporophytes grown at 25°C (Fig. 4) at which growth of sporophytes was better than at 15°C (data not shown). This suggests a similarity in the relationship between the organism growth and expression of the *DEL1* gene in *P. yezoensis* and *A. thaliana*.

Successful expression of fluorescent proteins in gametophytic cells

Since the transient gene expression system employing β -glucuronidase (GUS) and the AmCFP system are already established in *P. yezoensis* (Fukuda et al. 2008; Mikami et al. 2009), we examined the applicability of two other humanized fluorescent proteins, ZsGFP and ZsYFP, whose codon usage fits that of genes from *P. yezoensis*; this was carried out in addition to analysis of sGFP(S65T) (Niwa et al. 1999), which is widely used in land plants (Kosugi and Ohashi 2002; Tani et al. 2008). When expression plasmids of four fluorescent proteins, AmCFP, ZsGFP, ZsYFP and sGFP, under the direction of a promoter of the *P. yezoensis actin 1* (*PyAct1*) gene (Fig. 5A) were introduced into gametophytic cells by particle bombardment, fluorescent signals were clearly observed in the cytoplasm, as shown in Fig. 5B. The numbers of cells expressing ZsGFP and sGFP were similar and ca. 2 times higher than those expressing AmCFP, while the number of cells expressing ZsYFP tended to be lower (Table 2). Thus, these findings suggest that humanized fluorescent proteins and sGFP can be used as reporters of transient gene expression, although ZsGFP and sGFP are thought to be as good as

AmCFP (Mikami et al. 2009). Thus, we used AmCFP and ZsGFP in the following experiments.

Subcellular localization of transcription factors in gametophytic cells of *P. yezoensis*.

Next, we examined the subcellular localization of three transcription factors, PyDEL1, PyElf1 and PyMBF1, fused with fluorescent protein AmCFP or ZsGFP, as shown in Table 3. When expression plasmids (Fig. 6A) were introduced into gametophytic cells of *P. yezoensis* by particle bombardment, AmCFP-Elf1, AmCFP-MBF1, ZsGFP-Elf1 and ZsGFP-MBF1 were successfully expressed in gametophytic cells, strongly suggesting the nuclear localization of PyElf1 and PyMBF1 (Fig. 6B). To confirm this, signals from the fusion proteins and SYBR Gold staining were compared, indicating a complete overlap between signals (Fig. 6C). Therefore, we concluded that PyElf1 and PyMBF1 specifically localize to the nucleus, although the number of cells expressing MBF1-AmCFP was approximately half of those expressing AmCFP-MBF1 and no fluorescent signals from AmCFP-DEL1, ZsGFP-DEL1, DEL1-AmCFP and Elf1-AmCFP were observed (Table 3).

Discussion

Transcription factors play an important role in the regulation of gene expression (von Koskull-Döring et al. 2007; Alexandre and Henning 2008). Our long term goal is to elucidate the function of transcription factors in *P. yezoensis*. However, due to a lack of

effective experimental systems, little is known about their functions in red algal cells. Here, we confirmed the applicability of fluorescent proteins ZsGFP, ZsYFP and sGFP(S65T) in transient expression in gametophytic cells of *P. yezoensis* (Fig. 5, Table 2) and developed a system for visualization of the nuclear localization of transcription factors using fluorescent proteins in these cells. This is the first study to document the expression of GFP and YFP variants in red algae cells.

Since the GC content of *P. yezoensis* genes inferred from EST analysis reaches a high of 65.2% (Nikaido et al. 2000), optimization of codon usage of foreign genes, especially such as the AT rich-GUS gene, is important for high expression in *P. yezoensis* cells (Fukuda et al. 2008). However, the GC contents of ZsGFP, ZsYFP and sGFP(S65T) are high of 62.8 %, 61.9% and 61.4%, respectively. Moreover, optimization the codon usage of the coding sequence of sGFP(S65T) did not increase the number of expressing cells (data not shown). Therefore, the GC content value of 61.4% appears to be sufficiently high for expression of foreign genes in *P. yezoensis* cells. The reason why the expression of ZsYFP is inefficient in gametophytic cells remains unclear.

In this study, we cloned full-length cDNAs encoding transcription factors PyDEL1, PyElf1 and PyMBF1. PyDEL1 is a homologue of DEL/E2F7 protein, which localizes specifically in the nucleus and acts as a transcription repressor in plants and animals (Kosugi and Ohashi 2002; Vandepoele et al. 2002; Di Stefano et al. 2003). AtDEL1, which is a homologue of DEL/E2F7 from *A. thaliana*, is an important inhibitor of the endocycle (DNA replication without mitosis), preserving the mitotic state of proliferating cells by suppressing transcription of genes required for cells to enter the

DNA endoreduplication cycle (Vlieghe et al. 2005). The DEL1 homologue has been found in the marine unicellular green alga *Ostreococcus tauri* (Robbens et al. 2005) and the unicellular red alga *C. merolae* as a single gene; however, there is no DEL1 gene in the unicellular green alga *C. reinhardtii* (Bisova et al. 2005). PyElf1 is a homologue of Elf1 in *Saccharomyces cerevisiae*. Elf1 acts as a regulatory protein in transcription elongation and maintenance of a proper chromatin structure where transcription is active (Prather et al. 2005). PyMBF1 is a homologue of MBF1, a transcriptional coactivator involved in the regulation of diverse physiological processes such as endothelial cell differentiation, hormone-regulated lipid metabolism, central nervous system development and histidine metabolism (Takemaru et al. 1998; Brendel et al. 2002; Liu et al. 2003). MBF1 from *A. thaliana* (AtMBF1) predominantly localizes in the nucleolus (Sugikawa et al. 2005).

We demonstrated that AmCFP and ZsGFP can be used to monitor nuclear localization of transcription factors in *P. yezoensis* cells (Fig. 6). However, attention should be paid to the alignment order of transcription factors and fluorescent proteins. In this study, AmCFP-Elf1, AmCFP-MBF1, ZsGFP-Elf1 and ZsGFP-MBF1 were clearly expressed, although the expression of Elf1-AmCFP and MBF1-AmCFP were less or not detectable (Table 3). These findings suggest that the attaching of transcription factors at the C-terminal of the fluorescent protein is feasible for the monitoring subcellular localization. Attention should be also paid to the total length of fused cDNAs. In the present study, no fluorescent signals from AmCFP-DEL1 and ZsGFP-DEL1 were observed as well as DEL1-AmCFP (Table 3), suggesting that the length of cDNA fused to the fluorescent protein also influences the efficiency of

expression in *P. yezoensis* cells. Consistent with this, the number of cells expressing the fusion protein was significantly lower than those expressing the fluorescent protein itself (see Tables 2 and 3). The effect of the length of cDNA on detection of the expression was confirmed by the observation of no fluorescent signals from ZsGFP fused with cDNAs encoding sigma factors whose ORFs are 1.3 kbp.

From the above findings, it is clear that our system is useful for visualizing subcellular localization of desired proteins in *P. yezoensis* cells. However, we did find that overexpression of ZsGFP and sGFP had an inhibitory effect on the development of monospores (data not shown) as seen previously with AmCFP (Mikami et al. 2009). This prevents analysis of the molecular mechanism behind the development of monospores and gametophytes of *P. yezoensis* through observations of gene function. Particle bombardment itself does not appear to be the reason for this negative effect, since monospores overexpressing the GUS protein were developed normally (data not shown). Therefore, to increase the applicability of our system in future biological studies, it is necessary to remove the inhibitory effects of overexpressed fluorescent proteins on monospore development.

In conclusion, we showed for the first time, subcellular localization of transcription factors in the red algae *P. yezoensis* using various fluorescent proteins. Our system will provide new opportunities for functional analysis of transcription factors in *P. yezoensis* and other red algae through identification of the motifs involved in import into and export from the nucleus as well as co-transfection with desired promoter–reporter constructs for investigation of transcriptional regulation via transcription factors.

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Figure legends

Fig. 1. Structural characteristics of PyDEL1.

(A) Domain organization of PyDEL1. Conserved DNA binding domains 1 (DB1) and 2 (DB2) are represented by gray boxes. Numbers correspond to amino acid positions in relation with the first methionine residue.

(B) Comparison of amino acid sequences of DB1 and DB2 in DEL1 homologues.

PyDEL1, *Porphyra yezoensis* from the present study; HsE2F7, *Homo sapiens* (Hs

NP_976328); AtDEL1, *A. thaliana* (At NP_190399); CmE2F, *Cyanidioschyzon*

merolae (Cm CMT433C); OsDEL, *Ostreococcus tauri* (Os AAV68606). These

homologues were aligned using ClustalW. The amino acid sequence of CmE2F was

found in the “*C. merolae* Genome project” (<http://merolae.biol.s.u-tokyo.ac.jp/>).

Identical residues are boxed and conserved substitutions are highlighted in gray. Bars

represent gaps. Numbers indicate amino acid positions.

Fig. 2. Structural characteristics of PyElf1.

(A) Domain organization of PyElf1. The conserved C4 type-zinc finger domain is represented by a gray box. Numbers correspond to amino acid positions in relation with the first methionine residue.

(B) Comparison of amino acid sequences of Elf1 homologues. Py, *P. yezoensis* from the

present study; Sc, *Saccharomyces cerevisiae* (Sc NP_012762); Hs, *H. sapiens* (Hs

CH471106); At, *A. thaliana* (At NM_123971); Cr, *Chlamydomonas reinhardtii* (Cr

Chlre3 scaffold 13:1300308-1300837); Cm, *C. merolae* (Cm CMO247C). These homologues were aligned using ClustalW. The amino acid sequence of CrElf1 was derived from the website “*C. reinhardtii* Genome project” (<http://genome.jgi-psf.org/Chlre3/Chlre3.home.html>). Identical residues are boxed and conserved substitutions are highlighted in gray. The four conserved cysteines of the zinc finger domain are indicated by arrows above the alignment. Bars represent gaps. Numbers indicate amino acid positions.

Fig. 3. Structural characteristics of PyMBF1.

(A) Domain organization of PyMBF1. The conserved helix-turn-helix (HTH) domain is represented by a gray box. Numbers correspond to amino acid positions in relation with the first methionine residue.

(B) Comparison of amino acid sequences of MBF1 homologues. Py, *P. yezoensis* from the present study; Sc, *S. cerevisiae* (Sc NC_001147); Hs, *H. sapiens* (Hs NM_003792); At, *A. thaliana* (At NM_129829); Cr, *C. reinhardtii* (Cr XM_001699673); Cm, *C. merolae* (Cm CMJ111C); Nc, *Neurospora crassa* (Nc XP_960690). These homologues were aligned using ClustalW. Identical residues are boxed and conserved substitutions are highlighted in gray. The amino acid thought to be important for TBP binding is indicated by an arrowhead. Bars represent gaps. Numbers indicate amino acid positions.

Fig. 4. Expression of *PyDELI*, *PyElf1* and *PyMBF1* transcripts in *P. yezoensis* cells.

RNA prepared from gametophytic cells cultured at 15°C and 25°C (15G and 25G, respectively) and from sporophytic cells cultured at 15°C and 25°C (15S and 25S,

respectively) were used for RT-PCR. Lanes 1 to 4, *PyGAPDH* (869 bp); lanes 5-8, *PyDEL1* (1376 bp); lanes 9-12, *PyElf1* (255 bp); lane 13-16, *PyMBF1* (384 bp). M, size marker.

Fig. 5. Expression of AmCFP, ZsGFP, sGFP and ZsYFP in gametophytic cells of *P. yezoensis*.

(A) Schematic representation of structures of fusion genes used in particle bombardment. The 3.0-kbp long *PyAct1* promoter was fused to AmCFP (sky blue), ZsGFP (yellow green), sGFP (green) and ZsYFP (yellow) coding sequences, resulting in the construction of pPyAct1-AmCFP, pPyAct1-ZsGFP, pPyAct1-sGFP and pPyAct1-ZsYFP, respectively. NOST: terminator of a *nos* gene.

(B) Expression of humanized fluorescent proteins and sGFP. Gametophytic cells of *P. yezoensis* were transiently transformed with expression plasmids pPyAct1-AmCFP (a and b), pPyAct1-ZsGFP (c and d), pPyAct1-sGFP (e and f) and pPyAct1-ZsYFP (g and h) by particle bombardment. Gametophytes were then examined by fluorescent microscopy 3 d after transient transformation. Fluorescent images (b, d, f and h) are shown together with bright field images (a, c, e and g). Scale bar corresponds to 10 μ m.

Fig. 6. Nuclear localization of PyElf1 and PyMBF1 in gametophytic cells of *P. yezoensis*.

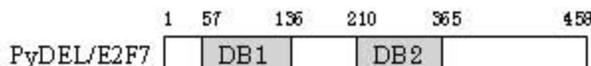
(A) Schematic representation of structures of fusion genes used in particle bombardment. The lengths of ORFs encoding PyDEL1 (light green), PyElf1 (pink) and PyMBF1 (blue) are 1374 bp, 255 bp and 384 bp, respectively.

(B) Expression of PyElf1 and PyMBF1 fused with humanized fluorescent proteins.

Expression plasmids pPyAct1-AmCFP-Elf1 (a and b), pPyAct1-AmCFP-MBF1, (c and d), pPyAct1-ZsGFP-Elf1, (e and f) and pPyAct1-ZsGFP-MBF1 (g and h) were introduced into gametophytic cells of *P. yezoensis*. Gametophytes were examined by fluorescent microscopy after 3d of transient transformation. Fluorescent images (b, d, f and h) are shown together with bright field images (a, c, e and g). Scale bar corresponds to 10 μm .

(C) Confirmation of nuclear localization of PyElf1 and PyMBF1. Gametophytic cells transiently transformed with expression plasmids pPyAct1-AmCFP-Elf1 (a to d) and pPyAct1-AmCFP-MBF1 (d to h) were fixed with 80% methanol then stained with SYBR Gold. (b and f) Expression of AmCFP fusions. (c and g) Signals of SYBR Gold staining. (d and h) Merged images of AmCFP and SYBR Gold. Fluorescent images (b, c, d, f, g and h) are shown together with bright field images (a and e). Scale bar corresponds to 10 μm .

A



B

DB1

PyDEL1	57	SRKQKSLGLLCEMFVNLV	GQTGSDGAGAA	85
HsE2F7	141	SRKQKSLGLLCKKFLARY	PSYP-----	162
AtDEL1	33	SRKQKSLGLLCTMFLALYN	-----	51
CmE2F	32	SRKQKSLGLLCEMFLSLV	GVLEQA-----	55
OsDEL	34	SRKQKSLWTLCEMFLTIY	G-----	52

RRXYD motif

PyDEL1	86	ADADGQPSDICTDAALRLH	WFRRIIYD	114
HsE2F7	163	--LSTEKTTISLDEVAVSL	GWERRRIYD	189
AtDEL1	52	---REGIEMVGLDDAASKL	GWERRRIYD	77
CmE2F	56	--RGNSSCEICLDEAATRL	GWERRRIYD	82
OsDEL	53	---DGSKEVSLDDAATRL	GWERRRIYD	78

PyDEL	115	WVLEALGVVVRKAKNRY	ITWIG	136
HsE2F7	190	WVLESLHLVSRVAKNQY	GWHG	211
AtDEL1	78	WVLESVGVLTFRKAKNQY	ITWIG	99
CmE2F	83	WVLESVGMVTRKAKNKY	ITWIG	104
OsDEL	79	WVLESVEVLETRKAKNQY	ITWIG	100

DB2

PyDEL1	210	RREKSLGLVLSDRFWQL	FLLGGTEMPPGDV	238
HsE2F7	282	RKDKSLRIMSDFVML	FLVSK----TKI	305
AtDEL	169	RREKSLGLLTDWFKL	FLCSEA----IRI	193
CmE2F	190	RREKSLGALSDFWQL	FLLAG----GDT	213
OsDEL	191	RREKSLGLLSDFWQL	FLLASK----MNV	214

PyDEL1	239	GTTSSAAGAPGSGP	SGGGVGDSSVGDT	267
HsE2F7	306	VTLDVANK-----	-----	313
AtDEL	194	ISLDDANK-----	-----	201
CmE2F	214	ISLEYAAS-----	-----	221
OsDEL	215	VSLETAAR-----	-----	222

PyDEL1	268	AGIVAPGAPGNRSST	GLADGGSGGVANGA	296
HsE2F7	314	--ILIEESQDA-----	-----	322
AtDEL1	202	--LLGDA-----	-----	207
CmE2F	222	--ILLGSGVGNR-----	-----	231
OsDEL	223	--IIMGE--DD-----	-----	229

PyDEL1	297	DGPGGSIVSLEAAAA	RLLGPSVGATPAAV	325
HsE2F7	323	-----	PD	324
AtDEL1	208	-----	HN	209
CmE2F	232	-----EAEEN-----	PL	238
OsDEL	230	-----	DD	231

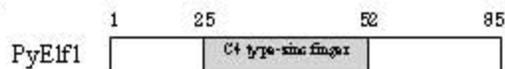
RRXYD motif

PyDEL1	326	AAKMTKVRRLYD	IANLLASLNITKRVHT	354
HsE2F	325	HSKFTKVRRLYD	IANVLTSLALIKKVHV	353
AtDEL1	210	TSIMTKVRRLYD	IANVLSMNLTEKTHIT	237
CmE2F	239	NGGMKTKVRRLYD	IANLLSSLGLTEKTHIT	267
OsDEL	232	EAKLTKVRRLYD	IANLLCSLRLLKRVHV	260

PyDEL1	355	ASRK---PAFRMLG	365
HsE2F	354	TEERGRKPAFKWIG	367
AtDEL1	238	LDSE--KPAFKMLG	250
CmE2F	268	EYRK---PAFVWCG	278
OsDEL	261	GETR--KPAFLMLQ	272

Fig. 1

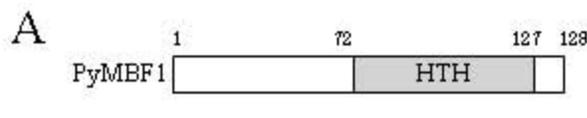
A



B

Py	1	MGRRKKATRPAPKAKAP-KLDTCFD	CHLLE	29
Sc	1	MGRRKKSTRKPTKRLVQ-KLDTKFN	CLFEN	29
Hs	1	MGRRKSKRKPPPKKMTGTLETQFT	CPFCN	30
At	1	MGRRKSRAPAPTKRMD-KLDTIFFS	CPFCN	29
Cr	1	MGRRKGGSKPPPKKARA-KLDTLFS	CPFCN	29
Cm	1	MGRRKSSKPPVVERVQP-KLDKRF	CPFCN	29
		C4 type-zinc finger		
Py	30	HEQCVDAVLLRSQK--VGSVECRV	CHVRHE	57
Sc	30	HEKSVSCTLDKKNM--IGTLSCKI	CGQSFQ	57
Hs	31	HEKSCDVKMDRARN--TGVLSCTV	CLEEFQ	58
At	30	HGSSVECIIDMKHL--IGKAACRI	CEESFS	57
Cr	30	SSKSVSINFDRDMG--RATAKCSQ	CSQKYE	57
Cm	30	QPKAVEVLLDRSTVPMRGRLECGV	CGASYQ	59
Py	58	MTITALDEPIDWYFSDMVD	EMVKVNGGEE--	85
Sc	58	TRINSLSQPVDWYFSDMFD	AVEEVMSGRGSD	87
Hs	59	TPITYLSEPVVDWYFSDMID	ACEAANQ-----	83
At	58	TTITALTEAIDWYFSEWIDE	CERVMTAEDDV	87
Cr	58	SRCTPLTDAVDWYFHDWLD	SCEEANK-----	82
Cm	60	CAINSLSEPVVDWYFSDMID	TIEEVMAAEPGD	89
Py		-----	-----	
Sc	88	TDDGDEGSDSDYESDSE	QDARTQMDGEIDS	117
Hs		-----	-----	
At	88	VQEEEEVEEEEEEEEEDE	EDDHVSVKRK	117
Cr		-----	-----	
Cm	90	S-----	-----	90
Py		-----	-----	
Sc	118	DEEEVDSDEERIGQVKR	GRGALVDS	145
Hs		-----	-----	
At	118	YNF-----	-----	120
Cr		-----	-----	
Cm		-----	-----	

Fig. 2



B

Py	1	MNQDWA-PVRI	RPK--TGSKKAD-----	20
Sc	1	MSDWDNTIIGSRARAGGSGPRANVAR	SQG	30
Hs	1	MAESDWDVTVLRKKGPTAAQAKSKQAIL-		29
At	1	MAGIGPITQDWEPPVIRKKPAMAAAKRDEK		30
Cr	1	MNMNSQDWD-TVVL	RKKQPTGAALKDEAA-	28
Cm	1	METQDWK-EV	VINPR-----AKRNQVSSQS	24
Nc	1	MSAWDTDAVKIGKNVSRGGAGPRETVVRGK		30
Py	21	--VSAAVRSG	GTVVAEKKHGAGSNS-----	43
Sc	31	QIN-AARRQGLVVSVDKKYGSTNTRGDN--		57
Hs	30	----AAQRG	EDVETSKKWAAGQNKQ-----	51
At	31	TVN-AARRSGADIE	TVRKFMAAGTNKAAS--	57
Cr	29	-VN-AARRQGA	AVE TSQKFMAGKNKPGAAQ	56
Cm	25	LTGPIT---	GAAAEK--KFGAGSNK-----	44
Nc	31	SALNAAQRSG	NIIAATEKKFAAGNTASKP--	58
Py	44	RTGDTNLSKLD	AETDVLAHATVLSLSKST	73
Sc	58	--EGQRLTKVD	RETDIVKPKKLDPNVGRAI	85
Hs	52	HSITKNTAKLD	RETEELHHDRTVLEVGKVI	81
At	58	SGTSLNTKMLD	DDTENLTHE RVPTELKKA	87
Cr	57	TVSGKPAAKLE	QETEDFHHERVSSNLKQQL	86
Cm	45	KMLVPNAKRLD	EAEHEPERLQSYPLSLAKRI	74
Nc	59	GVEGQRLTMVDR	SDDIVKPKTVSKEVGAAI	88
Helix-turn-helix				
Py	74	QKARI-----	DKKWSQAQLAQAIN	EPAVI 98
Sc	86	SRART-----	DKKMSQKDLATKINEK	PTVW 110
Hs	82	QQGRQ-----	SKGLTQKDLATKINEK	PQVI 106
At	88	MQART-----	DKKLTQSQLAQIINEK	PQVI 112
Cr	87	VQART-----	AKKMTQAQLAQAIN	EPQVI 111
Cm	75	QQARQ-----	QKQWTQTQLAQALGER	ARVW 99
Nc	89	QKARSAIMIGDK	AMTQKELATRCN	STQALI 118
▽ Helix-turn-helix				
Py	99	NQYESGKALP	NQIISKIERALG	TKVRSKK 128
Sc	111	NDYEAARAIP	QQVLSKLERALG	VKLRGMN 140
Hs	107	ADYESGRAIP	NMQVLGKIERAIG	LKLRGKD 136
At	113	QHYESGKAIP	NQIILSKLERALG	AKLRGKK 142
Cr	112	QHYEQGKAIP	NQVLSKLSRALG	VVLK-- 139
Cm	100	NDYERAAVPP	NPVIINKMEKALG	VRLRGQG 129
Nc	119	ACYERGEVPE	DQKLLGNLERV	LVNVKLRGSD 148
Py	-----	-----	-----	-----
Sc	141	IGSPLGAPKKK-		151
Hs	137	IGKPIEKGPRAK		148
At	-----	-----	-----	-----
Cr	-----	-----	-----	-----
Cm	130	STPAPSAK----		137
Nc	149	IGKPKFEKKEKK		160

Fig. 3

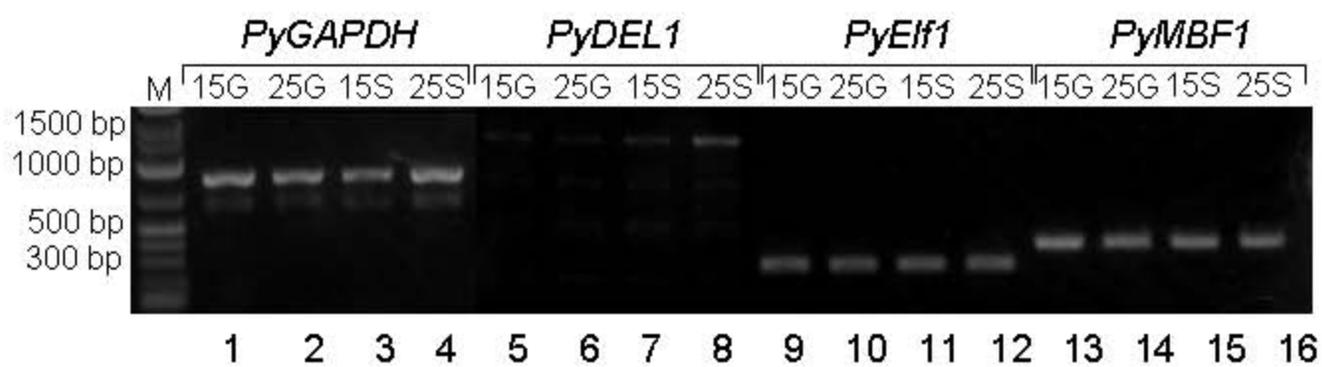


Fig. 4

A

pPyAct1-AmCFP	PyAct1 promoter	AmCFP	NOST
pPyAct1-ZsGFP	PyAct1 promoter	ZsGFP	NOST
pPyAct1-sGFP	PyAct1 promoter	sGFP	NOST
pPyAct1-ZsYFP	PyAct1 promoter	ZsYFP	NOST

B

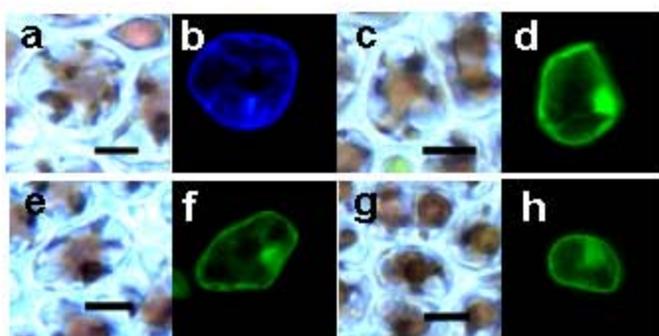
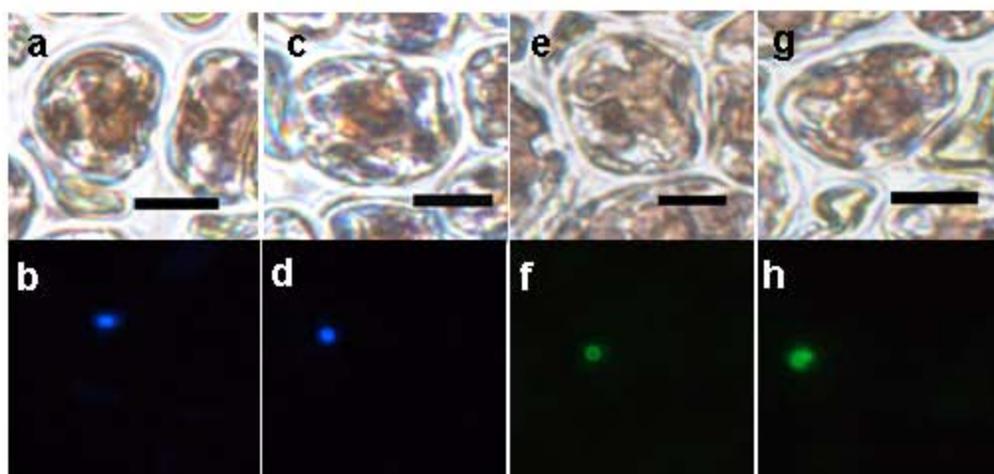


Fig. 5

A

pPyAct1-AmCFP-DEL1	PyAct1 promoter	AmCFP	PyDEL1	NOST
pPyAct1-AmCFP-E1f1	PyAct1 promoter	AmCFP	PyE1f1	NOST
pPyAct1-AmCFP-MBF1	PyAct1 promoter	AmCFP	PyMBF1	NOST
pPyAct1-DEL1-AmCFP	PyAct1 promoter	PyDEL1	AmCFP	NOST
pPyAct1-E1f1-AmCFP	PyAct1 promoter	PyE1f1	AmCFP	NOST
pPyAct1-MBF1-AmCFP	PyAct1 promoter	PyMBF1	AmCFP	NOST
pPyAct1-ZsGFP-DEL1	PyAct1 promoter	ZsGFP	PyDEL1	NOST
pPyAct1-ZsGFP-E1f1	PyAct1 promoter	ZsGFP	PyE1f1	NOST
pPyAct1-ZsGFP-MBF1	PyAct1 promoter	ZsGFP	PyMBF1	NOST

B



C

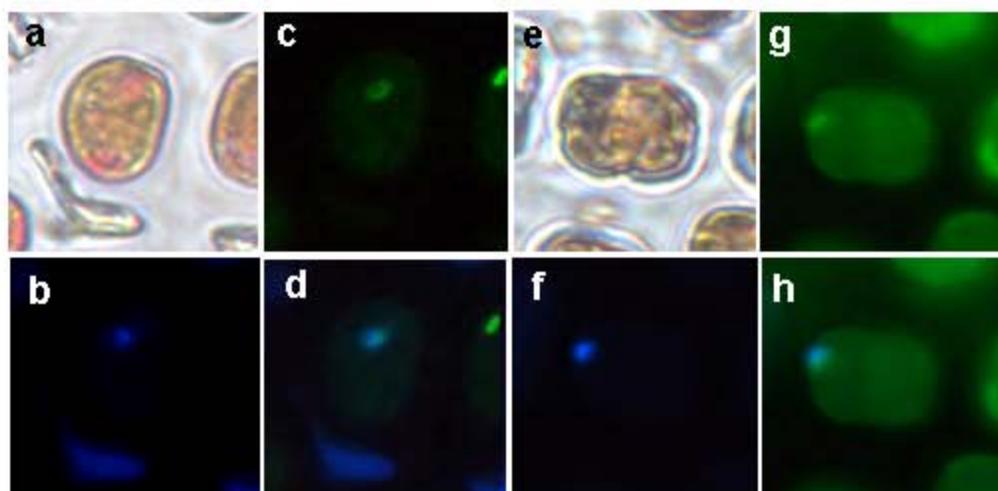


Fig. 6