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Full Length Research Paper

Characterization and expression of dehydrins in wild Egyptian pea (*Pisum sativum* L.)

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Pea genotypes exhibited significant genetic variation in drought tolerance. Polymerase chain reaction (PCR) screening for dehydrins in different pea genotypes indicated the amplification of at least six dehydrin gene fragments varied in length from 500 to 1000 bp. In this study, a dehydrin gene, *PsDhn1* (GenBank accession number JN182234), was isolated and characterized from wild Egyptian pea (*Pisum sativum* L.) using specific PCR. It belongs to the Y_2K_2 dehydrin subclass induced by drought stress. DNA sequence indicated an open reading frame which predicts a protein with 196 amino acids and molecular mass of about 21 kDa. Deduced amino acid sequence indicated the presence of the N-terminal consensus motif, Y-segment D(E/Q)YGNP, and the lysine rich domain, K-segment KIKEK(I/L)PG, common for all dehydrins, both of which are present in two copies. However, it lacks the track of serine residues (S-segment). Egyptian pea dehydrin (PsDHN1) contains two copies of a semi-conserved sequence of six amino acid residues in length (EYVREE), which lie in front of the lysine rich motif (K-segment), and common only for pea dehydrins. Northern blot analysis indicated two dehydrin mRNA transcripts detectable by *PsDhn1* probe. Western blot analysis using anti-dehydrin polyclonal antibody detected two corresponding dehydrins (29 and 21 kDa) accumulated in developing pea cotyledons. Both mRNA transcripts and dehydrins showed different patterns of expression and accumulation. The amino acid composition of dehydrins with high content of charged and polar residues may promote their specific protective functions under conditions of cell dehydration.

Key words: Dehydrins, late embryogenesis abundant (LEA) genes, drought tolerance, pea, desiccation.

INTRODUCTION

Dehydrins (DHNs) is a family of hydrophilic proteins, also known as Group II late embryogenesis abundant proteins (LEAs II) which accumulate during seed development and are associated with acquisition of desiccation tolerance in plant seeds (Dure III et al., 1981). In recent years, numerous studies have reported up-regulation of DHNs in diverse plant species in response to various abiotic stresses (Close, 1997; Kosova et al., 2007; Rorat, 2006; Tunnacliffe et al., 2010). DHNs are characterized by high glycine content and low secondary structure. They have some typical features like the unique lysine rich 15 amino acid domain called K segment (EKKGIMDKIKEKLPG) at the C-terminal of their molecule. Some DHNs also contain a track of serine residues (S-segment), and/or a consensus motif T/VDEYGNP called Y segment at the N-terminal. The number and order of the Y, S and K segments define different DHN subclasses (Close, 1996, 1997): YnSKn (low molecular weight, alkaline dehydrins induced by drought); YnKn (20 to 25 kDa, induced by drought); SKn (25 to 27 kDa molecules which are responsible for priming of the cells under chill and drought); Kn (related to cold tolerance) and KnS (which bind metals and have the ability to scavenge hydroxyl radicals). Pea DHNs have the N-terminal consensus motif (Y-segment) and the lysine rich domain (K-segment) at the C-terminal, both of which are present in two copies. However, they lack the track of serine residues (S-segment) as reported by Roberton and Chandler (1992).

Due to their hydrophilic property, DHNs are thought to protect plant tissues against stresses causing cellular dehydration such as drought, salinity and cold (Close,

Table 1. Cultivars, locations, origin and accession numbers of the studied *Pisum* genotypes.

Number	*Pisum* species	Genotype			
		Cultivar	Taxa location	Accession origin	Accession number
1	*P. sativum* L.	Vectory freezer			
2	*P. sativum* L.	Master			
3	*P. sativum* L.	Lincoln			
4	*P. sativum* L.	Little marvel			
5	*P. sativum* L.	Montana			
6	*P. sativum* L.	Local			
7	*P. sativum* L.	Billinder			
8	*P. sativum* L.		Ghazala		
9	*P. sativum* L.		El-Dabba		
10	*P. sativum* L.		Wadi El-Ramel		
11	*P. sativum* L.			Turkey	1023
12	*P. sativum* L.			Greece	1945
13	*P. sativum* L.			Cyprus	1749
14	*P. sativum* L.			Syria	2811
15	P. *sativum* L.			Algeria	876
16	*P. fulvum* L.			Syria (1)	2916
17	*P. fulvum* L.			Syria (2)	2917

1996; Garnczarska et al., 2008; Hu et al., 2010; Sarhan et al., 1997; Vaseva et al., 2011; Zhang et al., 2007). Some DHNs have also been implicated to serve as antioxidants scavenging free radicals (Hara et al., 2003; Sun and Lin, 2010). Additionally, the accumulation of DHNs is found to be associated with improved seed vigor and seed osmopriming (Chen et al., 2012). Thus, the diverse functions and distributions of DHNs enable them to respond not only to environmental but also to developmental cues (Hanin et al., 2011; Hara, 2010; Rorat, 2006). This work describes the characterization and isolation of a dehydrin gene from wild Egyptian pea (*Pisum sativum* L.). The nucleotide sequence, as well as deduced amino acid sequence of isolated dehydrin gene was compared with other pea dehydrins. Expression and accumulation of pea dehydrins in developing cotyledons was also investigated.

MATERIALS AND METHODS

Plant material

Seeds representing 17 samples belonging to two species of *Pisum* (*P. sativum* L. and *P. fulvum* L.) were obtained from different origins. Seven *P. sativum* L. cultivars (Vectory Freezer, Master, Lincoln, Little Marvel, Montana, Local and Billinder) were obtained from Horticultural Research Institute, Giza, Egypt. Three wild taxa of *P. sativum* L. were collected from different locations at the North West coastal region of Egypt. These locations are Ghazala, El-Dbba and Wadi El-Ramel (150 and 100 km eastern, and 20 km western Matrouh city). The three wild taxa were identified at the Cairo university herbarium. Seven accessions of *P. sativum* L. and *P. fulvum* L. were obtained from the International Center for Agriculture Research in Dry Areas (ICARDA), Aleppo, Syria (Table 1).

Pea plants were grown under standardized conditions in a growth cabinet and flowers were tagged on the day they opened. Pea cotyledons representing sample 8 (wild *Pisum sativum* L. collected from Ghazala, 150 km eastern to Matrouh city) were harvested at different days after flower opening (20, 24, 28, 32, 36 and 40 DAF). Testas and embryonic axes were carefully removed and developing seeds were kept for further analysis.

Polymerase chain reaction (PCR) conditions and southern blot analysis

Total genomic DNA was isolated from 1 g of *Pisum* leaves using cetyltrimethyl ammonium bromide (CTAB) buffer (2% CTAB, 100 mM Tris/HCl, pH 8, 20 mM EDTA, 1.4 M NaCl, 0.2% β-mercaptoethanol and 1% polyvinyl pyrrolidine PVP) as described by Giannino et al. (1989). Specific PCR reactions were conducted according to Williams et al. (1990) using pea DNA as template and dehydrin specific primers. For screening experiments, forward (*pea +ve*) and reverse (*pea -ve*) primers were designed corresponding to the pea dehydrin N-terminal consensus motif DEYGNP and the C-terminal lysine rich domain KIKEKLPG, respectively. These primers are provided as follows; Forward primer (*pea +ve*): 5'–GATGAATATGGAAACCCA –3', reverse primer (*pea -ve*): 5'–AGTACCAGGAAGCTTCTCCTTAAT– 3'. For isolation of pea dehydrin gene, forward (*dhn +ve*) and reverse (*dhn -ve*) primers were designed corresponding to the coding sequence of *pPsB12* dehydrin gene (Roberton and Chandler, 1992). These primers are provided as follows; Forward primer (*dhn +ve*): 5'-ATGTCTCAGTATCAAAACC–3', reverse primer (*dhn -ve*): 5'–CTAGTGTCCAGTACATCCTCC–3'.

PCR reactions were performed in 50 µl total volume of 10 mM Tris/HCl, pH 9, 50 mM KCl, 1.5 mM $MgCl_2$, 1% Triton X-100 and 200 µM of each of dATP, dGTP, dCTP and dTTP (Promega), containing 0.2 µM of each primer, 1.0 U Taq DNA polymerase

(Promega) and 50 ng template DNA overlaid with a drop of mineral oil. Amplification was carried out in a DNA thermocycler (Perkin Elmer Cetus 480, Norwalk, CT) programmed for 35 cycles, after initial denaturation for 3 min at 94°C, with the following temperature profile: 1 min at 94°C, 1 min at 52 or 62°C and 2 min at 72°C, followed by a final extension for 5 min at 72°C. Amplification products were visualized along with a λ phage DNA marker digested with Hind III (23.130, 9.416, 6.557, 4.361, 2.322, 2.027, 1.353, 1.078, 0.872, 0.603 and 0.310 kb) on 1.2% agarose gel, or 0.6% non-denatured polyacrylamide gels with 1× TAE buffer (10 mM Tris/acetic, pH 7.4 and 1 mM EDTA) and detected by staining with ethidium bromide (0.5 μg/ml) for 30 min (Sambrook et al., 1989). Fractionated PCR products were blotted on N-nylon membrane (Millipore) using trans-blot electrophoretic transfer cell (BioRad), and membrane immobilized DNA was hybridized with 100 ng α-^{32}p DNA labeled probe according to Sambrook et al. (1989). *pPsB12* dehydrin (*Dhn1*) gene fragment, available at the Agricultural Genetic Engineering Research Institute (AGERI) was labeled by the prime-It® II Random primer labeling kit (Stratagene) and used as a DNA probe. Hybridization was carried out in 50% (v/v) formamide, 6× SSC, 5× Denhardt's solution, 0.5% (w/v) SDS, 100 μg/ml sheared salmon sperm DNA and 10 μg/ml poly(A) at 42°C. Membranes were washed three times in 2× SSC, 0.1% (w/v) SDS at 65°C for 20 min each, followed by 0.1× SSC, 0.1% (w/v) SDS at 50°C for 20 min. Membranes were exposed to X-ray film with an intensifying screen at -80°C for the desired period of time.

Cloning and sequencing of PCR product

PCR product was cloned into the Sfr I site of pBlue-Script cloning plasmid (Stratagene) according to the manufacturer's instructions. Plasmid DNA was purified using High pure plasmid isolation kit (Promega) developed after Birnboim and Doly (1979). DNA was sequenced in both strands using T7 DNA polymerase and double-stranded DNA templates. The dideoxy chain termination protocol was carried out according to the Deaza sequencing kit (Pharmacia). Analysis and alignment of DNA and amino acid sequences were carried out using PC-gene program release (February, 1991) from Intelligenetics after Higgins and Sharp (1989).

RNA extraction and northern blot analysis

Total RNA was extracted from pea cotyledons with RNeasy mini kit (QIAGEN) according to the manufacturer's protocol. RNA concentration was calculated from the optical density of the samples at 260 nm using UV-1601 spectrophotometer (Shimadzu). Total RNA (20 μg) on nitrocellulose filters was hybridized with 100 ng of *PsDhn1* probe labeled with [^{32}P]-dCTP using the prime-It® II Random primer labeling kit (Stratagene). Hybridization and washing were carried out under same stringency conditions as described above. Dehydrin mRNA sizes were estimated against known rRNA (28S rRNA and 18S rRNA) mRNA sizes.

Protein extraction and western blot analysis

Pea cotyledons were ground with liquid nitrogen, and the powder (1 g) was homogenized on ice in two volumes of 20 mM Tris/HCl, pH 7.5, with 5% (v/v) glycerol, 10 mM β-mercaptoethanol and 35 μl protease inhibitor cocktail (Sigma) for 1 h. The homogenates were centrifuged at 13000 g for 20 min and the supernatants were collected and heated at 80°C for 15 min. After cooling on ice, samples were centrifuged at 13000 g for 20 min, and the supernatants containing thermostable proteins were collected for further analysis. Protein measurement was performed according to Bradford (1976). Equal amounts of proteins (20 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized along with protein markers (66, 45, 36, 29, 24, 20 and 14 KDa) on 12% SDS polyacryamide gels as described by Laemmli (1970). Separated protein bands were transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, USA) using a semi-dry transfer system (Sigma, USA). The transfer was carried out at 2 mA for each square cm for 1 h. Membranes were blocked in 5% non-fat milk solution overnight at 4°C. Then, membranes were incubated with a 1:2000 dilution of rabbit anti-dehydrin polyclonal antibody (Stressgen Biotechnologies Corporation, Canada) prepared against a synthetic peptide containing the conserved sequence EKKGIMDKIKELPG (Close et al., 1993) in Tris-buffered saline tween-20 (TBST) for 1 h. Goat anti-rabbit IgG antibody (dilution 1:4000) conjugated to alkaline phosphatase was used as the second antibody and was detected by 5-bromo-4-chromo-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NPT) substrate (Blake et al., 1984).

RESULTS AND DISCUSSION

Isolation of dehydrin gene (*PsDhn1*)

PCR screening for pea dehydrins was performed under annealing temperature (52°C) using pea DNA template and dehydrin specific primers (*pea +ve* and *pea -ve*) corresponding to the pea dehydrin N-terminal consensus motif DEYGNP and the C-terminal lysine rich domain KIKEKLPG. At least six PCR fragments representing putative pea dehydrin genomic DNA was amplified (Figure 1A). These DNA fragments were varied in length from 500 to 1000 bp. All of these DNA fragments hybridized positively with *pPsB12* dehydrin probe (Figure 1B). The highest number of PCR fragments (4) was found in the two wild *P. sativum* L. taxa collected from the North West coastal region of Egypt (Ghazala and El-Dabba), while the lowest number of DNA fragments was observed in cultivated pea and the two accessions representing *P. fulvum* L. The high number of DNA fragments indicated a dehydrin multigene family in Egyptian pea. Differences in length of pea dehydrin genes were also observed by Grosslindemann et al. (1998). They were able to identify four pea dehydrin genes with varied length (597, 697, 699 and 768 bp). They studied the sequence elements responsible for length differences between dehydrin alleles and between dehydrin loci in *P. sativum*. Moreover, to avoid primer mismatching, specific PCR reactions were conducted under high annealing temperature (62°C) using primer pair (*dhn +ve* and *dhn -ve*) corresponding to the coding sequence of *pPsB12* dehydrin gene. Pea DNA extracted from sample 8 (wild *Pisum sativum* L. collected from Ghazala, 150 km eastern to Matrouh city) was used as template. Data presented in Figure 2 indicated a single PCR fragment of about 591 bp corresponding to dehydrin gene. The amplified PCR product of 591 bp in length was further sequenced.

Nucleotide sequence of dehydrin gene *(PsDhn1)*

The isolated dehydrin gene fragment (591 bp) was

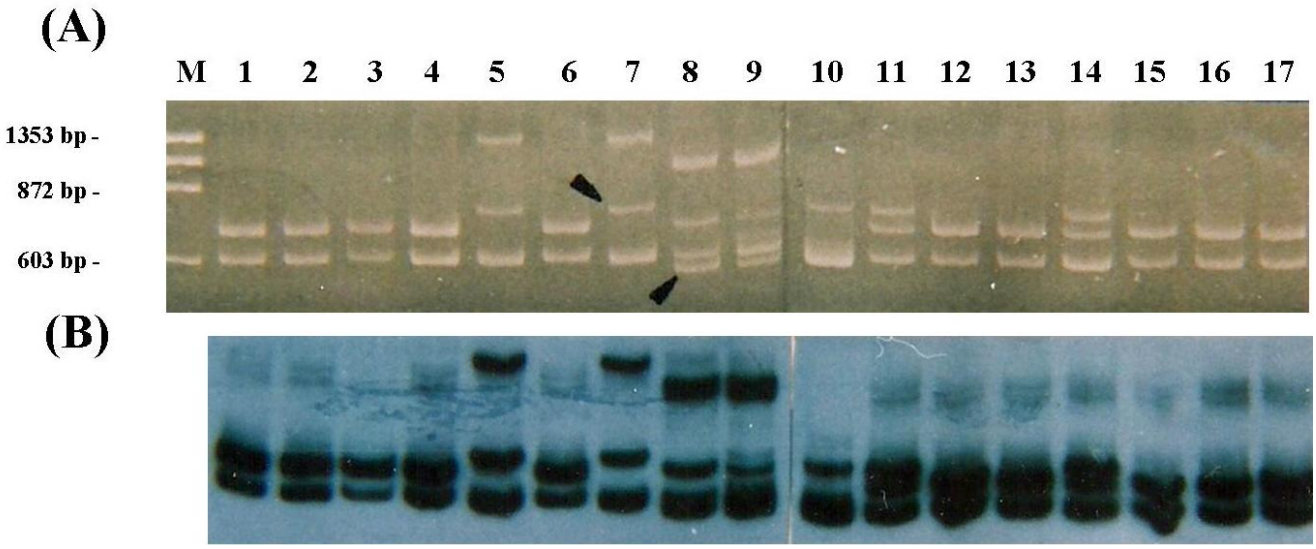

Figure 1. PCR amplified dehydrin products from pea genotypes as numbered in table 1 (A), PCR products fractionated on 6% polyacrylamide gel, and (B), southern blot analysis of PCR products with *pPsB12* gene probe.

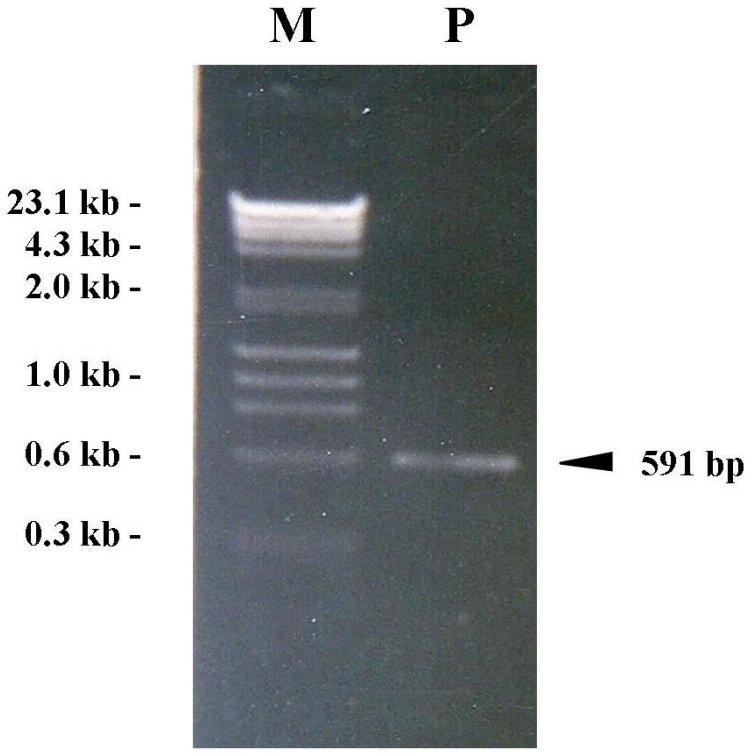

Figure 2. PCR amplification of *PsDhn1* gene fragment from wild Egyptian *Pisum sativum* L. taxon collected from Ghazala (P). M = λ phage DNA marker digested with hind III.

cloned into pBlue-Script plasmid, purified and sequenced in both strands. DNA sequence of cloned gene indicated 591 bp in length from the start codon (ATG) to the stop codon (TAG). This gene was designated *PsDhn1* and deposited in GenBank with an accession number (JN182234). Sequence alignment with the coding

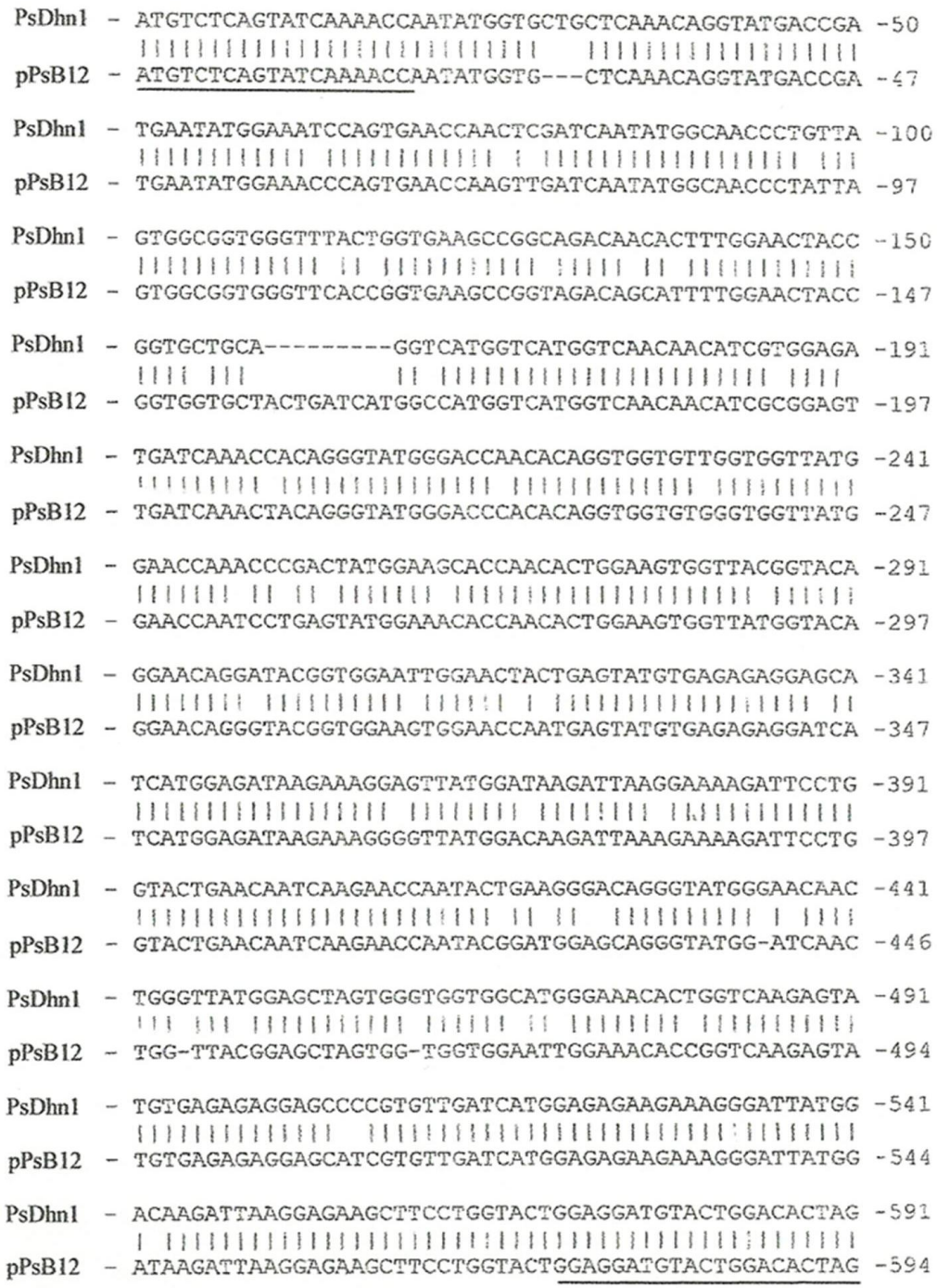

Figure 3. DNA sequence alignment between PsDhn1 gene and the coding sequence of pPsB12 gene showed 91.88% similarity. Sequences of forward and reverse primers are underlined. pPsB12 sequence contained 4 gaps, while PsDhn1 sequence contained only one gap.

sequence of *pPsB12* dehydrin gene (594 bp) isolated by Roberton and Chandler (1992) indicated 91.88% similarity (Figure 3). Number of gaps inserted in *pPsB12* gene is four, while only one gap is inserted in *PsDhn1* gene. *PsDhn1* gene indicated an open reading frame which predicts a protein with 196 amino acids and molecular mass of about 21 kDa. Alignment of the deduced amino acid sequence with that of pPsB12 dehydrin (197 amino acids) indicated 86.73% similarity with two gaps inserted in pPsB12 dehydrin and only one gap in PsDHN1 dehydrin as shown in Figure 4. Egyptian pea dehydrin (PsDHN1) was found to belong to the Y_2K_2

Figure 4. Alignment of the deduced amino acid sequences of PsDHN1 and pPsB12 dehydrins showed 86.73% similarity. pPsB12 sequence contained 2 gaps, while PsDHN1 sequence contained only one gap. The sequences of Y-segment D(E/Q)YGNP, K-segment KIKEK(I/L)PG, and the six amino acids semi-conserved sequence (EYVREE) are enclosed in boxes.

subclass (20 to 25 kDa) induced by drought as reported by Close (1996, 1997). It contains the N-terminal consensus motif (Y-segment) and the lysine rich domain (K-segment) at the C-terminal, common for all dehydrins, both of which are present in two copies as shown by Roberton and Chandler (1992). The two copies of six amino acid residues Y-segment D(E/Q)YGNP were present near the N-terminus at residues from 17 to 22 and from 27 to 32, respectively. The two copies of eight amino acid residues K-segment KIKEK(I/L)PG are found near the middle of the protein residues 124 to 131, and at the C-terminus, residues 182 to 189, both of PsDHN1 and pPsB12 lack the track of serine residues (S-segment) as reported by Roberton and Chandler (1992). Egyptian pea dehydrin (PsDHN1) contains two copies of a semi-conserved sequence of six amino acid residues in length (EYVREE), which lie in front of the lysine rich motif (K-segment). This six amino acid residues semi-conserved sequence, which is only common for pea dehydrins, was present also in pPsB12 dehydrin, and is responsible for length variation in pea dehydrins as suggested by Roberton and Chandler (1992) and Grosslindemann et al. (1998). They suggested that larger pea dehydrins could arise from three or more copies of this sequence.

Expression and accumulation of dehydrins in developing cotyledons

Expression and accumulation of dehydrins was studied in developing cotyledons representing sample 8 (wild *Pisum sativum* L. collected from Ghazala, 150 Km eastern to Matrouh city) using Northern and Western blot analyses (Figure 5). Only two mRNA transcripts were detected by *PsDhn1* probe (Figure 5A) and two dehydrins (29 and 21 kDa) were accumulated and hybridized positively with anti-dehydrin polyclonal antibody (Figure 5C). Similar data were obtained by Garnczarska et al. (2008). Their results indicated the accumulation of two dehydrins (30 and 22 kDa) during pea seed development. However, Roberton and Chandler (1992) identified one more dehydrin of 27 kDa accumulated during pea seed development, which was not detectable in this study. In this study, one large dehydrin mRNA transcript has an estimated size of about 1200 to 1300 nucleotides which was detectable between 20 to 40 DAF (Figure 5A). This mRNA transcript was abundant at 20 DAF and decreased at 28 DAF, then reached maximum abundance at 40 DAF. A corresponding dehydrin protein band of 29 kDa was also detectable between 20 to 40 DAF and hybridized positively with anti-dehydrin polyclonal antibody as shown in Figure 5C. This mRNA transcript and its corresponding dehydrin (29 kDa) seem to be expressed and accumulated early before 20 DAF (data not shown). The second mRNA transcript has an estimated size of about 800 to 900 nucleotides which was detectable at 28 DAF and reached maximum abundance at 40 DAF (Figure 5A). Its corresponding dehydrin protein band of 21 kDa was detectable between 32 to 40 DAF. Such expression and accumulation pattern during later stages of pea seed development suggests a role in

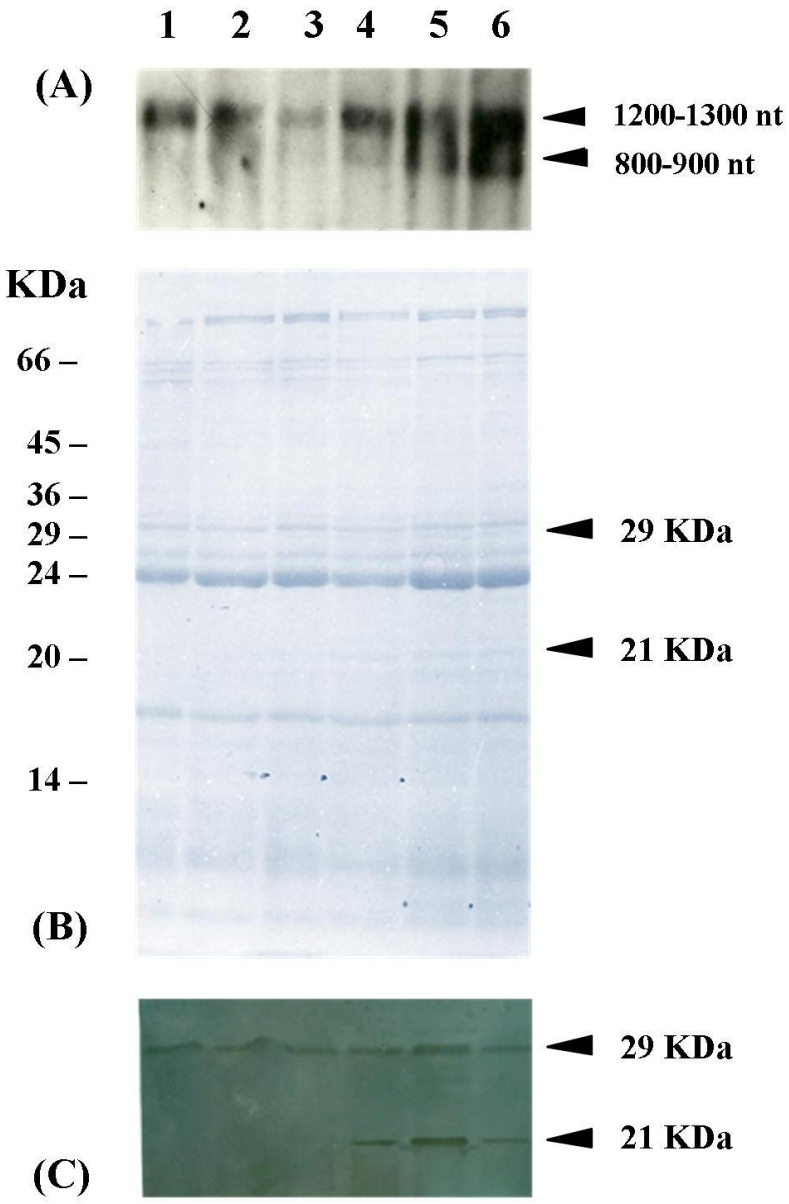

Figure 5. Expression and accumulation of dehydrins in pea cotyledons at specific intervals after flower opening (1: 20, 2: 24, 3: 28, 4: 32, 5: 36 and 6: 40 DAF). (A) Northern blot of total RNA using *PsDhn1* probe, (B) Electrophoretic profile of thermostable proteins and, (C) Western blot using anti-dehydrin polyclonal antibody. Detectable mRNA transcripts and dehydrin bands are indicated by arrows.

drought tolerance. The two mRNA transcripts as well as the two dehydrins (29 and 21 kDa) showed different pattern of expression and accumulation during pea seed development. This may be due to differences in the regulation of dehydrin expression suggested by Roberton and Chandler (1992). Similar size dehydrins were also observed in barley (Close et al., 1989), pea (Garnczarska et al., 2008), Bermudagrass (Hu et al., 2010), clover (Vaseva et al., 2011) and spinach (Chen et al., 2012).

Accumulation of dehydrins in plants is a common response to drought (Close, 1997). According to Cellier et al. (1998) and Farrant et al. (2004) dehydrin accumulation is associated with a tolerance mechanism leading to the maintenance of cellular turgor, suggesting that dehydrins might also be involved in preventing cellular dehydration. Western blot analysis indicated that accumulation of two dehydrins with molecular masses of 29 and 21 kDa correlated well with seed maturation and

desiccation. This suggests the involvement of these proteins in protective reaction promoting maintenance of embryo structures under conditions of water deficit (Allagulova et al., 2003). The amino acid composition of dehydrins with high content of charged and polar residues may promote their specific protective functions under conditions of cell dehydration (Allagulova et al., 2003; Rorat, 2006). It was also suggested that dehydrins are necessary for the prevention of protein aggregation and denaturation (that is, removal of un-functional and damaged molecules (Demirevska et al., 2008; Shinozaki and Yamaguchi-Shinozaki, 1996; Vaseva et al., 2010; Xoconostle-Cázares et al., 2011).

Conclusion

Pea (*Pisum sativum* L.) genotypes exhibited significant length variation in dehydrin genes. One of these genes *(PsDhn1),* which belongs to the Y_2K_2 dehydrin subclass, was isolated and characterized. This gene was 591 bp in length and coding for a 21 kDa dehydrin protein. Drought causes serious changes in cell metabolism and strongly stimulates dehydrin accumulation in plant tissues to protect cells from destruction and to improve their tolerance to stress conditions. Northern blot analysis indicated two mRNA transcripts and Western blot analysis detected two dehydrins (29 and 21 kDa) accumulated in developing pea cotyledons and showed different patterns of expression and accumulation

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