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Full Length Research Paper

Studies on new antifreeze protein from the psychrophilic diatom, *Fragilariopsis cylindrus*

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Antifreeze proteins (AFPs) are found in a wide range of species including fishes, plants, etc. They have very characteristic feature that inhibit the growth and recrystallization of ice that forms in intercellular spaces. Two expressed sequence tags (ESTs) were previously identified from salt stress cDNA library in *Fragilariopsis cylindrus* to have similarities with snow mold AFP. Using bioinformatics tools, we analysed these two AFP-ESTs. Accordingly, by using specially designed primers, the open reading frames (ORFs) subcloned into bacterial and plant expression vectors. The predicted gene product, AfpA, had a molecular mass of 27 kDa. Expression of *afpA* in *Escherichia coli* yielded an intracellular 27-kDa protein modified with His-tag. According to bioinformatics data, a comparison between AFP-A and carrot AFP has been carried out.

Key words: Antifreeze protein, *Fragilariopsis cylindrus*, recrystallization inhibition.

INTRODUCTION

Antifreeze proteins (AFPs) are family of proteins capable of protecting organisms from freezing or sub-freezing damage. The protective effect of AFPs is through lowering the freezing points of the organism's extracellular matrix and body fluids while leaving the melting point unchanged, a process termed thermal hysteresis (TH) (DeVries, 1971; Urrutia et al., 1992). AFPs have been identified from various organisms such as bitter-sweet nightshade (*Solanum dulcamara*) (Duman, 1994), winter rye (*Secale cereale*) (Antikainen and Griffith, 1997), carrot (*Daucus carota*) (Worrall et al., 1998), ryegrass (*Lolium perenne*) (Sidebottom et al., 2000), fish (Solomon and Appels, 1999; Tong et al., 2000; Li et al., 2001), insect (Li and Sun, 2002) and diatoms (Bayer-Giraldi et al., 2010). TH activity of plant AFPs is less than that of fish AFPs and significantly less than that of insect

AFPs. On the other hand, plant AFPs has strong ice recrystallization inhibition (RI) activity (Sidebottom et al., 2000). Ice recrystallization is the growth of large ice crystals in the expense of the smaller ones. Inhibition of this process has great effect on decreasing cellular damage (Knight et al., 1984).

In biotechnology, AFPs have wide range of applications. It is reported that the addition of fish antifreeze glycoproteins to vitrifying solutions increased post-thaw viability in cultured immature pig oocytes and embryos (Arav et al., 1993). Subzero cryopreservation of mammalian hearts for transplantation using fish AFPI or AFPIII was shown to be feasible, with good preservation of myocyte structure and mitochondrial integrity (Amir et al., 2003). Researchers found that antifreeze glycoprotein (AFGP) can provide a protective effect on platelets cooled to 4°C for 21 days (Tablin et al., 1996). It is therefore suggested that AFGP might inhibit leakage from platelets, thus, increasing their shelf life. In food industry, there is great promise for using AFPs in frozen food products to preserve their quality and texture (Venketesh and Dayananda, 2008). In fact, ice recrystallization is one major cause of loss of nutrients and smooth texture in frozen food.

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Abbreviations: AFPs, Antifreeze proteins; ESTs, expressed sequence tags; TH, thermal hysteresis.

Table 1. Deduced parameters of two AFP ESTs from *F. cylindrus*. ESTs sequences were analysed by ProtParam and Compute pI/M.W. web-based programs for ORF, pI and M.W. predictions.

EST Sequence	Length (nt)	ORF		Total a.a	pI	M.W.
		From	To			
FCyIESTA46A10.s1	948	65	886	273	4.47	27.9
FCyIESTA46C08.s1	886	48	845	265	7.79	27.3

EST, Expressed sequence tag; ORF, open reading frame; aa, amino acid; pI, isoelectric point; MW, molecular weight.

The function of AFPs in cold-adapted organisms is to help them avoid or reduce cellular damages caused by freezing. In the absence of AFPs, water molecules will be added to an ice lattice in an undercooled solution resulting in ice crystal growth. However, in the presence of AFPs, adsorption of AFPs to the ice surface makes it thermodynamically unfavorable for water molecules to be added to the ice lattice (Knight et al., 1995).

Expressed sequence tags (ESTs) from marine diatoms, *Fragilariopsis cylindrus* show similarities with antifreeze protein from snow mold, *Typhula ishikariensis* (Krell et al., 2008). Here, we analysed these sequences using bioinformatics tools, cloned and expressed it into *Escherichia coli* and cloned it into plant expression vector for plant biotechnology application on tomato.

MATERIALS AND METHODS

The ESTs library was made in pTriPLEX2 vector as previously described (Mock et al., 2005). Antifreeze-EST inserts were amplified using pfu DNA polymerase in PCR reaction and the following primers pairs: AFP-46A-F (5'-GCGAAGCTTATGATGAA CCTTAATCTATTTTTGATC-3') as forward primer and AFP-46A-R (5'-GCGCTCGAGTTATGCTACCTGTCCTCGTAGTCC-3') as reverse primer for amplification of FCyIESTA46A10.s1 and AFP-C-F (5'-CGCAAGCTTATGATGAACCTGAATCTCTTTTAA C-3') as forward primer and AFP-C-R (5'-GCGCTCGAGTTATGCTA CCTGTAGCCCC-3') as reverse primer for FCyIESTA46C08.s1 amplification. Both primer pairs have addition of HindIII site to the forward primer and XhoI to reverse primer for cloning into pET vector.

Bacterial and plant expressions

PCR products were analyzed and resolved on 1% agarose gel (w/v). Bands with expected lengths were excised from the gel with razor blade and purified using the QIAquick gel extraction kit (Qiagen, CA, USA). The purified DNA was then cloned into the pET-28a(+) vector (Novagen) according to the manufacturer's instructions. Ligation products were examined by PCR and double digestion for ligated vectors. Next, they were transformed into competent BL21 *E. coli* cells. Positive recombinant clones were authenticated by screening on LB agar ampicillin plates and by PCR. Positive colonies were cultured overnight in LB broth at 37°C and plasmids were extracted using the Fast Plasmid Mini Plasmid Purification Kit (Eppendorf, Hamberg, Germany). Plasmids containing inserts were randomly chosen from the positive recombinants and sequenced using T7 primers.

For plant expression applications, FCyIESTA46A10.s1 was amplified and cloned into pKYLX binary expression vector for tissue specific expression in tomato as previously described (Deng, 2003).

Sequence analysis and prediction of protein structure

Primary structural features of proteins were computed with ProtParam (<http://expasy.org/tools/protparam.html>) and Compute pI/Mw (<http://www.ncbi.nlm.nih.gov>). Secondary structure features of the protein coded by ORFs were computed with Scratch Protein Predictor (<http://scratch.proteomics.ics.uci.edu/>). The selected coding regions were subjected to domain and motif predictions using MyHits server (<http://hits.isb-sib.ch/cgi-bin/PFSCAN>).

RESULTS

Sequence analysis and protein structure prediction

We analyzed the FCyIESTA46A10.s1 and FCyIESTA46C08.s1 sequences using different bioinformatics web-based tools on NCI, Expaty and others for calculating the ORFs, pI and M.W. of deduced amino acid chains (Table 1).

The comparison of FCyIESTA46A10.s1 with carrot antifreeze protein revealed some interesting features. Table 2 summarizes the major differences between amino acid contents. FCyIESTA46A10.s1 was rich in Ala and Gly amino acid, while carrot AFP was rich in Leu (Table 2). Cys was absent in FCyIESTA46A10.s1 and was almost 4% in carrot AFP. On the other hand, FCyIESTA46A10.s1 was rich in Met when compared with carrot AFP.

The secondary structure prediction was conducted to figure out the reflection of these amino acids differences in the secondary structure of both FCyIESTA46A10.s1 and carrot AFP. The secondary structure of FCyIESTA46A10.s1 had 20% α -helix, 19% random coil and 35% extended strand. However, secondary structure of carrot AFP had 19% α -helix, 22% random coil and 15% extended strand. Carrot AFP is known to have LRR motifs, but analysis of motif structures in FCyIESTA46A10.s1 revealed the presence of phosphorylation sites, for protein kinase C.

For further characterization of candidate antifreeze proteins from *F. cylindrus*, we cloned the ORFs of FCyIESTA46A10.s1 and FCyIESTA46C08.s1 into pET-28

Table 2. Comparison of deduced amino acids content between AFPs from *F. cylindrus* and *D. carota* (carrot). ORFs were analysed by ProtParam and MyHitsweb-based programs for amino acids prediction and percentage.

A.A.	Predicted A.A. (%)	
	FCylESTA46A10.s1	Carrot
Ala (A)	12.5	3.9
Cys (C)	0.0	3.9
Gly (G)	11.0	6.0
Leu (L)	7.0	16.0
Met (M)	2.9	0.6
Thr (T)	9.9	5.1
Val (V)	8.4	2.4

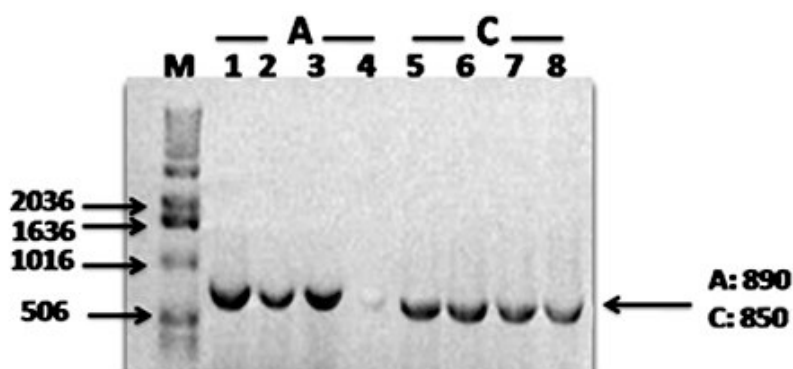


Figure 1. Cloning of ORFs for two AFP from EST library, from *F. cylindrus* bacterial expression vector. Lanes 1 TO 4 represent cloned FCylESTA46A10.s1 with molecular weight of 890 bp and lanes 5 to 8 represent cloned FCylESTA46C08.s1 with molecular weight of 850 bp into pET-28. M is 1 kb ladder (invitrogen).

bacterial expression vector. The amplified PCR products were then cloned into pET-28 vector and the recombinant pET-28 plasmids were verified for insert by PCR (Figure 1), and also by double digestion.

Recombinant plasmids were designated as pET-46A for cloned FCylESTA46A10.s1 ORF in pET, and pET-46C for cloned FCylESTA46C08.s1 ORF in pET for protein expression, and the pET-46-A and pET-46C were transformed into BL21 *E. coli* strain. *F. cylindrus* AFPs were induced by IPTG and total soluble proteins were extracted and separated (Figure 2).

For plant expression experiment, we cloned the FCylESTA46A10.s1 ORF into pKYLX with 35S promoter. The influence of the cloning process was confirmed by PCR (Figure 3) and double digestion.

DISCUSSION

Cold or low temperature stress is one of the major limiting environmental factors which affect the growth, development, productivity and distribution of almost all plants.

Additionally, cold stress or low temperature can affect plants not only by the effects of low temperature alone but by dehydration of the cells and tissues due to the crystallization of the cellular water (Beck et al., 2004; Pearce, 2001). Subsequently, the proteins and genes associated with freezing resistance have been widely studied from different organisms. We characterized two ESTs sequences from *F. cylindrus* which have been previously isolated from salt stress library (Krell et al., 2008) with similarity to AFP from *T. ishikariensis*, and we referred to them as AFP 46-A and 46-C. The two AFPs have more than 80% similarities. Due to the close phylogenetic relationship between plants and diatoms, we selected carrot AFP (Dc AFP) for comparison with *F. cylindrus* AFPs. In comparison with carrot AFP (Dc AFP), the predict pI has similar value with that of Dc AFP. On structure basis, it is not surprising to find the most abundant amino acid in Dc AFP Leu (16%) due to the presence of tandem LRR repeats (Worrall et al., 1998), while 46-A is rich in Ala, Gly and Thr. It is worthy to mention here that type I fish AFP is helical in structure with The residues arrayed on one side of the protein,

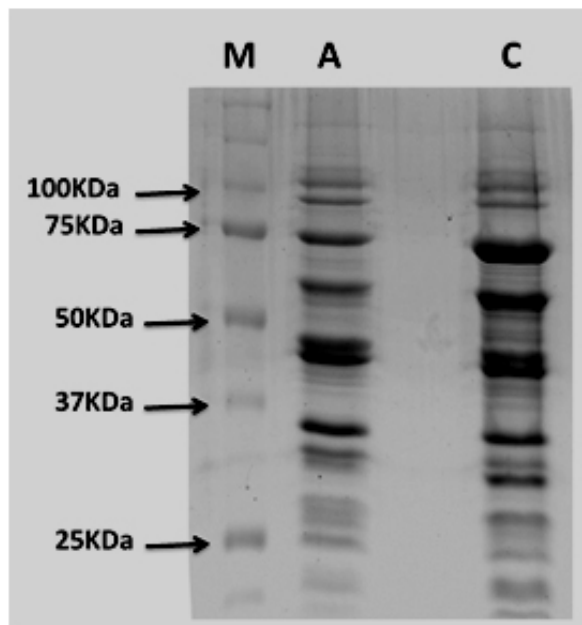


Figure 2. Bacterial expression of cloned ORFs for two AFP from EST library, from *F. cylindrus*. Lane A represents cloned FCyIESTA46A10.s1 with deduced molecular weight of ~29 KDa and lane C represents cloned FCyIESTA46C08.s1 with deduced molecular weight of ~28 KDa. M is protein marker.

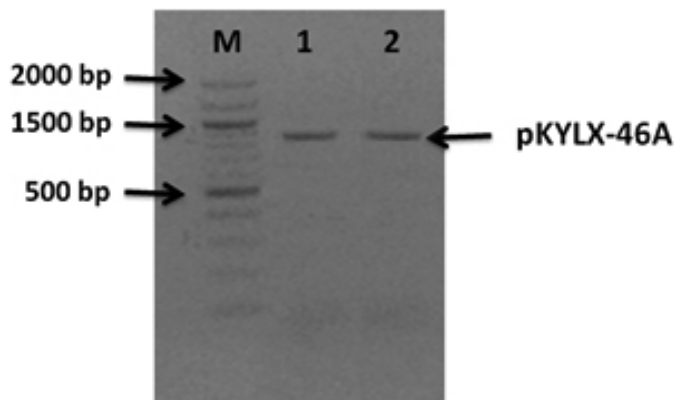


Figure 3. Cloning of ORFs for two AFP from EST library, from *F. cylindrus* into plant expression vector (pKYLX). Lanes 1 and 2 represent cloned FCyIESTA46A10.s1 with molecular weight of 890 bp into pKYLX. M is 100 bp ladder.

while AFGPs have Ala-Ala-Thr repeats (Zongchao and Davies, 2002). Deduced helical contents of both 46-A and Dc AFPs are almost similar but Dc AFP has more E structure. Unfortunately, no plant AFP crystal structures have yet been solved. Once available, these will help in comparing structures and will add interesting details to the structure–function puzzle.

Further characterization of *F. cylindrus* AFPs requires purified or at least partial, protein for TH and RI

measurements. Here, we took first steps of characterization by cloning and bacterial expression of 46-A and 46-C in BL21 bacteria (Figures 1 and 2). The expressed AFPs were found in total soluble extract and we observed good degree of resistance to freezing. We hope to measure both TH and RI because it was found that AFP with good TH is not necessarily equally good in RI. Plant AFPs are typically weak in their TH activity (Urrutia et al., 1992). This fits in with a freeze tolerance strategy; in

which supercooling should be avoided because it would ultimately lead to the rapid uncontrolled growth of ice (Urrutia et al., 1992). For example, AFP from ryegrass was better at RI than at TH (Sidebottom et al., 2000).

To achieve plant biotechnology application for the AFPs, we selected tomato to be transformed with the AFPs from *F. cylindrus*. Tomato is a very economically important crop; unfortunately, it is very prone to low temperature especially during flower developments, and in many cases, leads to dramatic reduction in the yield. Plant expression vector pKYLX harboring A-46 ORF was constructed and will be transformed to tomato plants to evaluate the A-46 expression and its effects on tomato protection against cold and drought stresses.

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