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Sequence analysis of a 24-kb contiguous genomic region at the *Arabidopsis thaliana PFL* locus on chromosome 1 [1]

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Abstract As part of the European Union program of European Scientist Sequencing *Arabidopsis* (ESSA), the DNA sequence of a 24.053-bp insert of cosmid clone CC17J13 was determined. The cosmid is located on chromosome 1 at the *PFL* locus (position 30 cM). Analysis of the sequence and comparison to public databases predicts seven genes in this area, thus approximately one gene every 3.3 kb. Three cDNAs corresponding to genes in this region were also sequenced. The homologies and/or possible functions of the (putative) genes are discussed. Proteins encoded by genes in this region include a polyadenylate-binding protein (PAB-3) and a GTP-binding protein (Rab7) as well as a novel protein, possibly involved in double-stranded RNA unwinding and apoptosis. Intriguingly, the gene encoding the PAB-3 protein, which is very specifically expressed, is flanked by putative matrix attachment regions.

Key words: *Arabidopsis thaliana*; *PFL1*; Sequence

1. Introduction

Genome sequencing provides information concerning novel genes, gene density, gene organisation as well as spacer sequence in-between genes. Recently, the full genome sequence of three bacteria has been obtained: *Haemophilus influenzae* [1], *Mycoplasma genitalium* [2], and *Methanococcus jannaschii* [3]. The sequence of the genome of the yeast *Saccharomyces cerevisiae* has recently been determined [4]. Other ongoing genome projects include various organisms such as several bacterial extremophiles, *Escherichia coli* [5], *Bacillus subtilis* ([6]; for an overview of microbial genome projects, see http://www.tigr.org/tdb/mdb/mdb.html), the human pathogen *Candida albicans* [7], *Caenorhabditis elegans* [8], *Drosophila melanogaster* [9], and *Homo sapiens* [10].

In plant molecular biology and genetics, *Arabidopsis thaliana* is well recognised as a model organism and a wealth of information is available concerning this plant's metabolism, environmental responses, and development. Furthermore, physical and genetic maps of *Arabidopsis* are well documented with chromosomes 2, 4, and 5 being covered best [11–13].

At the end of 1993, the European Union initiated a project called European Scientists Sequencing *Arabidopsis* (acronym ESSA) with the aim to sequence large fragments of the *A. thaliana* genome. Although most of the effort was concentrated on chromosome 4 (around the *FCA* and *AP-2* genes) smaller regions throughout the genome have also been sequenced [14,15]. Here we describe the sequencing data and analysis of one region, located around the *PFL* locus on the top arm of chromosome 1.

2. Materials and methods

2.1. DNA material and sequencing

The *A. thaliana pfl* mutant is a T-DNA-tagged mutant with the T-DNA inserted into the *RPS18A* gene [16] encoding the ribosomal S18 protein. The cosmid clone CC17J13 was kindly provided to us by Caroline Dean and Clare Lister (John Innes Institute, Norwich, UK) after they had screened the cosmid library of *A. thaliana* ecotype Columbia in vector pLAFR3 [17] with the *RPS18A* cDNA as a probe.

The S18 ribosomal protein gene family consists of three highly related members in *Arabidopsis*, which are *RPS18A*, *RPS18B*, and *RPS18C* [16]. Clone CC17J13 appears to contain the *RPS18A* gene as shown by PCR using gene-specific primers. This cosmid was subsequently selected to be fully sequenced.

The whole cosmid was sheared by sonication and subcloned in M13 [18]. DNA preparation of selected subclones was done using the Autogen 750 labstation (Kurabo, Osaka, Japan). With the M13 direct primer on an Applied Biosystems 373A automated DNA sequencer (Perkin-Elmer, Norwalk, CT), 850 dye-primer sequencing reactions on single-stranded M13 subclones were performed resulting in eight contigs. Primers were designed with the program Oligo (W. Rychlik, National Biosciences, Inc., Plymouth, MN) to close double-stranded and single-stranded gaps. The average coverage of the cosmid is 6. Expressed sequence tag (EST) clones were obtained through the Arabidopsis Biological Resource Center at Ohio State University (Columbus, OH).

2.2. Computer assembly and comparisons

Sequence assembly was done with an in house developed program [19]. Searches for homology in public databases were done using several mail and Web servers available on Internet. Other analyses were done with the Wisconsin Package (version 8; Genetic Computer Group, Madison, WI) and the Blast mail server (Genetic Computer Group).

Gene predictions were done with Grail (Uberbacher and Mural, 1991), Genefinder (P. Green, personal communication), and GeneMark [20]. Exon-intron boundaries were predicted by NetPlantGene [21].

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Abbreviations: EST, expressed sequence tag; MAR, matrix attachment region; ORF, open reading frame; PAB, poly(A) binding; SAR, scaffold attachment region

[1] The genomic sequence of cosmid 17J13 has been submitted to the Genbank under accession number Y12227, the three cognate cDNAs have accession numbers Y09821, Y09822 and Y09823.

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3. Results and discussion

3.1. Chromosomal location of the region

The *A. thaliana Pfl* mutant that has been described by Van Lijsebettens et al. [16] is characterised by the presence of a pointed first leaf, reduced fresh weight, and growth retardation. The T-DNA was inserted into the *RPS18A* gene and maps to chromosome 1, near the m235 and g3829 markers [16]. On the most recent recombinant inbred map from Dean and Lister [22], these markers map around position ±30 cM (the whole chromosome 1 map being 131 cM).

In view of the sequencing project, yeast artificial chromosome (YAC) clones in the area of the *PFL* locus have been identified. The *RPS18A* gene hybridised to YAC clone EG9F9. YACs CIC5F4, CIC8A12, CIC5G7, and CIC6F2 were shown by PCR screening to contain also the *RPS18A* gene. The two first YAC clones were known to contain markers m235 and g3829, whereas the last two did not (information: ATGC Web server) (Fig. 1). The size of these YACs is approximately 400–480 kb, indicating that the distance between the *RPS18A* gene and g3829 is smaller than 400 kb (Fig. 1). Using the *RPS18A* gene as a probe, several cosmid clones could be identified in the CC cosmid library (C. Dean and C. Lister, personal communication). CC17J13 was selected to be fully sequenced (see Section 2). The orientation of the CC17J13 cosmid sequence on the chromosome is, however, not known.

3.2. Gene organisation, structure, and sequence composition of the cosmid sequence

The 24 053-bp genomic sequence was analysed by the Munich Information Center for Protein Sequences (MIPS; Max-Planck-Institut für Biochemie, Martiensried, Germany) and also by the bioinformatics team of the Laboratory of Genetics (Gent, Belgium). The genomic DNA analysis performed at MIPS can shortly be summarised as follows: at the DNA level, all submissions were compared to public database entries, all available ESTs, and to a dataset containing small sequences, such as tRNAs. In a second step, analysis of all open reading frames (ORFs; no start codon required) of at least 300 bp was extracted with the program FIND-ORF (Susanne Liebl, MIPS). The extracted potential ORFs were compared to all available protein databases. In addition, sequences were analysed with programs that predict coding regions and precise gene structures.

3.2.1. The 24-kb region contains seven putative genes. Fig. 1 and Table 1 present an overview of the putative genes in the 24 053-bp genomic region. When an EST sequence was identified that matched the genomic sequence, the corresponding cDNA was fully sequenced. Exon and intron positions for the (putative) genes can be found in the Genbank submission annotations of this sequence.

Besides the *RPS18A* gene itself [16], the genes in this region are new for *Arabidopsis*. Two genes can easily be identified due to their high homology: gene *2*, encoding a GTP-binding protein, and gene *4*, encoding a poly(A)-binding protein. In addition, a gene encoding a protein that could be involved in double-stranded RNA unwinding is predicted as well as three genes that show no homology to known database entries. Two of them (genes *5* and *3*) encode proteins that display characteristics of membrane-spanning proteins.

3.2.2. Gene organisation, gene structure, and sequence composition. The 24-kb region is relatively rich in genes with one gene every 3.3 kb. There is a clear preference for the Watson strand concerning the presence of genes, as all genes can be found on this strand. On the Crick strand only six ORFs can be found larger than 100 amino acids. No gene predictions are made by either Genmark or Genefinder for this strand.

Genes account for 62% of the sequence and the average gene size (start to stop) is 2131 bp. Average base composition is 37.2% GC. The smallest internal exon is 27 bp (gene *Rab7*) and the largest 1857 bp (gene *1*), whereas introns range from 71 bp (gene *PAB-3*) to 514 bp (gene *PAB-3*).

3.2.3. Gene 1 encodes a new Arabidopsis protein possibly involved in double-stranded RNA unwinding. The first putative gene in the sequence is probably incomplete, lacking the 5′ end. There is one large ORF encoding a (partial) protein of 618 amino acids. The available sequence shows an internal repeat (1–300, 310–619), each repeat being itself an imperfect repetition of a basic module. Each half of this protein shows homology to the C-terminus of a 469-amino acid long protein encoded by the mouse *MA-3* gene [23] (identity 30.7% and 29.3% for each repeat). This gene is induced upon apoptosis [23], but has also been isolated independently in mouse as a gene suppressed by a topoisomerase inhibitor [24] and in human as encoding a nuclear antigen [25]. In plants, a homo-

Table 1
Gene order, function, and structure on the 24-kb genomic fragment

Gene	Name or putative function of protein	Cognate ESTs	Identical or similar database entries		Corresponding cDNA	Intron	Position (start–stop)	Gene structure predicted by
			Genbank	SP/PIR				
1	Double-stranded RNA unwinding	–	D50465	Q04637	–	ND	>0–1857	Homology+GM
2	GTP-binding Rab7	151O2	Z73940	P31022	Y09821	6	2325–3572	cDNA
3	Membrane protein	34F9 144D4 E10E1	–	–	Y09823 Y09822	8	3989–5791	cDNA
4	Poly-A-binding PAB-3	–	–	Q05196	–	6	8676–11877	Homology, GM, NPG
5	Membrane protein	–	D40642	–	–	12	15476–20525	EST, GM, NPG
6	RPS18A	165P20 79E12 many others	Z23165[a]	P34788[a]	Z28701	3	21068–22241	known
7	Unknown	–	–	–	–	ND	23349–24053>	GM, NPG

[a]Identical. Abbreviations: ND, not determined because sequence of gene is probably partial; SP/PIR, Swiss Protein database/PIR International Protein database; EST, expressed sequence tag; NPG, NetPlantGene [21]; GM, GeneMark [20].

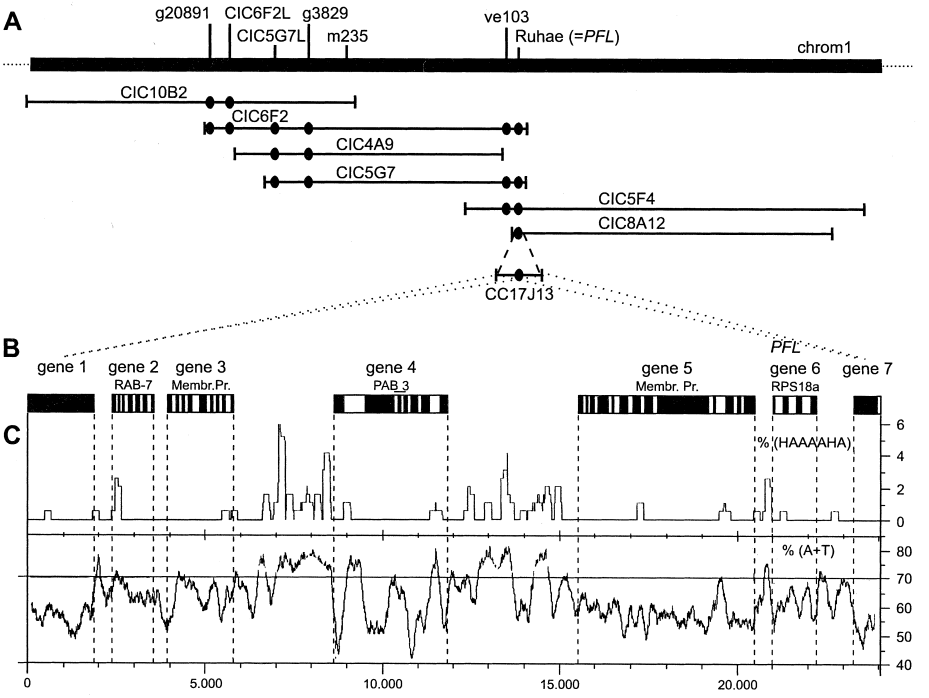

Fig. 1. A: Chromosomal location of PFL and schematic overview of the YAC clones in this area. ● indicates positive identification by the marker above (information from ATGC Web server). The region represents approximately 1.2 Mb. The sequenced cosmid CC17J13 is indicated; orientation is unknown. B: Gene order and structure (black, exons; white, introns). All genes are lying on the direct strand. C: Composition of the region (% HAAAHA and % AT) to help predict the MAR elements. The bottom line gives the length of the cosmid in bp.

logue of this *MA-3* gene seems to be induced upon apoptosis as well (S. Chamnongpol, personal communication). This protein also shows homology to the eukaryotic initiation factor IF-4 γ [26], in which the basic module is also found repeated. To our knowledge, this module has not been described before. From the observed homologies, we suggest that the gene encoding this protein may be involved in double-stranded RNA unwinding. No EST clone in the database is currently matching with this gene.

3.2.4. A new GTP-binding protein is encoded by gene 2. The second gene encodes a protein that shows high homology to GTP-binding proteins, more precisely to the Rab7 members (83.7% and 81.6% similarity with pea [27] and *Volvox carteri* Rab7 [28], respectively as calculated by GAP). For this gene, a full-length cDNA clone has been sequenced. Several other GTP-binding proteins are being identified in the *Arabidopsis* genome project. It is now clear that the consensus oligonucleotide that has been used by several groups in an effort to isolate GTP-binding proteins in plants (based on the *ras* motif DILDTAGQEEYSAM) does not really fit the plant consensus motif: only the core is conserved (QIW**DTAGQE**R(Y/F)R(A/S/T)I) (for a review, see [29]). The encoded Rab7 protein shows the typical C×C-terminus that is known to be involved in prenylation and membrane anchoring [30].

3.2.5. A gene with unknown function displays alternative splicing. The third gene is well identified because three ESTs are available that match this gene. Two of these (34F9 and 144D4) have been fully sequenced. Both cDNAs have an identical coding sequence but show different polyadenylation sites. More interesting is that the third EST (E10E1) shows alternative splicing, resulting in a last exon starting either at position 5686 for 34F9 and 144D4 or 5739 for E10E1, and coding for a C-terminus of 6 amino acids (KVKSKG) or 16 amino acids (AGDMYERTHSAALRIM), respectively. These different C-termini might be related with different cellular localisations. The 6-amino acid terminus shows some consensus to the mammalian peroxisomal targeting signal *PTS-1*, namely KSKL [31]. However, the function of this target C-terminal sequence has not yet been extensively tested in plants, although a similar sequence (ARL) is known for targeting the tobacco glycolate oxidase to the peroxisomes [32]. The encoded protein shows no homologies to known database entries. A search for transmembrane domains using servers available on the World Wide Web indicates features of a membrane-spanning protein with probably seven transmembrane domains.

3.2.6. Gene 4 encodes a new poly(A)-binding protein and is flanked by two putative matrix attachment regions. The fourth gene encodes a protein showing clear homology to poly(A)-binding (PAB) proteins [33]. It is most homologous to two other *Arabidopsis* PAB proteins (75.3% identity and 85.8% similarity with PAB5-At [34] and 55.1% identity and 69.8% similarity with PAB2-At [35]). Several PAB proteins have been identified in *Arabidopsis*, each of which seems to be expressed in a tissue-specific manner [34,35]. By comparing the sequence to unpublished data it could be concluded that this *PAB* gene is in fact PAB-3 (D. Belostotsky and R. Shaw, personal communication). The gene contains a long A-rich sequence which is commonly observed in *PAB* genes. These tracts have been shown to be involved in translational regulation of PAB proteins by an autocatalytic feedback mechanism [36].

The 24-kb region has been analysed for the presence of Matrix or Scaffold Attached Regions (MAR/SAR). These regions have been postulated to be DNA elements that define

the binding of chromatin to the chromosome protein matrix. These elements can be interpreted as dynamic structural subunits of chromosomes (for a review, see [37,38]). Furthermore, they have been shown to normalise transgene expression in plants [39,40]. Although there are no absolute criteria to predict the location of MARs in a sequence [40–42], most SARs, including the plant ones, are AT-rich stretches ranging from 200 bp to several kb, associated with additional features, which are not individually critical, such as inverted repeats, unwinding elements, homooligonucleotide repeats, topoisomerase II, and DNA I sites. We selected for AT-rich stretches (length > 200 bp; A+T ≥ 70%) with several of these features (data not shown; percentage HAAAAHA as example). Two regions, each approximately 2 kb in size, clearly fulfil these criteria: they flank the PAB-3 protein (Fig. 1). It is noteworthy that this protein is showing a very specific expression pattern (ovule and pollen) comparable to that of *PAB-5* (D. Belostotsky and R. Shaw, personal communication). It could be postulated that the MAR elements establish a gene-specific module for the *PAB-3* gene. The same MAR search was done for comparison with other *Arabidopsis* contigs, giving evidence for similar occurrence at a mean of one MAR element every 10 kb (P. Rouzé, unpublished results).

3.3. Conclusion

This 24-kb contig probably contains seven genes. Three of them have no clear function, although for two, genes *3* and *5*, corresponding *Arabidopsis* ESTs or homologous ESTs from other species (gene *3*, gene *5*) can be found. One gene (gene *7*) is only predicted, i.e. no similar EST sequences and no homologues are known. Apart from the known *RPS18A* gene, three other genes can be assigned a putative function based on their homology: the *PAB-3* gene, a novel GTP-binding protein gene, and a gene encoding a protein probably involved in double-stranded RNA unwinding. Intriguingly, the sequenced region is very gene dense, except for gene *4* encoding the poly(A)-binding protein, where the gene is flanked by putative MAR sites, thus possibly establishing a gene-specific expression module.

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