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Research article

A thylakoid-localised FK506-binding protein in wheat may be linked to chloroplast biogenesis

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ABSTRACT

Plant chloroplasts contain a large proportion of immunophilins, comprising the FK506-binding proteins (FKBPs) and cyclophilins (CYPs), which are members of the peptidyl-prolyl *cis*/*trans* isomerase (PPIase) family of proline-folding enzymes. Some of the chloroplastic immunophilins are known to chaperone certain photosynthetic proteins, however the functions of a majority of these proteins are unknown. This work focussed on characterisation of genes encoding the chloroplast-localised FKBP16-1 from wheat and its progenitor species, and identification of its putative promoters, as well as investigations into the effects of light regulation and plant development on its expression. The work identified several alternatively spliced *FKBP16-1* transcripts, indicating expression of FKBP16–1 may be post-transcriptionally regulated. FKBP16-1 was expressed in both green and etiolated tissues, and highest levels were detected in developing tissues, indicating a role in chloroplast biogenesis. We also report a novel transcription module, designated 'chloroplast biogenesis module' (CBM) in the *FKBP16-1* promoter of cereals that also appears to be involved in the regulation of additional genes involved in chloroplast biogenesis or other aspects of plant development. The results point to considerable potential for a role for FKBP16-1 in early chloroplast development, architecture of photosynthetic apparatus and plant development.

1. Introduction

The peptidyl prolyl *cis*/*trans* isomerase (PPIase) enzymes catalyse the rotation of peptide bonds preceding proline residues between *cis* and *trans* configurations during protein folding processes. The PPIase superfamily comprises the immunosuppressant drug-binding immunophilins and the parvulins [reviewed in [12]]. The immunophilins consist of the FK506-binding proteins (FKBPs) [20,42] and the unrelated cyclosporin A-binding cyclophilins (CYPs) [19], both characterised by their conserved catalytic domains. FKBPs and CYPs are ubiquitous and usually occur as large protein families distributed throughout the cell in eukaryotes [12].

Higher plant genomes typically encode about twenty FKBP-type and thirty CYP-type immunophilins [reviewed in [38]]. A number of these are known to have PPIase activity, however some isoforms show severely reduced activity [4,29] while many others lack any such activity [24,40]. The operation of numerous immunophilins as molecular chaperones with specific partner proteins suggests a primary function for the immunophilins that may not actually involve PPIase activity. Higher plants employ the chaperone activity of certain immunophilins in their response to changing environmental conditions, for example the rice isoforms OsFKBP1 [17] and OsFKBP20 [34], and ROF1 and ROF2 in Arabidopsis [31]. Plant development processes also require immunophilin chaperones, such as the transcription factor transporter FKBP72 (PAS1) involved in cell division [46], and the glycoprotein chaperone AtFKB42 (TWD1) that regulates cellular auxin efflux [2]. AtCYP19-4 (Cyp5) and AtCYP20-1 (ROC7) may also regulate development processes through interactions with a nucleotide exchange factor and protein phosphatase, respectively [37].

A large proportion of the immunophilin repertoire of higher plants is targeted to the chloroplast, with eleven FKBPs and five cyclophilins localised to the thylakoid membrane and lumenal space in Arabidopsis [38,21].The operation of most of the thylakoid-localised immunophilins is uninvolved with proline isomerisation, as only AtCYP20-2 and AtFKBP13 show PPIase activity [40]. Moreover, PPIase activity is dispensable for normal chloroplast function [23]. The functional significance of the immunophilin population within this compartment may more likely involve regulating the assembly and/or maintenance of the multi-protein photosynthetic complexes [10] through chaperone interactions. AtCYP38 regulates phosphorylation-mediated regeneration of photosystem II (PSII) through

Abbreviations: FKBP, FK506- and rapamycin-binding protein; CYP, cyclophilin; PPIase, peptidyl prolyl *cis*/*trans* isomerase; CBM, chloroplast biogenesis module; SV, splice variant.

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interaction with a thylakoid phosphatase [10,43]. AtFKBP20-2 is also important for accumulation of PSII supercomplexes [29]. AtFKBP16-2 was recently identified as part of the NAD(P)H dehydrogenase (NDH) complex and is vital for stability of that complex in the thylakoid membrane [35], while AtCYP20-2 has been detected in both PSII [38] and NDH complexes [44]. AtFKBP13 is known to interact with the Rieske protein, a subunit of the electron transfer complex [18]. Although they await further characterisation, the existence of numerous immunophilins in the thylakoid may be justified by their operation as chaperones for individual photosynthetic proteins.

Light-induced maturation of chloroplasts, from proplastids in developing meristematic tissue or etioplasts in dark-adapted tissues, enlists the cooperation of a multitude of proteins expressed from both nuclear and plastid genomes [reviewed in [33]]. In the developing chloroplast, construction of the thylakoid membranes and incorporation of photosynthetic complexes are vital, although not well understood. Recently, CYPs and FKBPs were detected in the wheat etioplast proteome alongside numerous photosynthetic subunits and biosynthetic enzymes [1], suggesting a role for immunophilins during chloroplast biogenesis. In this report, we investigate the wheat genes encoding an FKBP putatively targeted to the thylakoid lumen. The results show a conserved gene family, unique mechanisms of transcriptional as well as post-transcriptional regulation and potential roles in early chloroplast development.

2. Materials and methods

2.1. Plant materials and growth conditions

Seeds of common wheat (*Triticum aestivum* L.) (AABBDD) cv. Cranbrook (AWCC accession number 22660), durum wheat (*Triticum turgidum* var. *durum*) (AABB) Cyprus 14 (12 818), and diploid progenitors of wheat, *Triticum urartu* (AA) (27 032), *Ae. speltoides* (BB) (19 607) and *Ae. tauschii* (DD) (21 927) were kindly provided by the Australian Winter Cereals Collection (AWCC), Tamworth, Australia. All genotypes were used for genomic DNA (gDNA) analysis, while *T. aestivum* cv. Cranbrook was used for RNA analysis. Seedlings were grown typically under 16 h light/8 h dark photoperiods, 25 °C and 70% humidity. Plants were grown either for 14 days (for DNA extractions and for RNA extraction from developing tissues), 6 weeks (for RNA from mature tissues) or 8 days (for all other RNA extractions). For reverse transcriptase PCR (RT-PCR; see below), individual leaf, stem and root tissues were harvested from 14 day-old plants ($n = 3$) and leaves also from 6 week-old plants ($n = 3$). All other nucleic acid extractions were from total aerial tissues. All tissues were snap-frozen in liquid nitrogen and stored at −80 °C.

The total aerial tissues from 8-day-old *T. aestivum* grown under normal conditions were harvested as control ($n = 3$), pooled, snap-frozen and stored frozen as above. Other normally grown plants were exposed to 4 °C (cold-stress) or 40 °C (heat stress) for 1 h or 8 h. Total aerial tissues were harvested from three plants at each time point, from each temperature and stored. Etiolated plants ($n = 20$) were grown in complete darkness at 25 °C and 70% humidity for 8 days and total aerial tissues from three randomly selected plants were harvested as etiolated controls, pooled and stored. For light-induced expression analysis, some of the 8 day etiolated plants ($n = 15$) were introduced into typical conditions for 1, 8 or 24 h. Aerial tissues from three randomly selected plants were harvested at each time point, pooled and stored. Growth of plants under stress (temperature and etiolation) was repeated as above to assess accuracy of RT-PCR.

2.1.1. Genomic DNA isolation and amplification of FKBP16-1 gene sequences

Leaf tissues from three plants each of the 2n, 4n and 6n wheat were pooled and used for gDNA extraction with the Wizard Genomic DNA Purification Kit (Promega, Australia). The near-full-length *FKBP16-1* genes were amplified from gDNAs with the TaFKBP16-1 F1/R1 primers (forward 5′ CCCGTGCGCCTCTCCGTTTC 3′ and reverse 5′ CAGGACTTTGAGCAGCTGAACC 3′) designed based on conserved regions in an alignment of *T. aestivum FKBP16-1* expressed sequence tags (ESTs) (see below; alignment not shown). The amplifications were carried out in 20 μl volumes, typically consisting of 100 ng of gDNA, 0.05 μg of each primer and 10 μl 2X BioMix (Bioline, Australia; contains *Taq* polymerase, dNTPs). Cycling involved initial denaturation at 94 °C for 5 min, then 30 cycles of denaturation (94 °C, 30 s), annealing (68 °C, 30 s) and extension (72 °C, 2 min), then a final extension (72 °C, 5 min). Amplification of the 5′ upstream region of *TaFKBP16-1* using primers TaFKBP16-1Ups (5′ CTGGCGCCTTAGACCAACTCG 3′) and TaFKBP16-1R2 (5′ CAAGCTTTACTCCACTGTCCAGC) was conducted similarly except at an annealing temperature of 63 °C.

2.1.2. Cloning and sequencing of FKBP16-1 genes

The above PCR products were purified using Perfectprep Gel Cleanup Kit (Eppendorf, Australia) with slight modifications, then cloned into pGEM-T Easy (Promega, Australia), transformed into *E. coli* JM109 and plated on plates containing ampicillin/IPTG/X-gal. Up to ten recombinant (white) colonies per ligation were grown overnight in Luria Bertani broths containing ampicillin, and plasmids purified with the Wizard *Plus* SV Minipreps DNA Purification System (Promega, Australia). The plasmids were sequenced using T7 primer and BigDye termination chemistry v3.1 (Applied Biosystems, USA) as per the protocol supplied the Australian Genome Research Facility (AGRF, Melbourne) and analysed by capillary separation at AGRF.

2.1.3. Isolation of the putative FKBP16-1 promoter with inverse PCR

For amplification of the upstream regions of *FKBP16-1* genes by inverse PCR (IPCR), 2.5 μg of gDNA of Cranbrook was digested with 50 units of KpnI at 37 °C overnight, the reaction then stopped by incubation at 70 °C for 10 min. Ligations consisted of approximately 100 ng of digested DNA and 3 Weiss units of T4 DNA ligase (Promega, Australia) in 100 μl volume, conducted overnight at 4 °C, then stopped by heating at 70 °C for 10 min. IPCR (first round) was performed using TaFKBP16-1GW1 primers (forward 5′ GTAGGCTATATCGACGAAGAGCTTGCAG 3′ and reverse 5′ GCTCTGAGTCCGCGGCATGTCGACCTGG 3′) designed to anneal to conserved areas in the last and first exons of *TaFKBP16-1,* respectively, according to an alignment of *TaFKBP16-1* ESTs (see below). The reactions consisted of 2.5 ng circularised (*Kpn*I-digested, then ligated) gDNA, 0.1 μg of each primer and 25 μl of 2× BioMix, in 50 μl volumes. PCR involved initial denaturation, then 35 cycles of denaturation (94 °C, 45 s), annealing (60 °C, 45 s) and extension (72 °C, 150 s), then a final extension. To test the success, a nested (second round) PCR was conducted with 2 μl of first round product and the TaFKBP16-1GW2 primers (forward 5′ GCTGGTGTTTGAGGTTCAGCTGCTCA 3′ and reverse 5′ GGTGGATGCGGGCGTCTGATTGGATTGGG 3′) under the same conditions. The products of second round IPCR were cloned and sequenced as above.

2.1.4. RNA isolation and cDNA synthesis

Each sample comprised pooled tissues harvested from three individual plants, stored as above. The tissue was ground to a powder under liquid nitrogen and total RNA extracted using the-Trisure reagent (Bioline, Australia). RNA was precipitated with 100% ethanol, resuspended in diethyl pyrocarbonate (DEPC) treated MilliQ water and stored at −80 °C. RNAs were quantified spectrophotometrically and inspected for DNA contamination by agarose electrophoresis. Where necessary, DNA was removed by combining 10 μg RNA with 10 Weiss units of DNase I (Promega, Australia), 2 units