**BIGYIN, an orthologue of human and yeast FIS1 genes functions in the control of mitochondrial size and number in Arabidopsis thaliana**

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Abstract

Reverse-genetics was used to evaluate the role of an Arabidopsis homologue of the human and yeast FIS1 genes, which are both involved in mitochondrial fission. Two independent T-DNA insertion mutants of gene At3g57090 were identified and genetically transformed to express mitochondria-targeted GFP to enable visualization of mitochondria in vivo. Plants homozygous for either of the recessive T-DNA mutant alleles, termed bigyin1-1 (bgy1-1) and bigyin1-2 (bgy1-2), displayed an abnormal mitochondrial morphology. Disruption of BIGYIN leads to a reduced number of mitochondria per cell, coupled to a large increase in the size of individual mitochondria, relative to wild-type. It is concluded that BIGYIN is an Arabidopsis FIS orthologue and is part of the Arabidopsis mitochondrial division apparatus.

Key words: Dynamin, FIS1, mitochondria, mitochondrial division, morphology, organelle fission.

**Introduction**

Mitochondria undergo continual cycles of division and fusion. These two antagonistic processes, which operate concurrently, regulate the number, size, and shape of mitochondria in a cell (Sesaki and Jensen, 1999). The process of division is of particular importance since mitochondria cannot be created de novo, and must therefore be formed by the division of an existing organelle. In yeast and humans, several of the genetic components that control mitochondrial division and fusion have been identified and their function elucidated (for a review see Logan, 2003; Okamoto and Shaw, 2005).

For example, in budding yeast (Saccharomyces cerevisiae) three genes encode the primary components of the mitochondrial division machinery. DNM1 encodes a protein that is structurally similar to the dynamin-related GTPase proteins involved in membrane scission during endocytosis (Hermann et al., 1997; Otsuga et al., 1998). Yeast Dnm1p is targeted to mitochondrial division sites and is believed to act as a mechano-enzyme, constricting and/or severing the mitochondrial membranes. This dynamin-related protein requires two other interacting proteins to effect yeast mitochondrial division. Firstly, Mdv1p which, like Dnm1p, localizes to the outer mitochondrial membrane at division sites (Fekkes et al., 2000; Mozdly et al., 2000; Tieu and Nunnari, 2000; Cerveny et al., 2001). Secondly, Fis1p which is evenly distributed across the mitochondrial outer surface (Mozdly et al., 2000; Tieu and Nunnari, 2000). These three proteins form a complex on the yeast outer mitochondrial membrane and function in concert to effect division of the organelle (Cerveny and Jensen, 2003). All three genes are required for effective mitochondrial division in yeast, since knocking out any one of the three leads to a similar abnormal mitochondrial phenotype (a net-like sheet of mitochondrial tubules). In humans, normal mitochondrial division requires the dynamin-related DRP1 and hFIS1, which function analogously to their yeast orthologues, DNM1 and FIS1 (Smirnova et al., 2001; James et al., 2003; Youle and Karbowski, 2005). Searching the databases using the BLAST algorithm fails to identify cognate homologues of MDV1 in multicellular organisms: limited protein level similarity is restricted to members of the WD-40 repeat family of proteins (Logan, 2003; Stojanovski et al., 2004).

Research into the mitochondrial division apparatus in higher plants is at a rudimentary stage. Recently, it has been shown that two non-redundant Arabidopsis thaliana...
Dnm1p/DRP1 homologues are involved in mitochondrial division. Disruptions in either DRP3A or DRP3B lead to an altered mitochondrial phenotype where the organelles are both larger and fewer in number, suggesting both proteins are required for the normal division of mitochondria (Arimura and Tsutsumi, 2002; Arimura et al., 2004; Logan et al., 2004). The identification of two mutant alleles of an Arabidopsis hFIS1/FIS1 orthologue (At3g57090) is reported here. These mutants, which were named bigyn1-1 and bigyn1-2, have an aberrant mitochondrial phenotype characterized by an increase in the size of individual mitochondria and a concomitant decrease in the number of mitochondria per cell. These results demonstrate that BIGYIN is an Arabidopsis hFIS1/FIS1 orthologue and is the first member of only the second protein family known to be involved in plant mitochondrial division.

Materials and methods

Plant materials and growth conditions

Searches of the Arabidopsis genome, using the BLASTp algorithm (Gish and States, 1993) and yeast Fis1p and human hFis1 protein sequences, identified two homologues: At3g57090 (yeast 7e-7, human 6e-8) and At5g12390 (yeast 7e-6, human 2e-8) (Logan, 2003). The SAIL (Sessions et al., 2002) and SALK (Alonso et al., 2003) T-DNA insertion-line databases were searched to identify independent lines with an insertion in At3g57090. SAIL_1171_G11 is predicted to contain a T-DNA insertion in the first exon (5e-45) and SALK_086794 is predicted to contain an insertion in the final (fifth) exon (5e-15) (Fig. 1). SAIL seed was obtained from Syngenta Biotechnology Inc. (Research Triangle Park, NC, USA) and SALK seed from the Nottingham Arabidopsis Stock Centre (NASC, University of Nottingham, UK). Mitochondrial-GFP wild-type (Col-0 background) seed used in the study was of line 43C5 (Logan and Leaver, 2000). Seeds were surface-sterilized and germinated on MS-agar plates (Murashige and Skoog, 1962) containing 8% sucrose, and 0.5% (w/v) agar (Type M, Sigma Chemical Co., St Louis, USA), 2% (w/v) MES pH 5.8. For maximum synchronous germination, plates were kept in the dark at 4 °C for 3 d before transfer to a controlled environment growth room (16/8 h day/night, 25 °C). After a further 2 weeks, seedlings were transplanted to compost (Levington F2, Scotts, Marysville, USA) and grown in a greenhouse under supplementary lighting and constant 25 °C.

GFP transformation

Bulked seed from each insertion line were sown on a 2:1 mixture of compost/vermiculite in separate pots and germinated in the greenhouse. At 3-weeks-old, these plants were genetically transformed to express mitochondria-targeted GFP (mito-GFP) using the Agrobacterium floral dip method (Clough and Bent, 1998). SAIL_1171_G11 was generated using pCSA110 (Sessions et al., 2002) conferring resistance to glufosinate ammonium (BASTA) in planta and so was transformed with the mito-GFP vector pBINmpgfp5-atpase, which confers resistance to kanamycin (Logan and Leaver, 2000). SALK_086794 was generated with pROK2 (Alonso et al., 2003) conferring resistance to kanamycin in planta and so was transformed with the mito-GFP vector pMLBARTmpgfp5-atpase, which confers resistance to BASTA (Logan et al., 2004). Mito-GFP T1 seedlings of SAIL_1171_G11 were first selected by germination on MS-agar plates containing 50 mg l⁻¹ kanamycin, while mito-GFP T1 seed of SALK_086794 were sown on compost and the seedlings sprayed twice with 120 mg l⁻¹ BASTA (when 2 and 3-weeks-old). Resistant seedlings were screened by epifluorescence microscopy for expression of mito-GFP.

PCR analysis of putative insertion mutants

Both SAIL and SALK lines contain T-DNA insertions at known sites in the Arabidopsis genome and the SIGnAL iSECT tool (http://signal.salk.edu/isects.html) creates custom gene-specific forward and reverse PCR primers to check the nature of an insertion (absent, hemizygous, homozygous) in any individual plant. For SAIL_1171_G11, gene-specific forward (5'-GTATAACATC-TAACGTGGAAAGAT-3') and reverse (5'-TTCACACGAGAAA-GCACGAAAAAC-3') primers flanking the insertion site were used in combination with a primer to the T-DNA left border (LB3 5'-TAGCATCTGAATTTCGATACAC-3') to confirm the nature of the insertion (Sessions et al., 2002). For SALK_086794, the gene-specific LP (5'-TCTGGTGACCTGGG-CATTTC-3') and RP (5'-ATCCAGGT-TCTCATCCACCTC-3') primers were used in conjunction with the T-DNA left border primer LBb1 (5'-GGCTGGAACCCTGCTGCAA-3') (Alonso et al., 2003).

Microscopy and image analysis

GFP-positive plants were examined using either an Olympus BX-40 (Olympus Optical Co., UK) or Zeiss Axioskop 2 (Carl Zeiss Ltd., UK) epifluorescent microscope fitted with cubes for GFP (Olympus model 41001, excitation 455–495 nm, emission 510–550 nm; Zeiss model 488013, excitation 470–520 nm, emission 505–530 nm). Visualization of mitochondria was performed at ×1000 using an oil-immersion objective (Olympus ×100 Universal Plan Fluorite, numerical aperture=1.3; Zeiss ×100 Plan-Apochromat, numerical aperture=1.4). Epifluorescent micrographs were captured using a monochrome digital camera (F-View, Soft Imaging System GmbH, Münster, Germany) coupled to a PC running the analySIS software package (Soft Imaging System GmbH, Germany) for image analysis and storage.

Protoplasts were isolated from 2-week-old seedlings under aseptic conditions. Briefly, leaf blades were dissected, chopped, and incubated overnight at room temperature in a standard enzyme solution (0.33% w/v cellulase, 0.17% w/v pectinase, 3 mM MES, 7 mM CaCl₂ in 0.4 M mannitol). The digested material was sequentially filtered through 100 μm and 40 μm nylon mesh, harvested at 50 g and resuspended in 0.5 M mannitol to an approximate concentration of 1×10⁶ protoplasts ml⁻¹. Aliquots of the protoplast suspensions were mounted on a microscope slide under a glass cover-slip immediately prior to microscopy. Single images were captured of each of 25 intact protoplasts, chosen at random, from each of the three experimental lines. The plan area of individual mitochondria and their number per protoplast-field-of-view were measured in mito-GFP-positive wild-type, SAIL_1171_G11 and SALK_086794 protoplasts using the analySIS software package.
Results and discussion

Arabidopsis homologues of yeast FIS1 and human hFIS1

The Arabidopsis thaliana genome contains two homologues of the yeast FIS1 and human hFIS1 genes. Analysis of their protein sequence using the EMBOSS pairwise alignment program (http://www.ebi.ac.uk/emboss/align/index.html) shows that At3g57090 shares highest similarity with hFis1 (26.7% identity, 48.3% similarity) and other FIS1-type genes from multicellular organisms, such as the Caenorhabditis elegans FIS-2 gene (Fig. 2). Conversely, At5g12390 shares highest similarity with yeast Fis1p (27.0% identity, 43.8% similarity) (Fig. 2). As the Arabidopsis gene symbol FIS is already in use, At3g57090 was named BIGYIN, on the basis of the mitochondrial phenotype in the T-DNA mutants.

In silico analysis of the protein structure of BIGYIN or At5g12390 using InterProScan (http://www.ebi.ac.uk/cgi-bin/iprscan/iprscan) reveals a conserved tetratricopeptide repeat (TPR)-like binding domain (residues 51–139 of BIGYIN), common to all Fis1-type proteins (Suzuki et al., 2003, 2005). In addition, all Fis1-type proteins contain a single C-terminal putative transmembrane domain (residues 142–164 in BIGYIN) with a topology predicted to leave the N-terminal region exposed to the cytoplasm (Mozdy et al., 2000; Tieu and Nunnari, 2000; Suzuki et al., 2003; Yoon et al., 2003). The C-terminal structure of hFis1 has been demonstrated to be essential for mitochondrial localization: the transmembrane region is located within the outer-mitochondrial membrane with the C-terminal tail localized in the intermembrane space (Yoon et al., 2003). The cytosolic N-terminal region of hFis1, containing the conserved TPR motifs, has been shown to participate in the interaction with the dynamin-like protein, DLP1, or a DLP1-containing complex (Yu et al., 2005).

The second FIS1/hFIS1 homologue in Arabidopsis (At5g12390) sets it apart from yeast and humans, which contain only a single copy of a FIS1-type gene. However, the presence of multiple homologues of mitochondrial division genes appears to be a feature of the Arabidopsis genome. For example, while yeast, humans, and nematodes have a single dynamin-related gene involved in mitochondrial fission, there are at least two dynamin-related genes associated with this function in Arabidopsis (Arimura and Tsuchumi, 2002; Arimura et al., 2004; Logan et al., 2004). A similar analysis of the role of At5g12390 in mitochondrial fission is currently hampered by the lack of T-DNA mutants of this gene. Future research using alternative reverse genetics approaches will focus on the role of At5g12390 in mitochondrial dynamics and should reveal any redundancy between the two Arabidopsis Fis1-type genes.

There are fewer, but larger, mitochondria in the bigyin mutant

Mitochondria in yeast FIS1 gene knockouts do not have a normal branched-tubular structure, but instead form a mitochondrial net. A similar phenotype was observed in knockdowns of hFIS1 in COS-7 cells, although some mitochondria formed extended tubules. These phenotypes are thought

Fig. 2. Sequence homology between BIGYIN and other FIS-1-type proteins. BIGYIN has a higher sequence similarity to the FIS-1-type proteins from multicellular organisms, such as human (hFis1) and Caenorhabditis elegans (FIS-2), than to yeast (Fis1p). The regions of highest similarity represent the TPR-like binding domain (residues 51–139 in BIGYIN) and the C-terminal transmembrane domain (residues 142–164 in BIGYIN).
to result from reduced mitochondrial fission caused by an inability to recruit Dnm1p/Drp1 (Mozdy et al., 2000; Tieu and Nunnari, 2000; Stojanovski et al., 2004). To determine if BIGYIN is a functional orthologue of Fis1p/hFis1, the mitochondrial phenotype of two independent homozygous T-DNA mutants was analysed, SAIL_1171_G11_mito-GFP and SALK_086794_mito-GFP, bearing insertions within locus At3g57090 (Fig. 3). The mean mitochondrial plan area in wild-type plants was 0.366 ± 0.003 SE (n = 1793; Fig. 3B). In plants homozygous for either the SAIL or SALK mutant allele, named bigyin1-1 (bgy1-1) and bigyin1-2 (bgy1-2), respectively, mitochondrial size was greatly increased (Fig. 3B). In bigyin1-1 (SAIL_1171_G11_mito-GFP), the mean mitochondrial plan area was 0.629 ± 0.012 SE (n = 1065), while in bigyin1-2 (SALK_086794_mito-GFP), the mean plan area was 0.710 ± 0.018 SE (n = 1018) (Fig. 3B). In addition to an increase in mitochondrial plan area in bigyin mutants, there is a concomitant decrease in the number of mitochondria per cell. Wild-type protoplasts contained an average of 70.8 ± 6.2 SE mitochondria per protoplast-field-of-view, compared with means of 42.7 ± 4.9 SE and 39.7 ± 4.8 SE for bigyin1-1 and bigyin1-2, respectively (Fig. 3C). The net effect of the increase in size of individual mitochondria, coupled to the decrease in number of mitochondria per cell in bigyin, relative to wild-type, is that the total mitochondrial area per protoplast varies little between lines. For example, the average total mitochondrial plan area for wild-type is 25.6 µm², compared with 27 µm² and 28.4 µm² for bigyin1-1 and bigyin1-2, respectively. This result suggests that the total mitochondrial volume per cell is homeostatically controlled, under conditions whereby the normal equilibrium of mitochondrial fission and fusion is shifted towards fusion, and that BIGYIN is not necessary for the maintenance of total mitochondrial volume. A similar homeostatic control mechanism for mitochondrial volume was implied in an analysis of mitochondria in developing barley or wheat leaves: the volume fraction of mitochondria within mature mesophyll cells was found to be maintained at 0.6–1% of total cell volume (Bowsher and Tobin, 2001). Unfortunately, although it is well accepted that the equilibrium between mitochondrial fission and fusion controls mitochondrial shape, size, and number we can find no other analysis in the literature where the effect of a shift of this equilibrium on mitochondrial area, volume or mass has been quantified. However, similar correlations between organelle plan area and number have been reported in chloroplast arc mutants, where reductions in the number of chloroplasts per cell are linked to increases in organelle plan area (Pyke and Leech, 1991; Pyke and Leech, 1994; Aldridge et al., 2005). The mitochondrial phenotype of bigyin is similar to that in mutants of the Arabidopsis dynamin-like genes DRP3A (Arimura et al., 2004; Logan et al., 2004) and DRP3B (Arimura and Tsutsumi, 2002; I Scott, AK Tobin, DC Logan, unpublished results) and is indicative of a disruption of normal mitochondrial fission.
BIGYIN is a functional homologue of FIS1/hFIS1

Segregation analyses were performed to confirm whether or not the aberrant mitochondrial morphology phenotype was due to a homozygous T-DNA insertion in the BIGYIN gene. The mitochondrial morphology of 104 T2 individuals of line SAIL_1171_G11_mito-GFP was analysed by epifluorescent microscopy and 28 were identified as having an abnormal mitochondrial morphology phenotype. Statistical analysis of the result, using the chi-square ($\chi^2$) test, shows that the observed segregation ratio of 2.7:1 (wild-type:mutant) concurs closely with the 3:1 Mendelian segregation ratio expected for a single, recessive nuclear gene ($P > 0.6$, df=1, $n=104$). PCR analysis of the 104 T2 plants showed that all 28 individuals with aberrant mitochondrial morphology were homozygous for the mutant allele. The remaining 76 plants with wild-type mitochondrial morphology were either homozygous wild-type or hemizygous for the T-DNA insertion. A similar mitochondrial morphology analysis of the offspring from a T1 SAL-K_086794_mito-GFP hemizygote revealed a segregation ratio of 2.6:1 (wild-type:mutant), which also correlated with the expected 3:1 Mendelian segregation ratio for a single, recessive nuclear gene ($\chi^2$ test, $P > 0.6$, df=1, $n=58$). It is concluded that BIGYIN is an Arabidopsis functional orthologue of yeast FIS1 and human hFIS1 and is involved in mitochondrial fission.

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