The Plant Journal (2000) **24**(2), 219–229

A DNA helicase from *Pisum sativum* is homologous to translation initiation factor and stimulates topoisomerase I activity

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Summary

DNA helicases play an essential role in all aspects of nucleic acid metabolism, by providing a duplex-unwinding function. This is the first report of the isolation of a cDNA (1.6 kb) clone encoding functional DNA helicase from a plant (pea, *Pisum sativum*). The deduced amino-acid sequence has eight conserved helicase motifs of the DEAD-box protein family. It is a unique member of this family, containing DESD and SRT motifs instead of DEAD/H and SAT. The encoded 45.5 kDa protein has been overexpressed in bacteria and purified to homogeneity. The purified protein contains ATP-dependent DNA and RNA helicase, DNA-dependent ATPase, and ATP-binding activities. The protein sequence contains striking homology with eIF-4A, which has not so far been reported as DNA helicase. The antibodies against pea helicase inhibit *in vitro* translation. The gene is expressed as 1.6 kb mRNA in different organs of pea. The enzyme is localized in the nucleus and cytosol, and unwinds DNA in the 3′ to 5′ direction. The pea helicase interacts with pea topoisomerase I protein and stimulates its activity. These results suggest that pea DNA helicase could be an important multifunctional protein involved in protein synthesis, maintaining the basic activities of the cell, and in upregulation of topoisomerase I activity. The discovery of such a protein with intrinsic multiple activity should make an important contribution to our better understanding of DNA and RNA transactions in plants.

Keywords: DEAD-box protein, plant DNA helicase, DNA replication, topoisomerase I, translation initiation factor, unwinding enzyme.

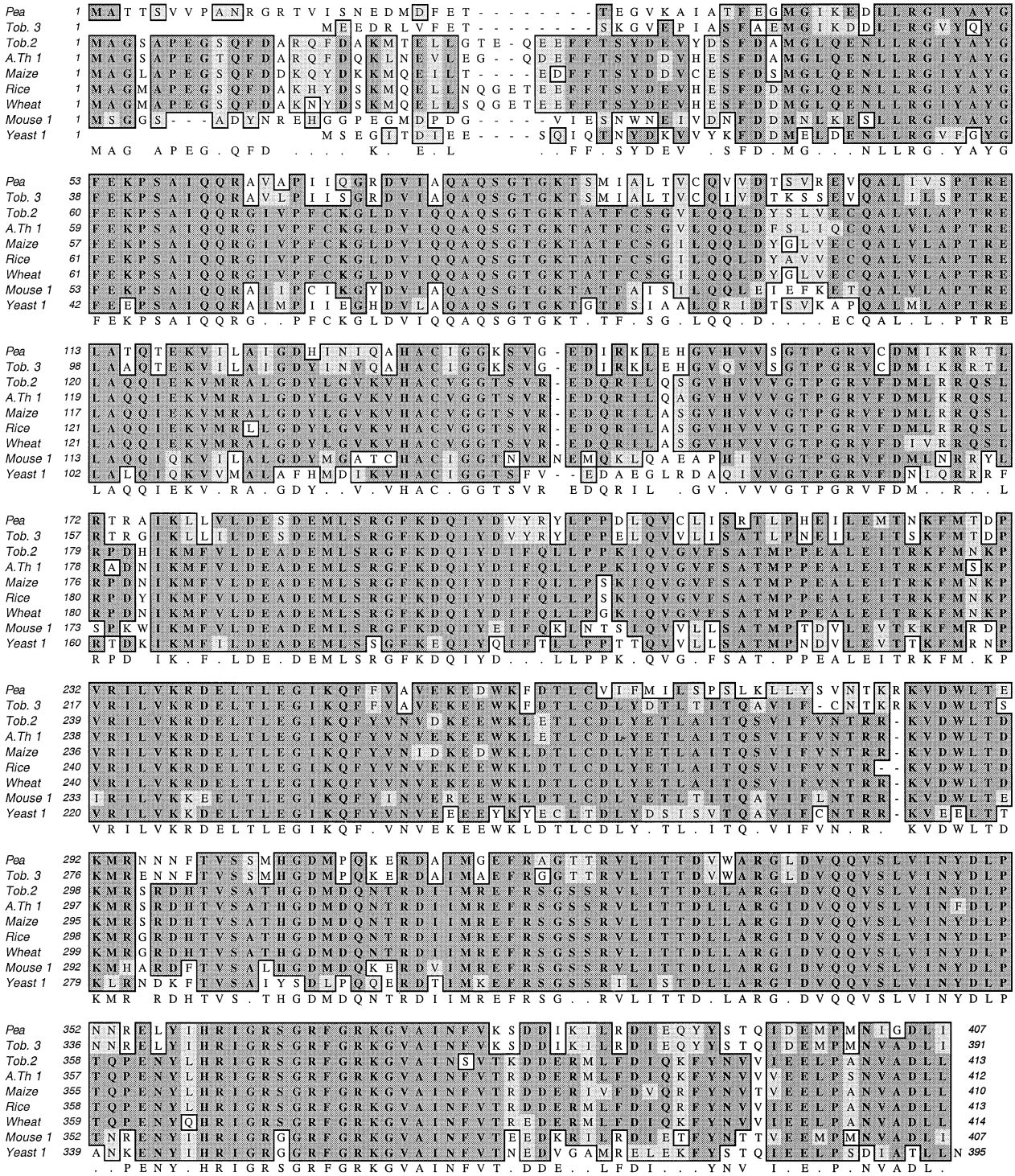
Introduction

Transient opening of duplex DNA is an essential pre-requisite for many biological processes including DNA replication, repair, recombination and transcription (Matson *et al.*, 1994; Tuteja, 2000). A special class of DNA-interacting enzymes called DNA helicases catalyse the unwinding of energetically stable duplex DNA in an ATP-dependent manner, and thus play an important role in all these basic genetic processes (Lohman and Bjornson, 1996; Matson *et al.*, 1994; Tuteja, 1997; Tuteja and Tuteja, 1996). Biochemical studies and computer analyses have revealed that many DNA and RNA helicases share a core region (approximately 400 amino acids) of highly conserved sequence motifs, and belong to the rapidly growing DEAD-box protein family (Gorbalenya *et al.*, 1989; Luking *et al.*, 1998; Pause and Sonenberg, 1992). The important helicase motifs are A/GXXGXGKT, DEAD, SAT and HRIGRXXR, which have been shown to be responsible, respectively, for initial ATP binding; hydrolysis of ATP; RNA unwinding; and ATP hydrolysis-dependent RNA binding (Gorbalenya *et al.*, 1989; Luking *et al.*, 1998; Pause and Sonenberg, 1992; Tuteja, 2000). However there is no guarantee that these conserved helicase domains may represent specific recognition features for all duplex-unwinding enzymes. Because these motifs are short and degenerate, their presence alone is not sufficient to confer significant sequence similarity on two proteins containing them. In general, DNA unwinding occurs with a unique directionality, usually defined as a 3′ to 5′, or 5′ to 3′, polarity relative to the strand of DNA that is bound by enzyme (Tuteja, 2000). Multiple DNA helicases have been

isolated from single cell types because of different structural requirements of the substrate at various stages of the DNA transaction (Lohman and Bjornson, 1996; Matson *et al.*, 1994; Tuteja and Tuteja, 1996). However, very little is known about DNA helicases from plant systems. The first plant DNA helicase purified to homogeneity and well characterized was reported from pea (*Pisum sativum*) chloroplast (Tuteja *et al.*, 1996).

Despite the ubiquitous presence of DNA helicases, their biological roles have been little investigated in plants and

(a)



many other eukaryotic systems. To our knowledge gene-encoding, biochemically active DNA helicase from plants has not yet been reported. Therefore a detailed molecular study of DNA helicase could be helpful for a better understanding of DNA transactions in plants. Here we report the cloning of the first plant DNA helicase gene, which encodes the biochemically active helicase protein. The helicase was overexpressed in bacteria and purified as 45.5 kDa protein. This helicase has been named PDH45, for 'pea DNA helicase 45.5 kDa in size'. The *PDH45* gene is expressed in different organs of the pea plant, and the encoded protein shows striking homology with the tobacco translation initiation factor NeIF-4A3. The antibodies against the pea helicase inhibit *in vitro* translation. We also show that the enzyme is localized in the nucleus and cytosol, and exhibits DNA and RNA helicase and ssDNA-dependent ATPase activities. It interacts with pea topoisomerase I (topo-I) and upregulates its activity. The results demonstrate that PDH45 could be a multifunctional protein.

Results

Cloning and sequencing of PDH45

Screening of the pea cDNA library revealed three positive clones: pBS-*PDH1*, pBS-*PDH2* and pBS-*PDH3*, with insert sizes of 1.6, 1.6 and 4.2 kb, respectively. For further study, only clones pBS-*PDH1* and pBS-*PDH2* were completely sequenced and were found to be identical (Accession No. Y17186). The sequence analysis shows that it encodes a full-length cDNA of 1630 bp with an ORF of 1224 bp, a 5′ untranslated region of 78 bp, and a 3′ untranslated region of 328 bp, including an 18 bp poly(A) tail. The deduced amino-acid sequence revealed a protein consisting of 407 amino-acid residues with a predicted molecular mass of approximately 45.5 kDa. The clone pBS-*PDH1* was selected for further study, and named pBS-*PDH45*.

The sequence contains all the known conserved helicase domains of the DEAD-box protein family (Gorbalenya *et al.*, 1989; Luking *et al.*, 1998). In contrast to all other DEAD-box family members, which have either a DEAD or a

(b)

	I	II	III	IV
Pea	QAQS**GTGKT**SMIA	LIVS**PTRELA**TQTE	HACI**GG**KSVGEDIR	VVSG**TPGRVCD**MIKR
Rad3	EMPS**GTGKT**VSLL	LMDY**RTKELG**YQED	RRMT**NG**QAKRKLEE	CPYF**IVRRMIS**LCNI
Rad15	IMPS**GTGKT**ISLL	GMDV**PTCEFH**DNLE	RREK**NG**NVVDARCR	EYGE**KTTRCPY**FTVR
ERCC2	EMPS**GTGKT**VSLL	GEKL**PFLGLA**LSSR	SKER**KG**TVVDEKCR	DDLK**ALGRRQG**WCPY
ERCC3	VLPC**GAGKS**LVGV	ACTV**RKRCLV**LGNS	KDKP**IG**CSVAISTY	----------
RECQL	VMPT**GGGKS**LCYQ	----------	----------	----------
P68T	IAKT**GSGKT**LGYL	LVLS**PTRELA**TQIQ	TCLY**GG**APKGPQLR	IVVA**TPGRLND**ILEM
A.th.	IAKT**GSGKT**LGYL	LVLS**PTRELA**TQIQ	TCLY**GG**APKGPQLR	IVVA**TPGRLND**ILEM
Spi.	AART**GQGKT**LAFV	LVLL**PTRELA**TQVL	CSVY**GG**APFHSQIS	IVVG**TPGRVKD**LLEK
P68H	VAQT**GSGKT**LSYL	LVLA**PTRELA**QQVQ	TCIY**GG**APKGPQIR	ICIA**TPGRLID**FLEC
VASA	CAQT**GSGKT**AAFL	VIVS**PTRELA**IQIF	GIVY**GG**TSFRHQNE	VVIA**TPGRLLD**FVDR
Yeast	IAAT**GSGKT**LSYC	LVLA**PTRELA**VQIQ	TCVY**GG**VPKSQQIR	IVIA**TPGRLID**MLEI

	V	VI	VII	VIII
	IKLL**VLDESDEM**LSRG	QVCLI**SRT**LPHE	ITTDV**WARGLD**VQQ	ELY**IHRIGRSGR**FGRK
	DSIV**IFDEAHNI**DNVC	TDALR**RAT**RGAN	AILLS**VARGKV**SEG	IDF**DHQYGRTVL**MIGI
	DCIV**VFDEAHNI**DNVC	QQVVA**FAT**LVAT	AVLLS**VARGKV**SEG	VDF**DHHYGRAVI**MFGI
	KAVV**VFDEAHNI**DNVC	GLREA**SAA**RETD	AVLLS**VARGKV**SEG	IDF**VHHYGRAVI**MFGV
	WGLM**ILDEVHTI**PAKM	CKLGL**TAT**LVRE	----------	GSR**RQEAQRLGR**VLRA
	FTRI**AVDEVHCC**SQWG	IGLTA**TAT**NHVL	----------	----------
	VSYL**VLDEADRM**LDMG	QTLMY**TAT**WPKG	VATDV**AARGLD**IKD	EDY**VHRIGRTGR**AGAS
	ISYL**VLDEADRM**LDMG	QTLMY**TAT**WPKG	VATDV**AARGLD**VKD	EDY**VHRIGRTGR**AGAT
	LLFR**VLDEADEM**LKMG	QTLLF**SAT**LPSW	VATNV**AARGLD**IND	EDY**IHRSGRTGA**AGNT
	TTYL**VLDEADRM**LDMG	QTLMW**SAT**WPKE	IATDV**ASRGLD**VED	EDY**IHRIGRTAR**STKT
	TRFV**VLDEADRM**LDMG	QTLMF**SAT**FPEE	IATSV**ASRGLD**IKN	DDY**VHRIGRTGC**VGNN
	VTYL**VLDEADRM**LDMG	QTLMW**SAT**WPKE	VATDV**AARGID**VKG	EDY**VHRIGRTGR**AGAT

Figure 1. Comparison of amino-acid sequence of PDH45 protein with translation initiation factor and other DEAD-box helicases.
(a) Multi-sequence alignment of deduced amino-acid sequence of PDH45 with translation initiation factor 4A (eIF-4A) from tobacco NeIF-4A3 (Tob.3), NeIF-4A (Tob.2), *Arabidopsis thaliana* (A.th.1), maize, rice, wheat, mouse (eIF-4AII) and yeast. Multiple alignment was done using the CLUSTAL W program. Identical amino acids at each protein are black-boxed, and similar ones are shaded. The accession numbers of the aligned sequences are Tob.3 (X61026), Tob.2 (X61205), Ath1 (X65052), maize (U17979), rice (D12627), wheat (Z21510), mouse 1 (S00986), and yeast 1 (X12813).
(b) Alignment of eight conserved helicase motifs of PDH45 protein from other DEAD/H-box proteins including DNA helicases: Rad3 (K03293), Rad15 (X60499), ERCC2 (X52470), ERCC3 (M31899), RECQL (L36140), and RNA helicases P68T (D16247), A.th (AB010259), spinach (X99937), P68H (X52104), VASA (X12945), and yeast (X52469).

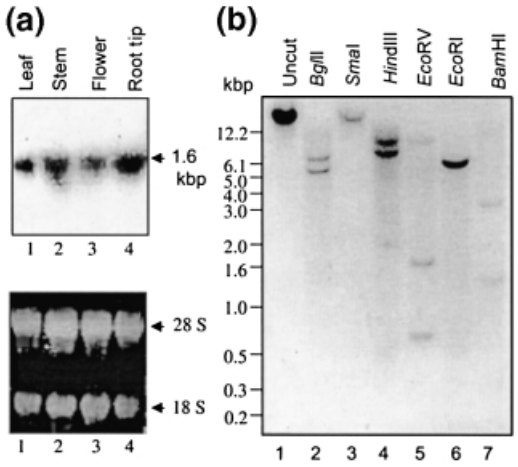


Figure 2. Northern and Southern analysis of the *PDH45* gene.
(a) Northern blot analysis. Total RNA (25 μg) in blot was hybridized with the ^{32}P-labelled full-length *PDH45* cDNA (1.6 kb). For equal loading of RNA in each lane the gel was examined after staining with EtBr, as shown in the bottom panel. Lanes 1–4 are RNAs isolated from leaf, stem, flower and root tips, respectively.
(b) Southern blot analysis. Pea genomic DNA (10 μg) was completely digested with the enzyme indicated and hybridized with the ^{32}P-labelled full-length *PDH45* cDNA. Lane 1, uncut; lane 2 *Bgl*II; lane 3, *Sma*I; lane 4, *Hin*dIII; lane 5, *Eco*RV; lane 6, *Eco*RI; lane 7, *Bam*HI.

DEAH box, PDH45 contains a DESD-box. The Ala–Ser substitution is a conservative amino-acid change and results from G–T transversion. There is also one amino-acid change in domain VI: instead of SAT (which is commonly present), PDH45 contains SRT. The Ala–Arg substitution is a result of G–C and C–G transition in the nucleotide sequence. A multiple alignment of amino-acid sequence homology search reveals that PDH45 shows 86% similarity with tobacco eIF-4A3 (Owttrim *et al.*, 1991), while with eIF-4A from other plant sources (tobacco NaIF-4A2, *Arabidopsis*, maize, rice, wheat) the similarity is approximately 58% (Figure 1a). However, the similarity of PDH45 with mouse and yeast eIF-4As is 59 and 54.5%, respectively (Figure 1a). The alignment of amino-acid sequences between PDH45 and other DNA and RNA helicases (Figure 1b) reveals regions of sequence identity only in the eight domains characteristic of helicases.

Northern and Southern blot analyses

A Northern blot analysis showed a single transcript of approximately 1.6 kb in size in pea leaf, stem, flower and root organs after washing the blot at both low stringency (data not shown) and at high stringency (Figure 2a). Southern blot analysis showed that under low and high stringency washing conditions, the *PDH45* cDNA probe hybridized to one major fragment of genomic DNA for *Eco*RI and *Sma*I; two fragments for *Bam*HI, and three fragments each for *Eco*RV, *Hin*dIII and *Bgl*II, respectively (Figure 2b). The *PDH45* cDNA sequence has one internal recognition site for *Bam*HI, and two sites each for *Eco*RV, *Hin*dIII and *Bgl*II, respectively, whereas there are no recognition sites for *Eco*RI and *Sma*I in the cDNA. The pattern of hybridizing bands suggests that *PDH45* exists as a single gene in the pea genome.

Expression, purification and localization of PDH45

SDS-PAGE analysis showed a highly expressed 45.5 kDa additional polypeptide that is IPTG-induced in *Escherichia coli* transformed with pET-*PDH45* (Figure 3a, lane 3), as compared to uninduced (Figure 3a, lane 2). A final ssDNA-cellulose chromatography step yielded a purified PDH45 which showed 45.5 kDa band on SDS-PAGE (Figure 3a, lanes 6 and 7). In Western blotting, the antibody detects PDH45 as a single band of 45.5 kDa after Ni^{2+}-NTA-agarose chromatography (Figure 3b, lane 3), heparin-sepharose chromatography (Figure 3b, lane 4), and ssDNA-cellulose (Figure 3b, lanes 5 and 6) chromatography. This shows that the preparation does not contain degradation products. This purification protocol routinely yielded 1–1.5 mg of homogeneous PDH45 from 1 l bacterial culture. The results of localization of PDH45 are shown in Figure 3(c). The anti PDH45 antiserum detected a protein band of approximately 45.5 kDa in the cytosol and in purified nuclear extract (Figure 3c, lanes 1 and 2) but not in the purified chloroplast lysate (Figure 3c, lane 3). As a control, the PDH45 was recognized by the antiserum (Figure 3c, lane 4). The pre-immune serum did not recognize any protein in the same preparation (data not shown).

Characterization of DNA unwinding activity of PDH45

The structure of the substrate used for characterization and in Figure 4(a–d) contains hanging tails of 15 bp on both the 5′ and 3′ ends, as shown in Figure 4(h). PDH45 enzyme required 150 mM KCl, 0.6 mM $MgCl_2$ and pH 8.0 for maximum DNA unwinding (data not shown). The activity is also ATP-dependent and showed maximum activity at 0.6 mM (Figure 4a). ATP was the best co-factor utilized by the PDH45, while dATP supported 87% of the activity (Figure 4b). The other NTPs/dNTPs, such as CTP, dCTP, GTP, dGTP, UTP and dTTP, supported 49, 51, 46, 51, 34 and 32% of the activity, respectively, as compared to ATP (Figure 4b). There was no activity if ATP was replaced by ATPγS or ADP or AMP, or if Mg^{2+} was replaced with other divalent cations such as Zn^{2+}, Cd^{2+}, Cu^{2+}, Ni^{2+}, Ag^{2+} and Co^{2+}, but with the exception of Mn^{2+} and Ca^{2+}; the overall divalent cation requirement was $Mg^{2+} > Mn^{2+} >> Ca^{2+}$(data not shown). The kinetics of the unwinding reaction of PDH45 at optimum assay conditions (0.6 mM ATP, 0.6 mM $MgCl_2$ and

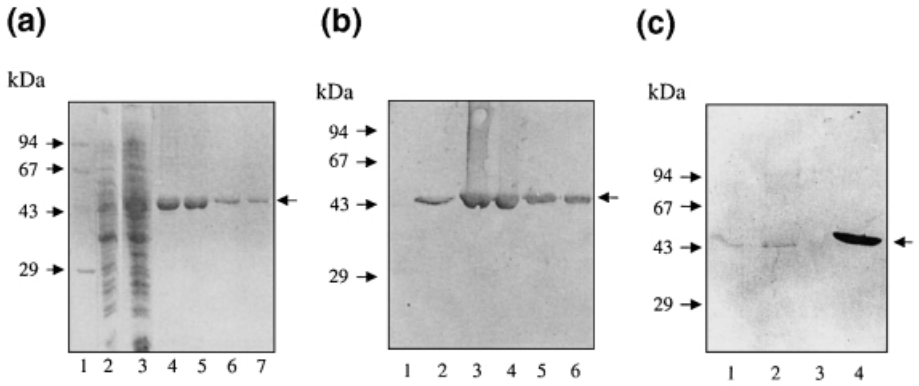


Figure 3. Expression, purification and localization of the PDH45 protein.
(a) The induction and purification of overexpressed PDH45 in *E. coli* is shown on SDS-PAGE. Lane 1 is a molecular weight marker. Lane 2, uninduced; lane 3, IPTG-induced; lane 4, after Ni^{2+}-NTA-agarose column; lane 5, after heparin-sepharose column; lanes 6 and 7, two different concentrations of purified PDH45 after ssDNA column.
(b) Western blot analysis of the protein fractions in (a) using polyclonal anti-PDH45 antiserum. The protein fractions in lanes 1–6 correspond to lanes 2–7 of (a).
(c) Localization of PDH45. Purified intact nuclei (lane 2), chloroplasts (lane 3) and cytosol (lane 1) were prepared from pea seedling and fractionated onto SDS-PAGE. Lane 4 is purified PDH45 (after ssDNA column). Proteins were transferred onto a nitrocellulose membrane and Western blot analysis performed using polyclonal anti-PDH45 antiserum. Protein size markers are indicated.
Arrows (right) represent 45.5 kDa bands.

150 mM KCl) using 40 ng purified PDH45 showed a linear rate up to 45 min (Figure 4c). On further incubation the linearity was deviated. Titration of unwinding activity with increasing amount of purified PDH45 showed linearity up to 40 ng protein (Figure 4d).

DNA-unwinding activity of PDH45 with a different fork-like structure of the substrates is shown in Figure 4(e–j). Almost the same activity was seen whether the substrate contained a 5′ tail (Figure 4f), a 3′ tail (Figure 4g), 5′ and 3′ tails (Figure 4h), or no tail (Figure 4e). However, if the duplex region of the substrate increased to 34 bp in the no-tail substrate (Figure 4i) or to 41 bp in the tailed substrate (Figure 4j) the PDH45 failed to unwind. It could not unwind blunt-ended duplexes (data not shown). To determine the direction of translocation of the PDH45, two substrates, one specific for the 3′–5′ direction (Figure 4k, top panel) and the other for the 5′–3′ direction (Figure 4l, top panel) were constructed. The release of radiolabelled 16-base DNA from the substrate of Figure 4(k) and of 17-base DNA from that of Figure 4l by the helicase indicates the movement in the 3′–5′ and 5′–3′ directions, respectively. PDH45 contained 3′–5′ direction unwinding activity (Figure 4k), and not 5′–3′ direction activity (Figure 4l).

ATPase and ATP-binding activities of PDH45

The release of radioactive phosphate (Pi) from [γ-^{32}P]ATP by PDH45 enzyme was calculated and plotted as a histogram (Figure 5a); the autoradiogram of the TLC is shown as an inset. ssDNA-dependent ATPase activity was present at a level of 1.98×10^3 pmol ATP hydrolysed at 37°C in 2 h by 80 ng purified PDH45 in the presence of 100 ng M13 ssDNA. There was no ATP hydrolysis without ssDNA and Mg^{2+} (2% background, Figure 5a, lanes 2 and 8). If the M13 ssDNA was replaced by the same amount of DNA helicase substrate, the ATP hydrolysis was observed to be 1.83×10^3 pmol (Figure 5a, lane 19), which was almost as observed with M13 ssDNA.

We further examined the effect of different DNA or RNA species and polynucleotides on the ATPase activity of PDH45. The data in Figure 5(a) show that the PDH45 had less ATPase activity ranging from 170 to 560 pmol in presence of the same amount (100 ng) of indicated DNA or RNA species (Figure 5a). Pea poly(A)+ RNA was more effective than pea total RNA (Figure 5a, lanes 11 and 12). Among DNA oligos, longer oligos were more stimulatory for ATPase activity of PDH45 than shorter oligos (Figure 5a, lanes 4–6). No activity was observed in the presence of poly[U] RNA (Figure 5a, lane 18) as compared to other polynucleotides (Figure 5a, lanes 15–17), indicating that the stimulation of ATPase activity by RNAs may require RNA secondary structure.

Figure 5(b) shows the autoradiogram of ATP-binding activity and Figure 5(c) shows the Coomassie-stained gel. The results showed radiolabelling of 45.5 kDa polypeptide of PDH45 (Figure 5a, lane 2), indicating that ATP does bind to the protein. As a positive control, *E. coli* DNA polymerase I (approximately 100 kDa) was also affinity-labelled to provide a 100 kDa band marker on SDS-PAGE (lane 1, Figure 5b,c). The labelling reaction was also performed in the absence of the protein PDH45 (Figure 5a, lane 3) in order to observe the background.

RNA helicase activity and translation inhibition

The result showed that the purified PDH45 protein also contained RNA-unwinding activity (Figure 6a, lane 2). This RNA unwinding was ATP-dependent, as the protein did not show unwinding activity without ATP (Figure 6a, lane 3). For studying the effect of PDH45 on translation, anti-PDH45 antibody was used in an *in vitro* transcription–translation coupled assay performed with pea calnexin mRNA (Ehtesham *et al.*, 1999) using a wheatgerm lysate system. Since PDH45 contains about 58% identity at the amino-acid level with wheatgerm eIF-4A (Figure 1a), the anti-PDH45 antibodies should react with wheatgerm eIF-4A. *In vitro* translation yielded 75 kDa pea calnexin (Figure 6b, lane 1). The presence of 1 μg purified anti-PDH45 antibodies (IgG) during *in vitro* translation resulted in the inhibition of translation (Figure 6b, lane 3). On increasing the concentration of the antibodies to 2 μg in the reaction, a complete inhibition of translation was observed (Figure 6b, lane 4). As a control, when the pre-immune IgG (2 μg) was included in the reaction, no inhibition of translation was observed (Figure 6b, lane 2).

Stimulation of pea topo-I activity by PDH45 and interaction of topo-I-PDH45

The result of stimulation of DNA relaxation activity of the purified pea topo-I (Reddy *et al.*, 1998) by PDH45 is shown in Figure 7(a). A normal DNA ladder formation is seen with 1 unit of topo-I (Figure 7a, lane 3) as compared to no topo-I (Figure 7a, lane 1). This activity was stimulated when 1 unit of topo-I was pre-complexed with 80 ng PDH45: as a result the DNA ladder moved up (Figure 7a, lane 4). The DNA ladder formation was unaffected when 80 ng heat-denatured PDH45 (pre-boiled for 3 min) was pre-complexed with topo-I (Figure 7a, lane 5). The pure preparation of PDH45 (80 ng) did not contain any topo-I activity (Figure 7a, lane 2). These results clearly indicate that PDH45 stimulates pea topo-I activity. This stimulation was also observed in the presence of ATP (data not shown). The topo-I and PDH45 preparations used here were pure, without any contamination of DNA or other proteins.

We have studied the topo-I–PDH45 interaction by protein hybridization and far-Western methods (see Experimental procedures). When ^{32}P-labelled pea topo-I (100 kDa) was hybridized to PDH45 and BSA on a blot, a radioactive band at the 45.5 kDa position in the PDH45 lane (Figure 7b, lane

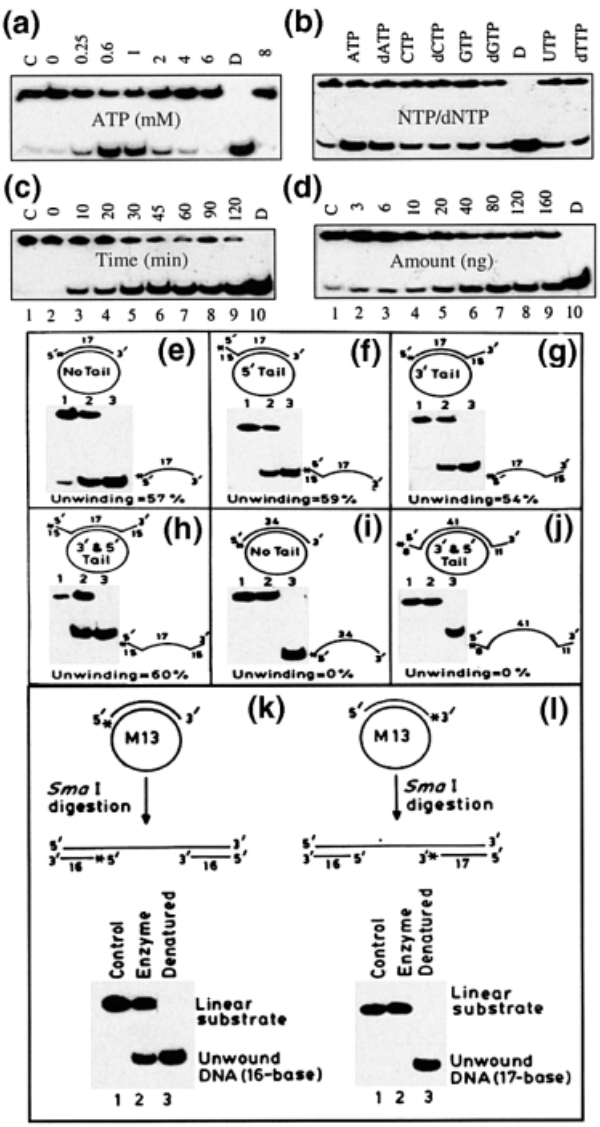


Figure 4. Characterization of the ATP-dependent DNA-unwinding activity of the PDH45 protein and its direction of unwinding.
The substrate used in (a–d) contains 15 bp hanging tails at both 3′ and 5′ ends, as shown in (h).
(a) ATP-dependent reaction. In each lane, 40 ng pure PDH45 protein with approximately 1 ng ^{32}P-labelled helicase substrate was used with varying concentrations of ATP (given at top).
(b) Preference of nucleotides for PDH45 activity. The standard helicase reactions were performed in the presence of one of the NTPs or dNTPs (0.6 mM). The different NTPs or dNTPs used are given at the top of each lane.
(c) Kinetics. For time dependence, 40 ng pure PDH45 was used for each time reaction. The times of reactions are given at the top of each lane.
(d) Concentration dependence. The various concentrations of pure PDH45 used in the 45 min reactions are given at the top of each lane.
Lanes C and D in all the above show reaction without enzyme and reaction with heat-denatured substrate, respectively.
(e–j) Unwinding activity of PDH45 with various forked and non-forked substrates. Each panel shows the schematic structure of the substrate at the top. Asterisks denote the ^{32}P-labelled end. In each autoradiogram lanes 1 and 3 are the reaction without enzyme and with heat-denatured substrate; lane 2 is the reaction with 40 ng pure pea PDH45. The percentage unwinding is shown below each autoradiogram.
(k,l) Direction of DNA unwinding by PDH45. The construction and structure of the linear substrates for 3′–5′ (k) and 5′–3′ (l) directions are given at the top. In each gel, lane 1 is the reaction without enzyme; lane 2 the reaction with 40 ng pure PDH45; lane 3 the heat-denatured substrate. Asterisk denotes the ^{32}P-labelled end.

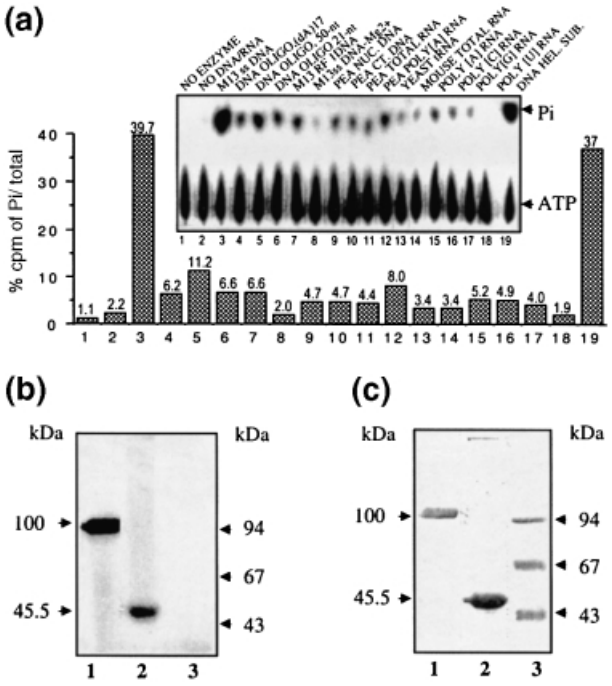


Figure 5. ATPase and ATP-binding activities of the PDH45 protein.
(a) Stimulation of ATPase activity of PDH45 by various DNA and RNA species. The radioactive phosphate (P_i) released from [γ-^{32}P]ATP by PDH45 was separated on a PEI-TLC plate and the percentage P_i plotted as a histogram. Percentage values are indicated at the top of each column. The autoradiogram of the TLC is shown as an inset. Lanes 1–19 of the histogram correspond to lanes 1–19 of the autoradiogram. Various DNA and RNA species used to stimulate ATPase activity are indicated at the top of each lane. Positions of P_i and ATP are marked on the right. Lane 1, reaction without enzyme; lane 2, reaction with enzyme in the absence of nucleic acids. 80 ng pure enzyme (PDH45) and 100 ng nucleic acids were used per reaction.
(b) UV cross-linking of ATP. Autoradiogram of photoaffinity-labelled *E. coli* DNA polymerase 1 as a positive control (lane 1) and PDH45 protein (lane 2) with [α-^{32}P]ATP. Lane 3, [α-^{32}P]ATP incubated without any protein.
(c) Coomassie-stained SDS-PAGE gel after the UV cross-linking reaction. Lane 1, *E. coli* DNA polymerase I; lane 2, is PDH45; lane 3, molecular weight protein marker.

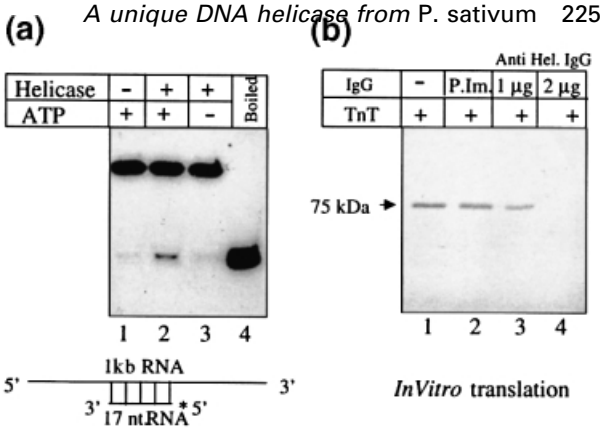


Figure 6. RNA unwinding activity of the PDH45 protein and *in vitro* translation inhibition.
(a) RNA-unwinding activity of PDH45. The model of the RNA duplex substrate used to detect RNA helicase activity is shown at the bottom of the autoradiogram. Asterisk denotes the ^{32}P-labelled end. Lane 1, reaction without enzyme; lane 2, reaction with PDH45 protein (40 ng); lane 3, reaction with PDH45 but without ATP; lane 4, heat-melted substrate (boiled).
(b) Inhibition of *in vitro* translation with anti-PDH45 antibodies. An *in vitro* translation reaction was performed using a wheatgerm lysate system and pea calnexin mRNA (Ehtesham *et al.*, 1999). A 75 kDa translated product of pea calnexin is shown by the arrow (left). Lane 1, standard reaction showing 75 kDa band; lane 2, reaction in the presence of rabbit pre-immune IgG (2 µg); lanes 3 and 4, are *in vitro* translation reaction in the presence of two different concentrations (1 and 2 µg) of anti-PDH45 IgG.

1) and not in the BSA lane (Figure 7b, lane 2) was observed, which confirmed the binding of topo-I to the helicase. The results of far-Western blotting showed that pea topo-I binds to pea DNA helicase, which was recognized by anti-topo-I antibodies (Figure 7c, lane 1). As a negative control, the BSA lane did not show any bands (Figure 7c, lane 2). These results indicate that pea DNA helicase interacts *in vitro* with pea topo-I, and this interaction may be responsible for stimulation of topo-I activity.

Discussion

The unwinding of duplex DNA by DNA helicase consists of a co-ordinated set of sub-activities including nucleotide binding, DNA binding, and DNA-dependent ATP hydrolysis (Lohman and Bjornson, 1996; Matson *et al.*, 1994). We have isolated the first plant full-length *PDH45* cDNA encoding functional DNA helicase. Biochemical analyses with purified PDH45 confirmed that it functions as a DNA helicase, RNA helicase and ATPase, as a component of translation initiation, and as an upregulator of topo-I activity. It is a single-copy gene and is expressed in different organs (leaf, stem, flower, root) of pea plants, suggesting its essential role in maintaining the basic activity of the cells.

The PDH45 protein sequence contained all eight conserved helicase domains, and therefore belongs to the DEAD-box protein family (Luking *et al.*, 1998). Most of the DEAD-box proteins have been identified as 'putative computer-predicted helicases'; however only few have been biochemically shown to contain unwinding activity. For example, yeast Rad3 (Deschavanne and Harosh, 1993); the 172 kDa protein encoded by the *DNA2* gene (Budd and Campbell, 1995); the *E. coli* RecQ gene product, a 74 kDa protein (Umezu *et al.*, 1990); a 72 kDa protein encoded by the human *REQL* gene (Puranam and Blackshear, 1994); and a product of excision repair gene *ERCC3* (Schaeffer *et al.*, 1993), are all known to contain DNA-unwinding activity, while human p68; mouse eIF-4A; *Xenopus*-an3 and Xp54; *Drosophila* VASA; and *Arabidopsis thaliana* DRH1 are known to contain RNA-unwinding activity (Aubourg *et al.*, 1999; Luking *et al.*, 1998). Several DEAD-box proteins have also been reported from plants includ-

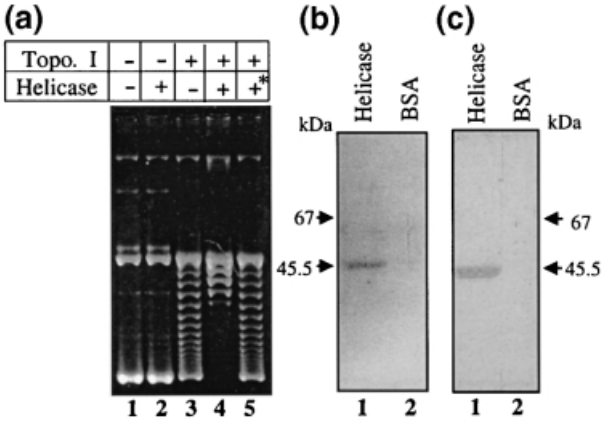


Figure 7. Stimulation of pea topo-1 activity by the PDH45 protein and protein–protein interaction of topo-I-PDH45.
(a) Stimulation of topo-I activity by PDH45. Supercoiled pBR322 DNA (0.6 μg) was used for each topo-I assay. Lane 1, reaction without any protein; lanes 2 and 3, reactions with PDH45 (80 ng) and pea topo-I (1 unit) proteins, respectively. PDH45 contained no topo-I activity (lane 2), while pea topo-I shows topo-I activity as the ladder formation (lane 3). Lane 4, DNA relaxation by pea topo-I pre-complexed with 80 ng pea PDH45, the DNA ladder moved up (stimulation). Lane 5, PDH45 (80 ng) was heat-inactivated (asterisk) by boiling for 3 min before the reaction with topo-I (buffer control). The topo-I and PDH45 preparations used were pure, without contamination of DNA or other activities.
(b) Protein hybridization of pea topo-I with PDH45. Lanes 1 and 2, PDH45 and BSA (as a negative control). The blot was hybridized with ^{32}P-labelled pea topo-I after renaturation of the protein. A radioactive band of 45.5 kDa PDH45 is shown in the autoradiogram.
(c) Far Western blot analysis. PDH45 (lane 1) and BSA (lane 2) were blotted and renatured on the membrane and incubated with pea topo-I protein, and later the interaction (topo-I on PDH45 band) was detected with anti topo-I antibodies by Western blotting. A 45.5 kDa band of PDH45 is shown on the immunoblot.

ing tobacco, rice and *Arabidopsis* (Aubourg *et al.*, 1999; Okanami *et al.*, 1998). The PDH45 is a unique member of this family because it contains DESD and SRT motifs instead of DEAD and SAT, and also contains both DNA and RNA helicase activities.

Distribution of PDH45 in various cellular compartments indicates that it may function in the cytosol and nucleus, and therefore could be a plurifunctional protein. The most striking feature of the PDH45 protein is its 86% similarity to the tobacco translation initiation factor NeIF-4A3 (Owttrim *et al.*, 1991), while it has 54–59% similarity to eIF-4A from other sources, including plants. We have shown that the antibodies against the PDH45 protein inhibit *in vitro* protein synthesis, which indicates that PDH45 might also be involved in translation. The eIF-4A is known to play a key role in unwinding inhibitory secondary structures (present in the 5′ UTRs of many proteins) during scanning, and therefore to facilitate translation of the mRNAs (Pause *et al.*, 1994). In plants, eIF-4A has been reported from tobacco, rice, maize and *Arabidopsis* (Owttrim *et al.*, 1991; 1994; Pause *et al.*, 1994). The DNA helicase activity of eIF-4A has not been reported from any other system. This is the first report to identify the DNA helicase activity of a translation initiation factor. The previous failure to detect the DNA helicase activity of eIF-4A might be ascribed to the lack of other helper components or a suitable helicase substrate. These findings suggest the possibility of a new function for PDH45 as a regulator of translational initiation.

The genomic hybridization banding pattern produced by the *PDH45* gene in Southern blotting was similar at both high (Figure 2b) and low stringency (data not shown), which suggests the presence of a single-copy gene in the pea genome. The number of bands present in the Southern blot (Figure 2b) is due to the presence of internal sites in the cDNA. These results are further supported by the fact that the gene shows a single transcript in Northern blotting, even with a low stringency wash (data not shown). A single transcript of tobacco eIF-4A was also reported (Owttrim *et al.*, 1991). The anti-PDH45 antibodies recognized only a single polypeptide in the nuclear extract and cytosol (Figure 3c), which again shows it to be a single-copy gene.

The PDH45 enzyme moves in the 3′–5′ direction along the bound strand in a manner similar to that of previously described pea chloroplast DNA helicases (Tuteja and Phan, 1998; Tuteja *et al.*, 1996); human DNA helicases I, II, III, V, VI and α (Tuteja and Tuteja, 1996); and SV-40 T antigen helicase (Borowiec, 1996). PDH45 contains limited unwinding activity and it could not unwind longer duplexes; probably it requires some additional accessory protein(s) in order to have processive unwinding activity. However, this is not unusual: *in vitro* a limited unwinding activity was reported for *E. coli* Rep protein helicase, which required CisA protein and SSB for processive unwinding (Matson *et al.*, 1994). Human MCM4, -6 and -7 protein complex, which also plays role in initiation of DNA replication, has limited helicase activity (Ishimi, 1997). PDH45 required ATP or dATP as a co-factor for optimal activity, although dATP showed 87% of the activity, and all the other NTP or dNTP could be utilized with less efficiency. This property is similar to human DNA helicase II (Tuteja and Tuteja, 2000; Tuteja *et al.*, 1994). The ATPγS, a poorly hydrolysable analogue of ATP, could not replace ATP, which clearly showed that the hydrolysis of ATP is an absolute requirement for the unwinding reaction of PDH45. PDH45 contained ATPase activity which was maximally stimulated by ssDNA. ss-DNA-dependent ATPase activity has been reported to be the intrinsic activity of all the helicases (Lohman and Bjornson, 1996; Matson *et al.*, 1994; Tuteja, 2000), and is also required for translocation of the helicase protein on the DNA (Tuteja and Phan, 1998). The RNA-dependent ATPase activity of PDH45 helps to limit its RNA helicase activity. There are only few proteins known that are endowed with both DNA and RNA helicase activity, for example human DNA helicase I and IV (Tuteja and Tuteja, 1996) and SV-40 T antigen (Borowiec, 1996).

Another striking property of PDH45 was its stimulation of pea topo-I activity. Topoisomerases are ubiquitously present and play a major role in cellular DNA metabolism, including replication, repair, transcription and recombination, by resolving the topological constraints imposed on DNA during these processes (Wang *et al.*, 1990). It is possible that both types of enzyme may function in concert, as topoisomerases probably relieve helicase-induced DNA torsional tension. Type I topoisomerases need ssDNA in order to catalyse a strand passage event; the DNA helicase produces the ssDNA from the normally duplex DNA. The stimulation of pea topo-I activity by PDH45 could be due to the direct interaction of both proteins. We have shown that both proteins *in vitro* interact with each other. The physical interaction of topo-I with SV-40 T antigen helicase (Haluska *et al.*, 1998) and nucleolin helicase (Bharti *et al.*, 1996) has been reported previously. Recently, Harmon *et al.* (1999) also reported that *E. coli* RecQ helicase stimulates the *E. coli* topoisomerase III (a type I topoisomerase) activity, and proposed that this shared activity was responsible for control of DNA recombination.

A physical association between PDH45 and topo-I may have additional relevance in plant DNA replication. During the progression of PDH45 through the duplex DNA, a local overwinding (positive supercoiling) could be created into the DNA region downstream from the helicase (or ahead of replication fork). This local superhelicity should be removed in order to obtain extensive unwinding. PDH45 and pea topo-I are both localized in the nucleus and interact/bind to each other, which might result in an allosteric stimulation of topo-I activity, relieving the tension of local overwinding ahead of the replication fork during DNA replication.

Although many helicases have been characterized biochemically, it is often difficult to determine the *in vivo* role of a specific helicase. However, the biological roles of only a few DNA helicases have been determined. For example, the DnaB, PriA protein, Rep protein and helicase II from *E. coli* and the SV-40 large T antigen helicase have been shown to play a role in DNA replication (Borowiec, 1996; Lohman and Bjornson, 1996; Matson *et al.*, 1994). A DNA-repair helicase has been shown to be a component of basic transcription factor 2 (TFIIH) (Schaeffer *et al.*, 1993). In plants, the isolation of the first DNA helicase gene, described in this study, is the first step towards elucidating the DNA transaction mechanism in plants.

This paper reports the cloning of a unique plant DNA helicase. The encoded protein contains both DNA and RNA helicase activities, and is homologous to eIF-4A. PDH45 also interacts and stimulates topo-I activity, which may have an important role in controlling DNA transaction processes. The DNA-dependent ATPase motifs of PDH45 are conserved from yeast to plant cells, suggesting that they play an important role in cellular DNA replication. PDH45 thus could be an important multifunctional protein involved in DNA and RNA metabolism, in the regulation of protein synthesis, and in maintaining the basic activities of the cell. Its localization in the cytosol and nucleus further support the multifunctionality of this protein.

Experimental procedures

Construction and screening of P. sativum *cDNA library and DNA sequencing*

A cDNA library was constructed from 5 μg poly(A)+ RNA (isolated from the top four leaves of 7-day-old pea) in a Uni-Zap XR vector using a Zap-cDNA synthesis kit (Stratagene, La Jolla, CA, USA) following the manufacturer's protocol. The resulting phage library contained 1×10^9 plaque-forming units ml^{-1}. For screening the library, a 21 nt degenerate oligo (5′-ACTAGT(A/G/C/T)CT(A/G/C/T)GA(T/C)GA(G/A)GC(A/G/C/T)GA-3′), which corresponds to a conserved helicase domain LVLDEAD, was radiolabelled at the 5′ end and used as a probe. The cDNA sequencing was performed by using the sequenase version 2 kit (US Biochemicals, Cleveland, OH, USA). The sequence was analysed using the FASTA AND CLUSTAL W alignment programs.

Northern and Southern blot analyses

The RNA and genomic DNA blots were prepared as described (Reddy *et al.*, 1998) and hybridized with an [α-^{32}P]-labelled (nick-translated) 1.6 kb *PDH45* cDNA probe at 50°C in 5 × SSC, 5 × Denhardt's, 0.1% SDS and 100 μg ml^{-1} denatured salmon sperm DNA for 16–18 h. After hybridization the blots were washed twice for 15 min at low stringency (2 × SSC + 0.1% SDS at 45°C) and twice for 15 min at high stringency (0.1 × SSC + 0.1% SDS at 50°C), followed by autoradiography.

Expression and purification of PDH45 protein

The coding region of the helicase gene (pBS-*PDH45*) was amplified by PCR using the following two primers. Primer 1: 5′-AGAGGCATATGGCGACAACTTCTGTGG-3′ contains an *Nde*I site (underlined), and primer 2: 5′-GAGCTCGAGTTATATAAGATCOACCAATATTC-3′ contains an *Xho*I site (underlined). The amplified product was subcloned in frame with Hexa His-tag into *Nde*I and *Xho*I sites of T7-driven *E. coli* expression vector pET14b (Novagene, Madison, WI, USA). This resulted in the construction of plasmid pET-*PDH45*. Fresh overnight culture of BL21(DE3) containing pET-*PDH45* was grown, induced by IPTG, and harvested by centrifugation. All the purification steps were performed at 4°C. The bacterial pellet was resuspended in ice-cold lysis buffer containing 50 mM Tris–HCl pH 7.4, 200 mM KCl, 14 mM β-mercaptoethanol, 0.5% Triton-X100, 0.01 mg ml^{-1} lysozyme, 1 mM EDTA and protease inhibitors followed by sonication and centrifugation. The supernatant was applied onto an Ni^{2+}-NTA-agarose column, and the resin was washed with buffer A (50 mM Tris–HCl pH 7.4, 200 mM KCl, 1 mM EDTA, 10% glycerol plus the protease inhibitors) and buffer B (buffer A containing 10 mM imidazole). The recombinant protein was eluted with 3 column volumes of buffer C (buffer A containing 200 mM imidazole). The fractions containing PDH45 were pooled and

dialysed at 4°C against buffer D (buffer A containing 50 mM KCl). The PDH45 was further purified to homogeneity using a heparin-sepharose 6B (Pharmacia, Sweden) column, followed by ssDNA-cellulose (USB, OH, USA) column chromatography. The PDH45 was eluted at 200 mM KCl in buffer D from both columns. The purity of recombinant protein (histidine-tagged PDH45) was checked by SDS-PAGE and Western blotting using standard procedures, and this pure protein was used for all assays. The polyclonal antibody against PDH45 protein was raised in rabbit as described (Reddy *et al.*, 1998).

Preparation of substrates for DNA and RNA helicase

The sequences of various DNA and RNA oligonucleotides, and the preparation of the duplex substrates used in this study, have been described previously (Tuteja *et al.*, 1991; 1992; 1994; 1996).

DNA and RNA helicases, ATPase and topoisomerase I assays

The DNA helicase reaction was performed in a 10 μl reaction mixture consisting of 20 mM Tris–HCl pH 8.0, 0.6 mM ATP, 0.6 mM $MgCl_2$, 150 mM KCl or NaCl, 8 mM DTT, 4% (w/v) sucrose, 80 μg ml^{-1} BSA, approximately 1 ng ^{32}P-labelled substrate (1000–2000 cpm), and the helicase fraction. The reaction mixture was incubated for 45 min at 37°C, and the reaction was stopped and analysed as described previously (Tuteja *et al.*, 1994; Tuteja and Phan, 1998). The RNA helicase assay was performed as previously described (Tuteja *et al.*, 1994). The ATPase reaction condition was as described for the helicase reaction, except that the ^{32}P-labelled helicase substrate was replaced by 1665 Bq [γ-^{32}P]ATP, and the reaction was performed for 2 h at 37°C and analysed as described (Tuteja *et al.*, 1992). The topo-I protein was purified and activity was assayed as described (Reddy *et al.*, 1998). For stimulation of topo-I activity with PDH45, both proteins were pre-incubated on ice for 5 min prior to the topoisomerase reaction.

UV-mediated cross-linking of ATP

Affinity labelling of purified PDH45 with [α-^{32}P]ATP was performed as previously described (Tuteja *et al.*, 1993). As a positive control, *E. coli* DNA polymerase I was also used.

Preparation of pea nuclear extract and chloroplast lysate

Pea nuclei were isolated from 7–8-day-old pea seedlings grown as previously described (Datta *et al.*, 1985), and nuclear extract was prepared by using the conditions described by Dignam *et al.* (1983). The chloroplast lysate was prepared as described (Tuteja *et al.*, 1996).

In vitro *translation*

For *in vitro* transcription and translation of the pea calnexin gene (Ehtesham *et al.*, 1999), commercially available TnT-coupled wheatgerm lysate from Promega (Madison, WI, USA), along with [^{35}S]methionine, was used as described (Ehtesham *et al.*, 1999). Before the above reaction, the wheatgerm lysate was pre-incubated for 10 min at 4°C with either pre-immune IgG or anti-pea helicase (anti-PDH45) IgG. The IgG from rabbit sera was purified by protein A sepharose using standard procedures.

Protein–protein interaction

The pea helicase–topo-I interaction was performed using two methods: far-Western analysis and protein hybridization. For far-Western analysis the pea helicase and BSA (negative control) proteins were transferred onto a nitrocellulose membrane after SDS-PAGE. The proteins on the membrane were denatured and renatured by incubating in 6 M guanidine-HCl (made up in HSM buffer: 25 mM Hepes–KOH pH 7.7, 25 mM NaCl, 5 mM $MgCl_2$) for 2 × 5 min at 4°C, followed by incubation for 6 × 10 min in a serial dilution (1 : 1) of denaturation buffer in HSM buffer (containing 1 mM DTT) at 4°C. The membrane was blocked in HSM buffer containing 1 mM DTT, 0.05% NP-40 and 5% milk for 60 min at 4°C, followed by washing the membrane twice in the same solution containing 1% milk. A second protein (pea topo-I, 500 ng ml^{-1}) in HSM-2 buffer (20 mM Hepes–KOH pH 7.7, 75 mM KCl, 0.1 mM EDTA, 2.5 mM $MgCl_2$, 1% milk, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, 0.05% NP-40) was overlaid on the membrane for 4 h at 4°C, followed by washing the membrane in HSM-2 buffer for 3 × 10 min at 4°C. The binding of topo-I was detected by conventional Western blot analysis using anti-topo-I antibodies. For protein hybridization, the pea helicase and BSA proteins were transferred on Hybond-C nylon membrane (Amersham, UK) after SDS-PAGE, and the proteins were denatured, renatured, blocked with milk, and washed on the membrane as described above. The membrane was hybridized with ^{32}P-labelled pea topo-I protein in HSM-2 buffer for 4 h at 4°C. The topo-I was radiolabelled using casein kinase 2 (CK2), and [γ-^{32}P]ATP followed by gel filtration on Sephadex G-50 to remove the unincorporated radioactivity. After hybridization the membrane was washed 3 × 15 min in HSM-2 buffer at 4°C and exposed to the film.

References

Aubourg, S., Kreis, M. and Lecharny, A. (1999) The DEAD box RNA helicase family in *Arabidopsis thaliana*. *Nucl. Acids Res.* **27**, 628–636.

Bharti, A.K., Olson, M.O.J., Kufe, D.W. and Rubin, E.H. (1996) Identification of nucleolin binding site in human topoisomerase I. *J. Biol. Chem.* **271**, 1993–1997.

Borowiec, J.A. (1996) DNA helicases. In *DNA Replication in Eukaryotic Cells* (De Pamphilis, M.L., ed.). Cold Spring Harbor: Cold Spring Harbor Laboratory Press, pp. 545–574.

Budd, M.E. and Campbell, J.L. (1995) A yeast gene required for DNA replication encodes a protein with homology to DNA helicases. *Proc. Natl Acad. Sci. USA*, **92**, 7642–7646.

Datta, N., Chen, Y.R. and Roux, S.J. (1985) Phytochrome and calcium stimulation of protein phosphorylation in isolated pea nuclei. *Biochem. Biophys. Res. Commun.* **128**, 1403–1408.

Deschavanne, P.J. and Harosh, I. (1993) The Rad3 protein from *Saccharomyces cerevisiae*; a DNA and DNA:RNA helicase with putative RNA helicase activity. *Mol. Microbiol.* **7**, 831–835.

Dignam, J.D., Lebowitz, R.M. and Poedii, G. (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from mammalian nuclei. *Nucl. Acids Res.* **11**, 1475–1459.

Ehtesham, N.E., Phan, T.-N., Gaikwad, A., Sopory, S.K. and Tuteja, N. (1999) Calnexin from *Pisum sativum*: cloning of the cDNA and chracterization of the encoded protein. *DNA Cell Biol.* **18**, 853–862.

Gorbalenya, A.E., Koonin, E.V., Donchenko, A.P. and Blinov, V.M. (1989) Two related superfamilies of putative helicases involved in replication, recombination, repair and expression of DNA and RNA genomes. *Nucl. Acids Res.* **17**, 4713–4730.

Haluska, P., Saleem, A., Edwards, T.K. and Rubin, E.H. (1998) Interaction between the N-terminus of human topoisomerase I and SV-40 large T antigen. *Nucl. Acids Res.* **26**, 1841–1847.

Harmon, F.G., DiGate, R.J. and Kowalczykowski, S.C. (1999) RecQ helicase and topoisomerase III comprise a novel DNA strand passage function; a conserved mechanism for control of DNA recombination. *Mol. Cell*, **3**, 611–620.

Ishimi, Y. (1997) DNA helicase activity is associated with an MCM4-6 and -7 protein complex. *J. Biol. Chem.* **272**, 24508–24513.

Lohman, T.M. and Bjornson, K.P. (1996) Mechanism of helicase-catalyzed DNA unwinding. *Ann. Rev. Biochem.* **65**, 169–214.

Luking, A., Stahl, U. and Schmidt, U. (1998) Protein family of RNA helicases. *Crit. Rev. Biochem. Mol. Biol.* **33**, 259–296.

Matson, S.W., Bean, D. and George, J.W. (1994) DNA helicases: enzymes with essential roles in all aspects of DNA metabolism. *Bioessays*, **16**, 13–21.

Okanami, M., Meshi, T. and Iwabuchi, M. (1998) Characterization of a DEAD box ATPase/RNA helicase protein of *Arabidopsis thaliana*. *Nucl. Acids Res.* **26**, 2638–2643.

Owttrim, G.W., Hofmann, S. and Kuhlemeier, C. (1991) Divergent genes for translation initiation factor eIF-4A are coordinately expressed in tobacco. *Nucl. Acids Res.* **19**, 5491–5496.

Owttrim, G.W., Mandel, T., Trachsel, H., Thomas, A.A.M. and Kuhlemeier, C. (1994) Characterization of the tobacco eIF-4A gene family. *Plant Mol. Biol.* **26**, 1747–1757.

Pause, A. and Sonenberg, N. (1992) Mutational analysis of a DEAD-box RNA helicase; the mammalian translation initiation factor eIF-4A. *EMBO J.* **11**, 2643–2654.

Pause, A., Methot, N., Svitkin, Y., Merrick, W.C. and Sonenberg, N. (1994) Dominant negative mutants or mammalian translation initiation factor eIF-4A define a critical role for eIF-4F in cap-dependent and cap-independent initiation translation. *EMBO J.* **13**, 1205–1215.

Puranam, K.L. and Blackshear, P.J. (1994) Cloning and characterization of RECQL, a potential human homologue of the *Escherichia coli* DNA helicase RecQ. *J. Biol. Chem.* **269**, 29838–29845.

Reddy, M.K., Nair, S. and Tewari, K.K. (1998) Cloning, expression and characterization of a gene which encodes a topoisomerase with positive supercoiling activity in pea. *Plant Mol. Biol.* **37**, 773–784.

Schaeffer, L., Roy, R., Humbert, S., Moncollin, V., Vermeuen, W., Hoeijmakers, J.H.J., Chambon, F. and Egly, J.M. (1993) DNA repair helicase: a component of BTF2 (THIIH) basic transcription factor. *Science*, **260**, 58–63.

Tuteja, N. (1997) Unraveling DNA helicases from plant cells. *Plant Mol. Biol.* **33**, 947–952.

Tuteja, N. (2000) Plant cell and viral helicases: essential enzymes for nucleic acid transactions. *Crit. Rev. Plant Sci.* in press.

Tuteja, N. and Phan, T.N. (1998) A chloroplast DNA helicase II from pea that prefers fork-like structures. *Plant Physiol.* **118**, 1029–1039.

Tuteja, N. and Tuteja, R. (1996) DNA helicases: the long unwinding road. *Nature Genet*, **13**, 11–12.

Tuteja, R. and Tuteja, N. (2000) Ku autoantigen: a multifunctional DNA binding protein. *Crit. Rev. Biochem. Mol. Biol.* **35**, 1–33.

Tuteja, N., Rahman, K., Tuteja, R. and Falaschi, A. (1991) DNA helicase IV from HeLa cells. *Nucl. Acids Res.* **19**, 3613–1618.

Tuteja, N., Rahman, K., Tuteja, R., Ochem. A., Skopac, D. and Falaschi, A. (1992) DNA helicase III from HeLa cells: an enzyme that acts preferentially on partially unwound DNA duplexes. *Nucl. Acids Res.* **20**, 5329–5337.

Tuteja, N., Rahman, K., Tuteja, R. and Falaschi, A. (1993) Human DNA helicase V, a novel DNA unwinding enzyme from HeLa cells. *Nucl. Acids Res.* **21**, 2323–2329.

Tuteja, N., Tuteja, R., Ochem. A. *et al.* (1994) Human DNA helicase II: a novel DNA unwinding enzyme identified as the Ku autoantigen. *EMBO J.* **13**, 4991–5001.

Tuteja, N., Phan, T.-N. and Tewari, K.K. (1996) Purification and characterization of a DNA helicase from pea chloroplast that translocates in the 3′ to 5′ direction. *Eur. J. Biochem.* **238**, 54–63.

Umezu, K., Nakayama, K. and Nakayama, H. (1990) *Escherichia coli* RecQ protein is a DNA helicase. *Proc. Natl Acad. Sci. USA*, **87**, 5363–5364.

Wang, J.C., Caron, P.R. and Kim, R.A. (1990) The role of DNA topoisomerases in recombination and genome stability. *Cell*, **62**, 403–406.

GenBank accession number Y17186.