J. Mol. Microbiol. Biotechnol. (2000) 2(2): 195-198.

A Novel Ubiquitous Family of Putative Efflux Transporters

Kevin T. Harley and Milton H. Saier, Jr.*

Department of Biology, University of California at San Diego, La Jolla, CA 92093-0116, USA

Abstract

We describe a novel family of putative efflux transporters (PET) found in bacteria, yeast and plants. None of the members of the PET family has been functionally characterized. The bacterial and yeast proteins display a duplicated internal repeat element consisting of an N-terminal hydrophobic sequence of about 170 residues, exhibiting six putative transmembrane α–helical spanners (TMSs), followed by a large (230 residue), C-terminal, hydrophilic, cytoplasmic domain. The plant proteins exhibit only one such unit, but they have a larger C-terminal cytoplasmic domain. *Arabidopsis thaliana* encodes at least seven paralogues of the PET family. The Gram-negative bacterial proteins are sometimes encoded by genes that are found in operons that also contain genes that encode membrane fusion proteins. This fact strongly suggests that PET family proteins are efflux pumps. The sequence, topological and phylogenetic characteristics of these proteins as well as the operonic structures of their encoded genes when relevant are described.

We previously described a family of "membrane fusion proteins" (MFPs) (Dinh *et al.,* 1994) and one of "outer membrane factors" (OMFs) (Paulsen *et al.,* 1997) that, together with an integral cytoplasmic membrane permease of the ABC, RND or MFS superfamily (Pao *et al.,* 1998; Saurin *et al.,* 1999; Tseng *et al.,* 1999), are believed to comprise transenvelope export systems for drugs, heavy metals and proteins in Gram-negative bacteria (Letoffe *et al.,* 1996; Binet *et al.,* 1997; Zgurskaya and Nikaido, 1999). Recently, MFPs have been identified in Gram-positive bacteria, but their functions in these cells are not understood (Franke *et al.,* 1999; Harley *et al.,* 2000). In general, the MFPs are encoded by genes that are found in operons together with the cytoplasmic membrane efflux permeases with which they form a complex and function. The same is sometimes (but not always) true for genes encoding the OMFs (Paulsen *et al.,* 1997).

One of the MFP/OMF pairs identified in our earlier study was found to occur in an operon with a gene encoding a putative 12 TMS protein (typical of many cytoplasmic membrane permeases), but no ABC, RND or MFS permease-encoding gene was found in this operon. We postulated that the 12 TMS protein was an efflux pump, but no further analyses were conducted (Paulsen *et al.,* 1997).

In this report we conduct sequence, structural, and phylogenetic analyses of the homologues of this putative 12 TMS protein and analyze the operons in which these proteins occur. We show that they exhibit a topology different from any previously characterized transporter with two repeat units probably consisting of six N-terminal TMSs plus a large C-terminal cytoplasmic domain. Proteins with these duplicated units are found in yeast and bacteria, but only one such unit is present in the protein homologues found in plants. By analogy with other well-characterized transporters, we postulate that the monomeric internally duplicated bacterial and yeast proteins function as monomers, but that the plant proteins are functional dimers in the membrane.

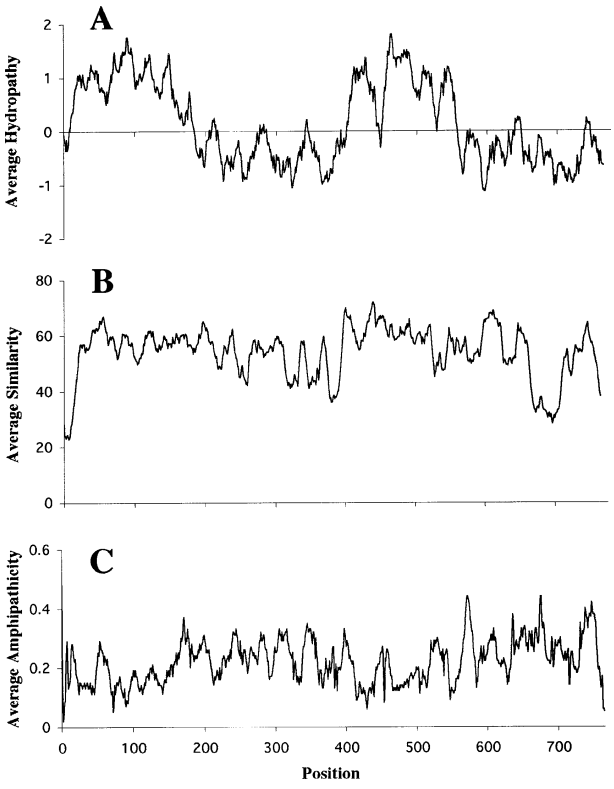

Figure 1. Average hydropathy (A), similarity (B) and amphipathicity (C) plots for the six full-length bacterial proteins presented in Table 1 (Kyte and Doolittle, 1982; Le *et al.,* 1999). The TREE program was used to generate the multiple alignment upon which these plots as well as the phylogenetic tree shown in Figure 4A were based (Feng and Doolittle, 1990). Hydropathy values were as reported by Kyte and Doolittle (1982). The program for average amphipathicity has been described by Le et al. (1999). Part of the multiple alignment upon which these plots are based is reproduced in Figure 5.

Received January 28, 2000; accepted January 28, 2000. *For correspondence. Email msaier@ucsd.edu; Tel. (858) 534-4084; Fax. (858) 534-7108.

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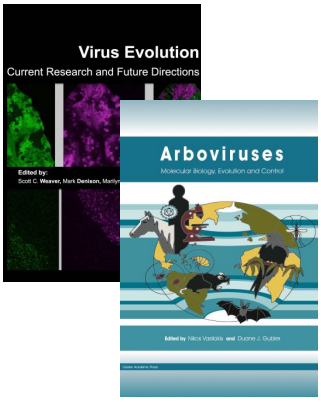

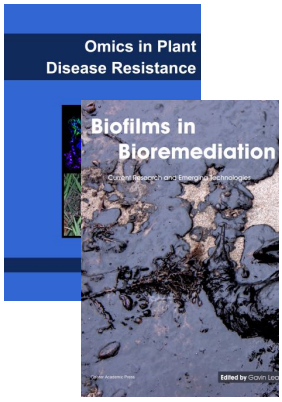

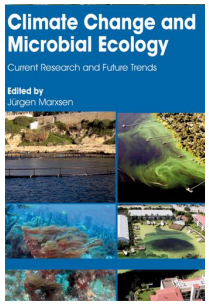

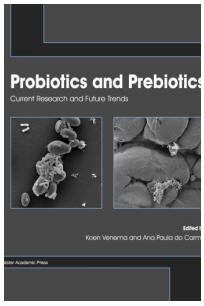

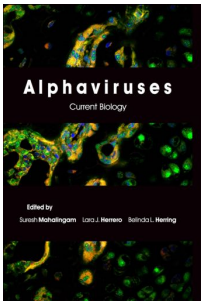

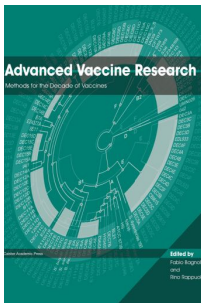

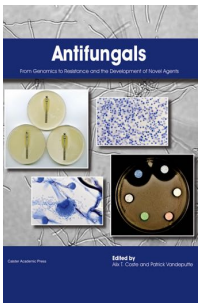

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Table 1. Proteins of the Putative Efflux Transporter (PET) Family

Abbreviation	Database Description	Organism	# Residues	Accession #
Bacterial Proteins				
YjcQ Eco	Hypothetical 76.1 kD protein in FDHF ALSK	*Escherichia coli*	683	spP32715
YccS Eco	Hypothetical 82.0 kD protein in SULA-HELD intergenic region	*Escherichia coli*	720	spP75870
YccS Hin	Hypothetical protein, HI1680	*Haemophilius influenzae*	718	spP44289
YhfK Eco	Hypothetical 79.5 kD protein in CRP-ARGD intergenic region	*Escherichia coli*	696	spP45537
Yc98 Spc	Hypothetical 84.3 kD protein 3LR1278	*Synechocystis PCC6803*	755	spP72831
YhcP Eco	Hypothetical 73.6 kD protein in ARGR-CAFA intergenic region	*Escherichia coli*	655	spP46481
Yor2 Ngo	Hypothetical protein, Orf2 (fragment)	*Neisseria gonorhoeae*	417	spO33369
Plant Proteins				
Orf1 Ath	Putative protein, Orf1	*Arabidopsis thaliana*	560	gbAL021889
Orf2 Ath	Open reading frame, Orf2	*Arabidopsis thaliana*	507	gbAF013294
Orf3 Ath	Hypothetical protein similar to T18A10.3	*Arabidopsis thaliana*	538	gbAC007584
Orf4 Ath	Hypothetical protein similar to T18A10.3	*Arabidopsis thaliana*	548	gbAC002329
Orf5 Ath	F24J5.16	*Arabidopsis thaliana*	533	gbAC008075
Orf6 Ath	Hypothetical protein	*Arabidopsis thaliana*	506	gbAC006233
Orf7 Ath	TEGT protein homologue; probable fragment	*Arabidopsis thaliana*	262	gbZ97343
Yeast Protein				
Ydg8 Spo	Hypothetical 111.4 kD protein C26F1.08C	*Schizosaccharomyces pombe*	977	spQ10495
Protozoan Protein				
Orf1 Pfa	Asparagine-rich protein (clone 25C4)	*Plasmodium falciparum*	669	pirS14535

The proteins of the putative efflux transporter (PET) family are tabulated in Table 1. The bacterial proteins are of 655-755 amino acyl residues in length; the yeast protein is larger (977 residues), but all of these proteins exhibit two repeat units as noted above. The plant proteins have a single such unit and are substantially smaller (506-560 residues). Their C-terminal hydrophilic domains are larger than those found in bacteria. The plant proteins show maximal sequence similarly with the bacterial proteins in their transmembrane domains. A plasmodium protein (Table 1) exhibits homology only to the hydrophilic portions of PET family members and may not be a transporter.

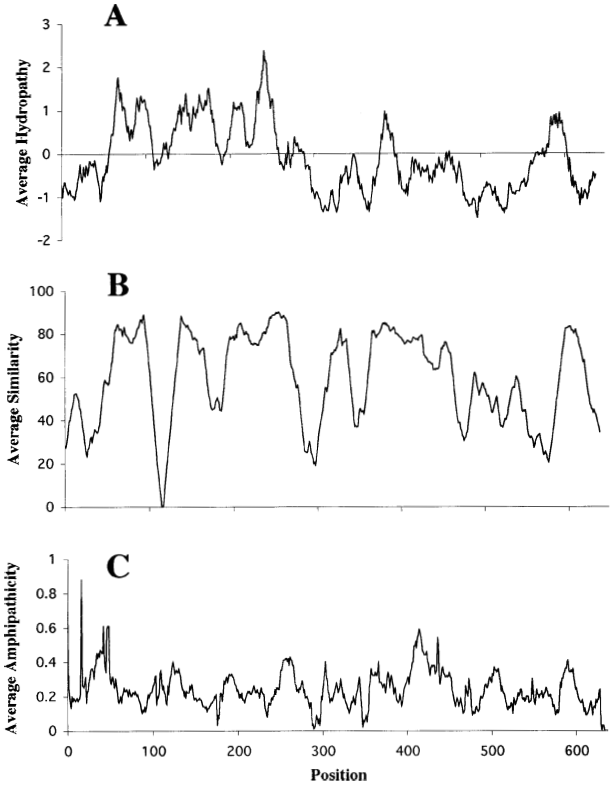

Figure 2. Average hydropathy (A), similarity (B) and amphipathicity (C) plots for the six full-length *Arabidopsis* proteins listed in Table 1. The programs and format of presentation are as described in the legend to Figure 1. Part of the multiple alignment for these proteins is shown in Figure 6, and the corresponding phylogenetic tree is shown in Figure 4B.

Figures 1A-C show the average hydropathy, similarity and amphipathicity plots for the bacterial proteins. As noted above, six peaks of hydrophobicity are followed by an extended hydrophilic domain, and this pattern is repeated. The first and second halves of these proteins were shown to be homologous. For example, employing the GAP program (Devereux *et al.,* 1984), a comparison score of 10.4 S.D. was obtained when the first half of YhcP Eco was compared with the second half of YhfK Eco (22% identity, 34% similarity, one gap of one residue). Such results establish that the two halves of these proteins share a common evolutionary origin. The yeast protein exhibits a similar topology (data not shown). As noted above, the *P. falciparum* protein exhibits a sequence that is in part homologous to the soluble portions of the cytoplasmic domains of the plant proteins but does not show the characteristic 6 TMS integral membrane domain. It may therefore not be a transporter, or it may be only a partial sequence. Consequently, it will not be discussed further.
The average similarity plot for the bacterial proteins (Figure 1B) reveals that the transmembrane regions are slightly better conserved than the hydrophilic domains, and the two halves of these bacterial proteins are about equally well conserved. The average amphipathicity plot (Le *et al.,* 1999) (Figure 1C) reveals that the hydrophilic domains display a much greater degree of amphipathicity than do the transmembrane domains when the angle is set at 100° as is appropriate for an α-helix.

Corresponding plots for the plant proteins are shown in Figures 2A-C. It is immediately apparent that these proteins exhibit only one set of six N-terminal hydrophobic peaks (residue positions 50-260) and that these peaks are followed by an extended hydrophilic region (residue positions 260-650; Figure 2A). The average similarity plot (Figure 2B) shows that the transmembrane domain is better

Figure 3. Alignments of segments of the two halves of bacterial PET family members (A) and of a segment of a bacterial homologue with a plant family member (B). In A, the first repeat unit of *E. coli* YhcP is aligned with the second repeat unit of *E. coli* YhfK. The vertical lines indicate identities while two dots or one dot indicate(s) a close or distant similarity, respectively. Residue numbers are indicated in parentheses. This alignment and the one shown in Figure B were generated with the GAP program (Deveraux *et al.,* 1984). In B, the transmembrane domain of the *A. thaliana* Orf2 protein is aligned with the second transmembrane domain of the *E. coli* YccS protein. The conventions of presentation are the same as in A.

conserved than the presumed cytoplasmic domain in agreement with the result observed for the bacterial proteins. The average amphipathicity plot revealed a pattern similar to that of the bacterial proteins except that the first putative TMS is preceded by a potential strongly amphipathic helix (angle = 100°). This region may have biogenic significance, functioning to facilitate insertion of the protein into the membrane (Yamada *et al.,* 1991).

Although the two halves of the bacterial proteins as well as the homologous regions of the yeast and plant proteins proved to be very divergent in sequence, homology could be established (Saier, 1994). For example, when the first repeat unit of the YhcP protein of *E. coli* was compared with the second repeat unit of the YhfK protein of *E. coli*, a comparison score of 10.4 S.D. (500 random shuffles) was obtained (22.2% identity; 34.3% similarity; one gap of one residue). The alignment upon which these values are based is shown in Figure 3A. Further, when the transmembrane region of Orf2 Ath was compared with the second transmembrane region of YccS Eco, a comparison score of 12.9 S.D. (500 random shuffles) was obtained (25.6% identity; 38% similarity; one gap of one residue). The alignment upon which these values are based is shown in Figure 3B.

Phylogenetic trees for the bacterial and plant proteins are shown in Figures 4A and B, respectively. As expected, the *Arabidopsis* proteins were much more similar in sequence to each other than were the bacterial proteins. In Figure 4A, it can be seen that the four *E. coli* paralogues are very divergent in sequence, but the YccS protein of *E. coli* is similar to the YccS protein of *Haemophilus influenzae*. These two proteins are undoubtedly orthologues and probably serve the same function. The same is possible for the *Synechocystis* protein.

The phylogenetic tree for the plant paralogues (Figure 4B) reveals that Orfs 3 and 4 resulted from a very recent gene duplication event, that Orf 5 split off from these two ORFs somewhat earlier, and that all other paralogues are about equidistant from each other in the tree. The shorter branch lengths in Figure 4B compared to Figure 4A reflect the higher degree of sequence similarity between the plant proteins as compared with the bacterial proteins. One can postulate that all of the gene duplication events that gave rise to these *A. thaliana* paralogues occurred during evolution of the plant kingdom.

Figures 5 and 6 reproduce partial multiple sequence alignments for the bacterial and plant proteins, respectively. Although the alignment of the bacterial proteins reveals much less conservation than that of the plant proteins, the identities (indicated by asterisks above the alignments) and the consensus sequence (indicated below the alignments) clearly reveal that these proteins are homologous. These alignments were used to derive signature sequences for each subfamily. These two sequences are respectively:

1 (R K) (L M T) (S A T L) (L I V) X_3 (L I V F) (G A C) X_{15} (L I V M)$_2$ (T M) X (L I V A) (L I V F) V X_7 (S T P) X_2 (R K) (L I V M)

2 G A T X_2 K (G C) (L I V F) N R (A G V) (L I V A) (G A) T X_2 A G (G S) L

The operons in which the genes encoding bacterial PET family proteins are found were analyzed for genes encoding membrane fusion protein (MFP) family members (Dinh *et al.,* 1994). In addition to the one reported by Paulsen et al. (1997) (*yjcP*, adjacent to *yjcQ*; see Table 1), the *yhcP* gene proved to be adjacent to the *yhcQ* gene. Both *yjcQ* and *yhcQ* encode MFP homologues. This fact provides further evidence that at least some, and probably all, of the PET family members are export permeases.

In this communication we have identified a novel family of putative efflux transporters which together comprise the

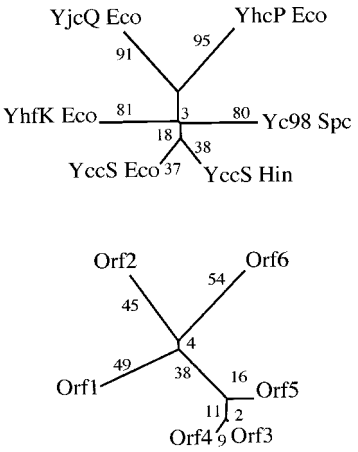

Figure 4. Phylogenetic trees for the full-length bacterial PET family members (A) and the full-length *A. thaliana* proteins (B). Numerical values for the branch lengths are expressed in arbitrary units but are comparable for the two figures. These values reveal that the plant homologues are much more similar in sequence to each other than are the bacterial homologues.

PET family. These proteins exhibit either 6 TMSs (the plant proteins) or 12 TMSs (the bacterial and yeast proteins). The latter, but not the former proteins, exhibit an internal repeat sequence, but the former proteins exhibit hydrophilic extensions that are longer than those in the bacterial proteins. Because some of the bacterial proteins are found in operons encoding known MFP and OMF protein homologues, proteins that always function in efflux (not uptake), we postulate that PET family proteins will prove to be a family of efflux pumps. However, this postulate has yet to be established, and the substrate specificities and energy coupling mechanism(s) of PET family members have yet to be determined. Experiments are currently underway to define the functions of representative members of the PET family.

```
                                                    *
YccS Eco (393) LFRHAVRMSLVLCFGYAIIQITGMHHGYWILLTSLFVCQPNYNATRHRLK  L
YccS Hin (382) LFRHAVRLSIVVFLCCAIVEFFQFNLGYWILLTTVFVCQPNYSATKVRLR  Q
YhfK Eco (363) ALRNAGRLSVMLSVASLMGTALHLPKSYWILMTVLLVTQNGYGATRLRIV  N
Yc98 Spc (394) LLRHGLRMALGGMVGATIAHLTQIPYGFWIVITLIFVLKPDFSLTFQRLS  N
YjcQ Eco (350) YMRYAVKTLLACLICYTFYSGVDWEGIHTCMLTCVIVANPNVGSSYQKMV  L
YhcP Eco (354) AMVNFWRTTLSCILGTLFWLWTGWTSGSGAMVMIAVVTSLAMRLPNPRMVAID

Consensus:     --R---R--L----------------G-WI--T---V--P----T--R-----

                  *                   *                         *
YccS Eco (444) RIIGTLVG IAIGIPVLWF VPSLEGQLVLLVITG  VLFFA        FRN
YccS Hin (433) RIIGTILG VVVGSLLPYL NPTLELKLGLVVLTS  TLFFF        FRS
YhfK Eco (414) RSVGTVVGLIIAGVALHFK IPEGYTLTLMLITTL  ASYLI        LRK
Yc98 Spc (445) RLLGTFLGVLVMSIALKLIQDPQLLSWLGILAIA   MGMAL        LRF
YjcQ Eco (401) RFGGAFCG AILALLFTLLVMPWLDNIVELLFVLA  PIFLLGAWIATSSERS
YhcP Eco (407) FIYGTLAA LPLGLLYFLVIIPNTQQSMLLLCISLAVLGFFLGIEVQ   KRR

Consensus:     R--GT--G----G--------P-------LL--------F-----------R-

                        *                           *
YccS Eco (485) VQYAHATMFITLLVLLCFNLLG EGFEVAL PRVIDTLIGCAIAWAAVSYIM
YccS Hin (474) NNYSFSTFFITLQVLLSFDVMG FDTAAALMPRLLDTLLGAAISWFAVSYLM
YhfK Eco (456) N YGWATVGFTITAVYTLQLLW LNGEQYILPRLIDTIIGCLIAFGGTVWLW
Yc98 Spc (487) H YSVAVFFITAFALILKAIDPSVPTEYALLSRLVCTLIGSAIALGLAFSFL
YjcQ Eco (451) S YIGTQMVVTFALATLENVFGPVYDLVEIRDRALGIIIGTVVSAVIYTFVW
YhcP Eco (456) L GSMGALASTINIIVLDNPMT FHFSQFL DSALGQIVGCVLAFTVILLVR

Consensus:     --Y-------T------------------L--R---T-IG--IA-------W
```

Figure 5. Partial multiple alignment of the bacterial members of the PET family. The TREE program (Feng and Doolittle, 1990) was used to generate the alignment as well as the tree shown in Figure 4A. Residue numbers are provided in parentheses following the protein abbreviations. Asterisks above the alignment indicate identities, while a residue that appears in the consensus sequence below the alignment is present in a majority of the proteins represented.

```
                *** *  **   *  ** *                          *
Orf3 Ath ( 86) GATLVKGFNRAIGTVSAGGLALGIARLSVLSRDFEQTIII TCIFLAG
Orf4 Ath (109) GATLVKGFNRAIGTVSAGGLALGIARLSVLSRDFEQTIII TCIFLAGC
Orf5 Ath ( 99) GATLVKGFNRALGTMLAGGLALGVAQLSVLAGEFEEVIIV ICIFLAG
Orf2 Ath (133) GATFCKCVNRVVATILAGSLGIAVHWVATQSGK AEVFVIGCSVFLFA
Orf1 Ath ( 94) GATLCKGLNRGLGTLIAGSLAFFIEFVANDSGKVLRAIFIGTAVFIIG
Orf6 Ath ( 68) GATLGKGLNRVAATLFAGGLGIGAHHLASMSGPTGEPILLAVFVFVQA

Consensus:     GATL-KG-NR--GT--AGGLA-G---L---SG-----I-I----FL-G-

                                      *    * **     *
Orf3 Ath (133)         FIASYSKLHP AMKPYEYAFRVFLLTFCIVLVSGNNTGDFFS
Orf4 Ath (157) RISLSSCFIASYSKLHP AMKPYEYAFRVFLLTFCIVLVSGNNTGDFFS
Orf5 Ath (156)         FGASYLKLYA SMKPYEYAFRVFKLTYCIVLVSGNNSRDFLS
Orf2 Ath (180)         FAATYSRFVPSFKARFDYGAMIFILTFSLVSVGGYRVDKLVE
Orf1 Ath (142)         AAATYIRFIPYIKKNYDYGVVIFLLTFNLITVSSYRVDSVIN
Orf6 Ath (106)         ALSTFVRFFPRVKARYDYSLLIFILTFALISVSGFREEQVVK

Consensus:             F-A-Y----P---K-Y-Y----F-LTF--V-VSG--------

                     *      *    *         * * *  **
Orf3 Ath (174) TAYYRFLFIVVGATTCLVVNIFIFPIWAGEDLHKLVANNFKSVANSLEG
Orf4 Ath (205) TAYYRFLFIVVGATTCLVVNIFIFPIWAGEDLHKLVANNFKSVANSLEG
Orf5 Ath (197) TAYYRILLIGLGATICLLVNVFLFPIWAGEDLHKLVAKNFKNVANSLEG
Orf2 Ath (222) LAQQRVSTIAIGTSICIIITVFFCPIWAGSQLHRLIERNLEKLADSLDA
Orf1 Ath (184) IAHDRFYTIAVGCGICLFMSLLVFPIWSGEDLHKTTVGKLQGLSRSIEA
Orf6 Ath (148) LTHKRISTVIIGGLSCVIISIFVCPVWAGQDLHSLIASNFEKLSFFLLG

Consensus:     -A--R---I--G---CL----F-FPJWAGEDLHKL-A-NF---A-SLEG
```

Figure 6. Partial multiple alignment of the plant members of the PET family. The conventions of presentation and the program used were the same as in Figure 5.

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