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Proteins related to St. John's Wort p27^{SJ}, a suppressor of HIV-1 expression, are ubiquitous in plants

Tekla Perera [a,1,2], Anne Berna [b,1], Ken Scott [a], Christelle Lemaitre-Guillier [c], François Bernier [b,*]

[a] *School of Biological Sciences, University of Auckland, Auckland, New Zealand*
[b] *Institut de Biologie Moléculaire des Plantes du C.N.R.S., Université Louis Pasteur, Institut de Botanique, 28 Rue Goethe, 67083 Strasbourg Cedex, France*
[c] *Plateforme Protéomique de l'Esplanade, 15 Rue René Descartes, 67084 Strasbourg Cedex, France*

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Abstract

Proteins belonging to the family of DING proteins are ubiquitous in animals and several of them are associated with various diseases. Their presence in a few plant species has previously been reported and the St John's Wort DING protein was recently described as an inhibitor of HIV replication and transcription. However, data about DING protein occurrence in plants and their biochemical properties remain almost nonexistent. We describe methods for the purification of DING proteins from plants that may have general applicability since they are not dependent upon specific affinity ligands, contrary to previously described protocols. Cibacron Blue chromatography, sometimes preceded by an ion-exchange chromatographic step, is suitable for most plant extracts. DING proteins were purified from various species and cell types and their identity was confirmed immunologically and, in some cases, by N-terminal sequence analysis, indicating that they are ubiquitous in the plant kingdom. They are associated with the cell wall and sometimes secreted in the medium for *in vitro* grown cells. High-molecular-weight DING precursors were often observed. Internal peptides were also sequenced, as a prelude to gene cloning experiments.

Keywords: *Arabidopsis thaliana*; *Hypericum perforatum*; *Ipomoea batatas*; *Nicotiana tabacum*; *Solanum tuberosum*; HIV; Phosphate-binding protein; DING protein

1. Introduction

Proteins belonging to the so-called DING family of proteins (named for their conserved N-terminus, which starts with DINGGG-) are still poorly studied although they are ubiquitous in animals (Berna et al., 2002). They are related to a prokaryotic phosphate-binding protein superfamily that includes: soluble extra-cellular components of phosphate ABC transporters (PstS proteins), a few *Pseudomonas* alkaline phosphatases and a few other *Pseudomonas* proteins of unknown functions predicted from genome sequencing. These latter proteins are those showing the strongest resemblance with animal DING proteins, including the DINGGG- N-terminus, with aminoacid identities around 70–75% (Berna et al., 2007).

Phosphate binding has not been extensively studied in animal DING proteins. It has nevertheless been evidenced for two of them and crystallographic data indicate that they share prokaryotic phosphate-binding proteins typical three-dimensional structure (Morales et al., 2006; Scott and Wu, 2005). In addition, available sequence data demonstrates perfect conservation of the eight aminoacid residues forming the phosphate-binding pocket (Morales et al., 2006; Berna et al., 2007).

* Corresponding author. Tel.: +33 3 90 24 18 35; fax: +33 3 90 24 19 21.
E-mail address: francois.bernier@ibmp-ulp.u-strasbg.fr (F. Bernier).
[1] Contributed equally to this work.
[2] Present address: Virionyx, Auckland, New Zealand.

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DING proteins are not merely phosphate-binding proteins. Animal DING proteins have been independently purified by several teams. Some of them stimulate cell proliferation, and this activity appears to be independent of phosphate binding (Hain et al., 1996; Adams et al., 2002; Scott and Wu, 2005). Many DING proteins are associated with various diseases such as rheumatoid arthritis, cancer, infections, lithiasis and atherosclerosis (Hain et al., 1996; Mehta et al., 2001; Weebadda et al., 2001; Belenky et al., 2003; Kumar et al., 2004; Renault et al., 2006). In most of the studies, DING proteins have been identified by affinity chromatography. The list of ligands that have allowed DING protein purification comprises the plant metabolites, oxalate, genistein and cotinine, the anticoagulant hirudin, some polysaccharides and proteins, viral particles and selenium (Bush et al., 1998; Mehta et al., 2001; Weebadda et al., 2001; Belenky et al., 2003; Kumar et al., 2004; Darbinian-Sarkissian et al., 2006; Renault et al., 2006; Du et al., 2007). Because of this surprising diversity of ligands and diseases associated with DING proteins, a common and precise function has yet to be associated with them (for reviews, see Berna et al., 2002, 2007).

DING proteins also exist in fungi since they have been purified from the Ascomycetes, *Candida albicans* and two *Colletotrichum* species (Berna et al., 2002; Chen et al., 2007) and the Basidiomycete, *Ganoderma lucidum* (Du et al., 2007). These observations make it likely that DING proteins are ubiquitous in eukaryotes. Based on N-terminal sequences, the presence of DING proteins in some plant species has indeed been reported (Berna et al., 2002). A proteomics study of plant cell walls also yielded a French bean protein, which could not be related to the DING family at that time (Robertson et al., 1997). Recently, a DING protein from St. John's Wort (*Hypericum perforatum*) was identified through its ability to suppress expression of the HIV-1 genome. This protein, named $p27^{SJ}$, acts by interacting with the transcriptional activators C/EBPβ and Tat thus preventing their nuclear localization (Darbinian-Sarkissian et al., 2006).

Quite oddly, genes and mRNAs for DING proteins are almost totally lacking from databases even though their products are ubiquitous (Berna et al., 2007). This lack of molecular genetics data have considerably hampered the work toward understanding DING protein functions. The only extensive eukaryotic sequence information has thus come so far from direct protein sequencing of two DING proteins, one from monkey renal cells and human urine and the other from human plasma (Kumar et al., 2004; Morales et al., 2006).

Much more biochemical characterization will thus be required to understand DING protein properties and functions. Because they are widespread in eukaryotes, an especially intriguing question is whether all DING proteins share the ability of $p27^{SJ}$ to suppress expression from HIV, or other viruses. Progress in this direction has been slowed down by the lack of a reliable and universal purification protocol: basically, all DING proteins have so far been purified by affinity chromatography, and each one with a different ligand (see above).

In this paper, we describe purification protocols that do not rely on binding to a specific ligand and which should be of general use for plant DING proteins. In addition, we provide evidence for the widespread expression of DING proteins in plants, by purification of the proteins from a range of species and tissues. Biochemical characterization of these plant DING proteins allows interesting parallels to be drawn with animal DING proteins. In addition, sequence comparisons evidence a very strong conservation between $p27^{SJ}$ and other plant DING proteins as well as between some plant and human proteins.

2. Results and discussion

2.1. Identification of a tobacco protein with DING N-terminus

As part of our strategy to study *A. thaliana* germin-like proteins, one of them, named AtGER3, was overexpressed in transgenic tobacco plants (Membré et al., 2000; Bernier and Berna, 2001). AtGER3 is purified from *Arabidopsis* as homo-oligomers but some very stable hetero-oligomers were obtained in transgenic tobacco plants. The biochemical basis for this hetero-oligomerization is unknown, but they contained a 40 kDa subunit in addition to the expected 23 kDa subunit (not shown). The N-terminus of this protein was determined as DINGGGATLPQKLYQTAGVLTARF–. This sequence does not correspond to any part of germin-like proteins and matched only one of the known or predicted plant proteins: 15 of its 20 first residues are identical to those of a French bean cell wall protein for which the N-terminus is DVNGGGHTLPQPLYQTTVVL– (Robertson et al., 1997). It also differed at only three residues from N-termini of human DING proteins (Berna et al., 2002, 2007).

2.2. Purification of DING proteins from various plant species

To date, all purification procedures for DING proteins have relied on an affinity-chromatography step. Since very diverse ligands were used (see Introduction), it seemed unlikely that any of these protocols could be of general application. Indeed, although good purification of human DING proteins was obtained with a single hirudin-agarose affinity column step (Bush et al., 1998; Adams et al., 2002), preliminary experiments indicated that plant DING proteins did not bind to this matrix (not shown). We thus set out to develop a purification scheme for plant DING proteins. Since plant and human DING proteins share a common N-terminus, the human DING N-terminal peptide antibody could be used to screen plant material for cross-reacting proteins. An antiserum to the SDS–PAGE purified tobacco DING protein was also produced, and used in some experiments.

Sweetpotato (*Ipomoea batatas*) sprout extract was shown to contain a DING cross-reactive band at approximately 100 kDa (not shown) and was thus chosen as starting material for purification. Two successive DEAE-52 chromatography steps yielded a fraction enriched in a 40 kDa protein cross-reacting with the human DING antiserum (not shown). This fraction was further purified by Sephadex G-100 gel filtration. A single major protein peak eluted from the column, containing two major proteins, of approximate M_r of 70 and 40 kDa. The smaller protein reacted strongly with the human DING antiserum (Fig. 1a, lanes 1 and 2). The identity of the 70 kDa protein was not defined: 40 kDa DING proteins are probably processed from larger precursors (see below) in which the N-terminal epitope could be masked. As a final step, the pooled protein solution from the major peak was passed through a Cibacron Blue column. A single major protein peak was substantially pure, with a protein of 40 kDa in SDS–PAGE, which cross-reacted with the human DING antiserum (Fig. 1a, lanes 3 and 4). Similar results were obtained with both sweetpotato cultivars.

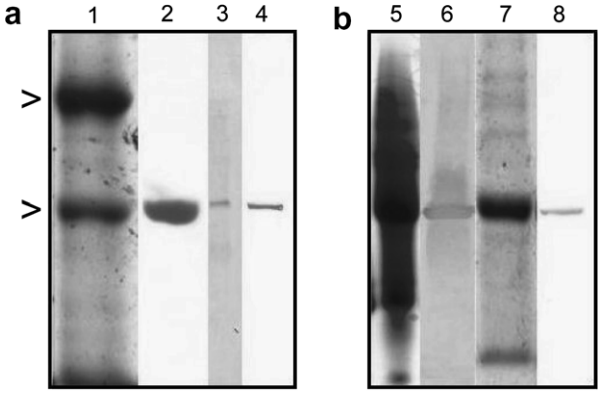

Fig. 1. Purification of DING protein from sweetpotato sprouts (a) and culture medium of *in vitro* grown *A. thaliana* cells (b). Protein extracts were prepared as described in Section 4. DING proteins were then purified by a succession of chromatographic steps and analyzed by SDS–PAGE followed by Coomassie staining (lanes 1, 3, 5, 7) or Western blotting with anti-N-terminal DING antibody (lanes 2, 4, 6, 8). Analysis of the single major protein peak (lanes 1 and 2) or of the low M_r fraction (lanes 5 and 6) eluted from the Sephadex G-100 column. These peaks were further purified on a Cibacron Blue column (lanes 3, 4, 7, 8). Upper arrow: 70 kDa; lower arrow: 40 kDa.

The identity of this 40 kDa protein was confirmed by N-terminal sequencing (DINGGGATLPQXLYXTP–). It appears that the sweetpotato DING protein is present as a protein of at least 100 kDa in freshly-extracted sprouts, but that, during the course of the purification, endogenous proteolysis converts it to a 40 kDa form.

Since human DING protein was purified from fibroblast-conditioned culture medium, and is therefore a secreted protein in human cells, we wanted to check whether plant tissue cultures also generated a DING protein. Western blotting of dialyzed, freeze-dried *A. thaliana* culture supernatant indicated diffuse DING cross-reacting proteins spread through much of the gel, with a stronger reaction at a position corresponding to 40 kDa (not shown). The culture supernatant was subjected to DEAE-52 chromatography, Sephadex G-100 gel filtration and Cibacron Blue affinity chromatography. Although not completely homogeneous, the 40 kDa DING band was by far the major component of the resulting fraction, and gave a strong response to the human DING antiserum in the Western blot (Fig. 1b). N-terminal sequencing confirmed the identity of the DING protein (DING-GGATLPQXL–).

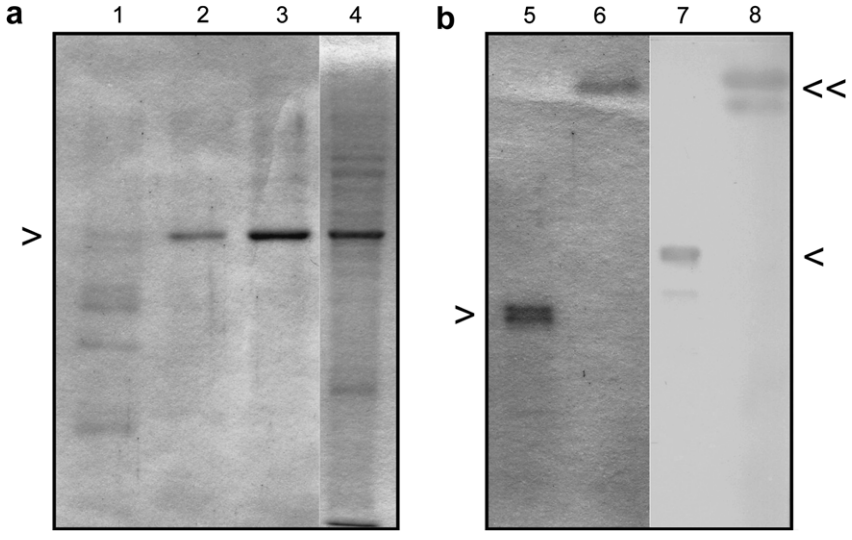

Fig. 2. Purification of DING protein from tobacco and potato using the simplified protocol. (a) SDS–PAGE analysis followed by Coomassie staining of DING proteins purified from intercellular washing fluid of wild-type tobacco leaves (lanes 1 and 2), potato sprouts (lane 3) or potato tuber skin (lane 4). Elution of the Cibacron Blue column by 200 mM (lanes 1, 3, 4) or 500 mM (lane 2) NaCl. (b) Oligomeric status of DING protein. Purified tobacco leaf DING protein was analyzed by SDS–PAGE followed by Coomassie staining (lanes 5 and 6) or by Western blotting with anti-DING antibody (lanes 7 and 8). Proteins samples were either boiled (lanes 5 and 7) or not heated (lanes 6 and 8) before loading on gel. Arrows: 40 kDa, double arrow: 200 kDa.

DING proteins were also analyzed during *A. thaliana* seed germination. All samples showed the presence of DING protein bands at about 40 kDa, as well as traces of larger proteins that reacted with the DING antiserum. There was only minor variation of the amount of DING protein over the period of 4–16 days after germination (not shown).

We have not further characterized the high-M_r sweetpotato or *Arabidopsis* proteins that cross-reacted with the human DING antiserum. However, we know that the antiserum will detect the DING antigen within the larger human synovial stimulatory protein (SSP), from which DING can be liberated by trypsin-like proteolysis (Hain et al., 1996; Adams et al., 2002). We surmise that plant DING proteins are also probably generated from a larger precursor protein, but addition of proteinase inhibitors to plant tissue extracts would be necessary to confirm this view. We have found no evidence for the further degradation of the 40 kDa DING proteins *in vivo*. However, lower MW bands were observed upon extended storage of the purified protein.

Based on its sequence, the suppressor of HIV expression purified from St John's Wort p27^{SJ} protein clearly belongs to the DING family of proteins although the authors did not identify it as such (see below). However, it represents the only described DING protein with a MW clearly different from 40 kDa. This could be explained by the fact that the coagulation proteinase, factor Xa, was used during p27^{SJ} purification. Indeed, examination of DING proteins sequences deduced from the few available DNA clones reveals the presence of a well-conserved consensus peptide sequence for factor Xa cleavage (Morales et al., 2006; our unpublished observations).

2.3. Use of a simplified purification procedure

A simplified purification protocol was devised that is especially suited for samples which are naturally rich in endogenous DING protein. In such cases, passage through a Cibacron Blue column without any previous chromatographic steps is generally sufficient to provide almost pure protein. By this approach, DING proteins could be purified from tobacco leaves, potato sprouts and tuber skin (Fig. 2a) as well as from wheat and rhubarb leaves and banana peel (not shown). The NaCl concentration required for optimal recovery of DING protein varied according to starting material between 200 and 500 mM.

Fully-denatured DING protein from all plant sources consistently ran as a 40 kDa band. However, when samples were not boiled before SDS–PAGE, DING proteins migrated at around 200 kDa as a doublet. The same phenomenon was previously observed with turkey LFPBP-40, another member of the DING family of proteins (Weebadda et al., 2001). The presence of two isoforms was also sometimes noted with fully-denatured samples (Fig. 2b). N-terminal sequencing of the 40 kDa band from a potato extract confirmed its identity (Table 1).

Table 1
Comparison of N-terminal and internal peptides of St. John's Wort p27^{SJ} (Darbinian-Sarkissian et al., 2006), potato DING protein (pot), human and monkey crystal adhesion inhibitor (CAI; Kumar et al., 2004), and human phosphate-binding protein (HPBP; Morales et al., 2006)

p27^{SJ}	DINGGGATLPQaLYQTSGVLTAGFAPYI
pot	DINGGGATLPQkLYQTSGVLTArFAPYI
CAI	DINGGGATLPQpLYQTSGVLTAGFAPYI
HPBP	DINGGGATLPQkLYlTpdVLTAGFAPYI
p27^{SJ}	aAFLnNDYtkFqaGv
pot	aAFLtNDYtk
CAI	lAFLnNDYsqFGTGT
HPBP	iAFLeNkYnqFGTdT
p27^{SJ}	LsATELSTYAsaK
pot	LTATELSTYAtnK
CAI	LTsTELSTYAstK
HPBP	LTATELaTYAadK
p27^{SJ}	LIQVPSVgTaVAIPFNK
pot	LIQVPSVATSVAIPF
CAI	LIQVPSVATSVAIPFNK
HPBP	LIQVPSVATSVAIPFrK
p27^{SJ}	taAVDLSVsELCGVFSGRITDWSGis
pot	aNAVDLSVsELCGVFSGRITDWSGly
CAI	tNAVDLSVdqLCGVFSG-ITtWdqlp
HPBP	aNAVDLSVkELCGVFSGRIaDWSGit
p27^{SJ}	GRTGaItVVYRsESSGTTELFTR
pot	GRTGpItVVYRsESSGTTELFTR
CAI	GRTGnIvVVYRnEa
HPBP	GRsGpIqVVYRaESSGTTELFTR
p27^{SJ}	tTYm-SPDfAAPTLAGLDDATK
pot	ITYpySPvyAAsTLAGLDDATK
CAI	ITYm-SPDyAAPTLAGL
HPBP	ITYi-SPDfAAPTLAGLDDATK
pot	HfGdtnNtqdAItANRFVPLPdNWK
CAI	HYGaSAlntnAIkANRFVPLPtaWK
HPBP	HYGtSANdnaAIeANaFVPLPsNWK
pot	AtITdNFVTASSALSIGkTNVCNGiGRgp
CAI	AAITsNFVTAtSAL
HPBP	AAvrasFlTASnALSIGnTNVCNGkGRpq

Aminoacid shown in lower case letters are those found at a specific position in only one of the sequences or those that correspond to residues in highly variable positions. *positions corresponding to residues involved in phosphate binding in bacterial PstS proteins.

2.4. Processing of DING proteins and interaction with cell surface

The probable synthesis of DING protein as a high M_r precursor was confirmed by studying accumulation of the protein from cultured tobacco cells (Fig. 3). In this system, part of the DING protein is weakly bound to the cell surface, whereas some is secreted into the medium, as was observed with cultured *Arabidopsis* cells (see above), and with animal DING proteins (Hain et al., 1996; Mehta

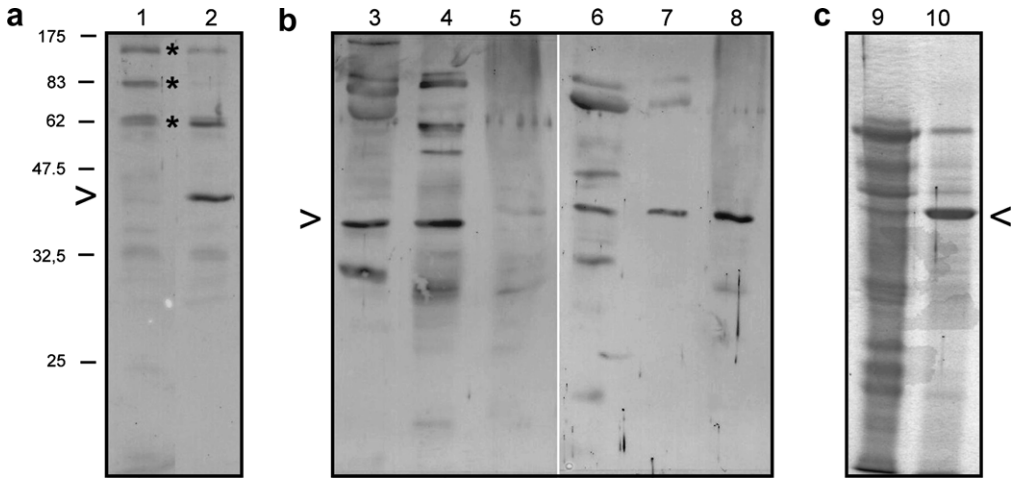

Fig. 3. Accumulation of DING protein from *in vitro* grown tobacco cells. (a) Cells were grown *in vitro* and proteins were washed from the cell surface with buffer (see Section 4) after 2 days (lane 1) or 3 days of culture (lane 2). Proteins were then separated on SDS–PAGE and reacted with anti-tobacco DING antibody. Positions of MW markers are indicated on the left. Estimated MW of the proteins marked by asterisks are (from top to bottom): 120, 83 and 62 kDa. (b) Proteins from *in vitro* grown tobacco cells were analyzed by Western blotting with an anti-tobacco DING antibody after 3 days (lanes 3–5) or 10 days of culture (lanes 6–8). At 10 days, cell division had ceased and cells were forming clumps. lanes 3 and 6: culture medium, lanes 4 and 7: surface proteins obtained by a buffer wash, lanes 5 and 8: surface proteins obtained by an additional wash with calcium (see Section 4). (c) SDS–PAGE analysis followed by Coomassie staining of total proteins from the culture medium (lane 9) and cell surface proteins obtained by a wash with water (lane 10) of actively growing cells. Arrows: 40 kDa.

et al., 2001; Weebadda et al., 2001; Adams et al., 2002; Belenky et al., 2003; Kumar et al., 2004). A gentle wash of the tobacco cells readily removed DING protein from their surface, as revealed by Western blot analysis, using the anti-tobacco DING antiserum (Figs. 3a and b). Cells that had been growing for two days displayed a series of high M_r DING-reactive proteins (62, 83 and 120 kDa), that were progressively replaced by a 40 kDa band starting from the third day of culture (Fig. 3a). We surmise that these bands represent successive stages in the extracellular proteolytic processing of a DING precursor protein. This could be self-processing since we have observed specific proteolysis of the purified protein (see Section 2.2).

Synthesis of DING proteins as larger precursors seems to be the rule in eukaryotes as high MW bands cross-reacting with DING protein antibodies have been observed in several instances (Hain et al., 1996; Adams et al., 2002; E. Chabrière: personal communication). On the contrary, no high MW precursors are known for prokaryotic DING-related proteins and none is predicted from any of the numerous gene sequences available for those (Berna et al., 2007).

To determine whether part of the DING proteins adhere more tightly to the cell surface, a wash with $CaCl_2$ was added. In actively-growing cells, this failed to yield additional DING protein (Fig. 3b). However, when cells had stopped dividing, $CaCl_2$ was required to extract the major part of DING protein, indicating a variable mode of association with the cell surface according to physiological conditions (Fig. 3b). A DING protein was also found to be tightly bound to French bean cell wall since successive washes with $CaCl_2$ and CDTA were required to extract it (Robertson et al., 1997).

In cultured tobacco cells, DING protein is synthesized in relatively large amounts: a gentle wash with buffer or even water will often yield a fraction highly enriched in DING proteins (Fig. 3c). Yield varied depending on growth conditions but, in some experiments, as much as one microgram of DING protein was purified from 60 mg (fresh weight) of cells. Two hypotheses might explain why a mild wash releases some of the wall-associated protein: first, since DING proteins are abundant, there might be an equilibrium between medium and cell surface DING protein. Second, it is possible that salts and sugars from the culture medium stabilize the wall itself and that their absence weakens the wall structure resulting in the release of some of its constituents, including DING protein.

It is quite odd that a protein of this abundance has been missed in all genomic and proteomic approaches except one (Robertson et al., 1997). However, the same is true for HPBP, an abundant human plasma protein which was only recently identified (Morales et al., 2006).

2.5. Internal peptide sequencing

Purified potato DING protein was subjected to tryptic digestion and resulting peptides were subjected to *de novo* sequencing (Table 1). Added to the N-terminal sequencing (Berna et al., 2002), 190 aminoacids are now known for this protein, a little bit over half of the number expected for a 40 kDa protein. Table 1 also shows a comparison of these peptides with corresponding parts of three other biochemically characterized eukaryotic DING proteins: St. John's Wort $p27^{SJ}$, monkey and human crystal adhesion inhibitor (CAI) and human phosphate-binding protein from plasma (HPBP).

CAI is a 39 kDa glycoprotein secreted from cultured monkey renal cells that has been identified by virtue of its ability to bind calcium oxalate and thus potentially prevent development of renal stones. It was also found in the cytosol and plasma membrane of cells from various rat organs as well as in normal human urine. Extensive protein sequencing work has yielded about 70% of the aminoacid residues (Kumar et al., 2004).

HPBP is also a member of the DING family and is the first plasma protein shown to bind phosphate ions. It has been crystallized: its structure almost perfectly matches the structure of bacterial PstS proteins, soluble extra-cellular proteins that participate in phosphate uptake together with transmembranar ABC transporters. The complete HPBP sequence was deduced from crystal data and confirmed by direct protein sequencing (Morales et al., 2006; Renault et al., 2006).

Pairwise comparisons between peptide sets from these four proteins are possible on a number of aminoacids ranging from 136 to 190. Conclusions can thus be drawn about overall similarities of these proteins, especially since these peptides are scattered over the entire sequences. Aminoacid identities range from 73% to 87%, not taking into account conservative changes and possible sequencing misinterpretations. Not surprisingly, the two plant proteins, $p27^{SJ}$ and potato DING protein, are the most closely related. However, it is interesting to point out that the two human proteins, CAI and HPBP, are more closely related to plant DING proteins (pairwise identities between 77% and 83%) than they are to each other (73% aminoacid identity). This clearly indicates that DING proteins are very well conserved between different eukaryotic kingdoms and that there are at least two different members of the DING family of proteins in human, as there probably are in plants (our unpublished observations).

3. Conclusion

DING proteins have first been identified in animals and more recently in fungi. In this paper, we have shown that they also exist in many plant species from various families, indicating they are probably ubiquitous in eukaryotes. All DING proteins are secreted glycoproteins, based on the presence of a well conserved putative glycosylation sequence (Morales et al., 2006), sometimes also found in the cytosol, and are synthesized as high MW precursors eventually processed down to 40 kDa proteins starting with DINGGG. They probably all possess the ability to bind phosphate. This is made possible by the conservation of eight aminoacid residues and a three-dimensional structure found in prokaryotic PstS proteins, extracellular proteins participating in solute uptake with ABC transmembrane transporters. DING proteins thus represent the first eukaryotic proteins related to these prokaryotic solute binding proteins (SBPs). However, there is no evidence that eukaryotic phosphate transporters interact with a phosphate-binding protein (Werner and Kinne, 2001), and evidence is accumulating in favour of a much wider spectrum of functions than phosphate transport. More biochemical studies will be required to clarify this point.

As a family, DING proteins present an astonishing array of binding abilities (see Section 1). The mechanism for this remains to be established. It is especially striking that plant metabolites such as genistein and cotinine have been identified as ligands for animal DING proteins. Interestingly, a *Pseudomonas* DING protein also displayed some affinity for cotinine (Scott and Wu, 2005). This raises the possibility that plant DING proteins might represent a novel type of cell-surface receptor and thus contribute to signal transduction pathways involved in the control of cell proliferation and cellular recognition, in agreement with proposed functions for animal DING proteins. Structure–function relationships between DING proteins and well-characterized prokaryotic SBPs suggest possible mechanisms for these functions. These proteins display the so-called ''Venus flytrap'' structure which allows important conformational changes upon ligand binding and also characterizes other proteins involved in signalling (Felder et al., 1999).

Now that general purification protocols have been devised, it will be possible to address more specifically the questions of the physiological functions of DING proteins. Another question deals with the effect of these proteins on viral genome expression. Since, we have shown that proteins related to St. John's Wort $p27^{SJ}$ are ubiquitous in plants, including edible ones, as well as in all eukaryotes, a major issue will be to determine whether all DING proteins affect transcription from viral genomes, whether this is specific for some viruses and how this is related to their cellular functions.

4. Experimental

4.1. Plant material

Sprout tissue from potato (*Solanum tuberosum*) or sweetpotato (*Ipomoea batatas*, Rekamaroa and Taputini cvs) was obtained by letting tubers or roots germinate in mild, damp, dark conditions.

Arabidopsis thaliana cv Columbia cells from callus tissue were grown in Murashige and Skoog salt and vitamin mixture (Murashige and Skoog, 1962), containing 3% sucrose and 2 mg/l 2,4-dichlorophenoxyacetic acid (pH 5.8). Centrifugation at 10,000 rpm for 1 h at 4 °C yielded conditioned suspension culture supernatant.

Nicotiana tabacum cv Xanthi cells were cultured in Gamborg B5 medium (Gamborg et al., 1968) supplemented with 1 mg/l naphthalene acetic acid, 736 mg/l $CaCl_2$, 92 mg/l $CaHPO_4{\cdot}12\ H_2O$, 250 mg/l NH_4NO_3, 2% sucrose (pH 5.7).

Leaves from tobacco (*Nicotiana tabacum*), wheat (*Triticum aestivum*) and rhubarb (*Rheum rhabarbarum*) were obtained from plants grown in soil.

4.2. Antibodies

The human DING antibody was previously raised in rabbits against a synthetic conjugated N-terminal DING peptide (Adams et al., 2002). The plant DING antibody was raised against tobacco 40 kDa DING protein band extracted from a SDS–PAGE gel. Western blotting with the anti-human DING was carried out as previously described, after separating proteins by SDS–PAGE (Berna, 1995; Adams et al., 2002). Specificity of the antibodies was tested by checking the absence of reaction of preimmune sera with crude extracts and purified DING protein.

4.3. Purification of DING protein from sprouts or culture medium

Purification was performed from 75 g of *I. batatas* sprout tissue or from ca 1000 *A. thaliana* cv Columbia washed seeds, ground in 100 ml of 30 mM Tris–HCl buffer, pH 7.5, and centrifuged at 10,000 rpm for 60 min at 4 °C. This supernatant or 100 ml of the conditioned suspension culture supernatant were used for further purification.

The first fractionation step was a DEAE-52 cellulose ion-exchange chromatography column. The matrix (20 g) was equilibrated with 30 mM Tris–HCl buffer, pH 7.5. The extract was applied, washed with 75 ml of the same buffer, and eluted with a linear NaCl gradient to 1.0 M. Pooled fractions identified by Western blotting with the human anti-DING antiserum were combined, and dialysed against 30 mM Tris–HCl buffer, pH 7.5, for rechromatography under the same conditions, with a shallower gradient to 0.4 M NaCl.

Protein fractions scoring positive with human anti-DING antiserum were pooled and dialysed against distilled water, freeze-dried and fractionated by Sephadex G-100 gel filtration on a column equilibrated in 30 mM Tris-HCl buffer pH 7.5. In last step, the pooled protein solution from the Sephadex column was passed through a 20 ml Cibacron Blue column, washed with 30 mM Tris–HCl buffer, pH 8.0, and eluted with 0.2 M NaSCN-30 mM Tris–HCl buffer, pH 8.0.

Gas-phase N-terminal aminoacid sequencing was carried out as previously described (Adams et al., 2002).

4.4. Preparation of samples and simplified purification protocol

The simplified protocol was performed on plant tissues or *in vitro* grown cells or conditioned culture medium. Plant tissue was ground in two volumes of 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.5, 100 mM CH_3COOK, 3 mM $(CH_3COO)_2Mg$. This extract or a liquid culture was filtered on nylon mesh. The filtrate was heated at 60 °C for 20 min and centrifuged at 17,000*g* for 10 min to obtain a soluble protein fraction. Material retained by the filter was first washed with either water or 100 mM 2-(*N*-morpholino) ethanesulfonic acid (MES) pH 5.6 and then extracted with 1 M $CaCl_2$, followed by extensive dialysis, to obtain a fraction of proteins tightly bound to the wall. Alternatively, leaves were vacuum infiltrated with 10 mM Tris–HCl, pH 7.5 to obtain an intercellular washing fluid. Soluble fractions concentrated by freeze-drying were then directly passed on a Cibacron Blue column in 50 mM Tris–HCl, pH 7.5, 50 mM NaCl. Elution was carried successively with 200 mM and 500 mM NaCl in 50 mM Tris–HCl, pH 7.5.

4.5. Internal peptide sequencing

The in-gel digestion procedure was carried out as described (Rabilloud et al., 2001). Briefly, gel spots were alternatively washed with 25 mM NH_4HCO_3 and acetonitrile (ACN) (3 cycles) before reduction (10 mM DTT/25 mM NH_4HCO_3 buffer at 56 °C for 1 h) and alkylation (25 mM iodoacetamide/25 mM NH_4HCO_3 buffer for 1 h, room temperature, in darkness). Afterwards, gel spots were submitted to another three cycles of washes. Following dehydration, the gel pieces were rehydrated by three volumes of trypsin (Promega, V5111), 12.5 ng/μl/in 25 mM NH_4HCO_3 buffer (freshly diluted) and incubated overnight at room temperature. Tryptic peptides were extracted form gel by shaking for 30 min in 5 μl of 35% H_2O/60% ACN/5% HCOOH.

The tryptic digest was analysed on nanoscale capillary liquid chromatography-tandem mass spectrometry (Cap-LC, Micromass, Altrincham, UK/Q-TOF II, Micromass, Altrincham, UK). Chromatographic separations were performed on a reversed-phase capillary column (Pepmap C18, 75 μm i.d., 15 cm length (LC Packings, Amsterdam, the Netherlands)) under a 200 nl/min flow rate. Fragmentation was performed using argon as the collision gas and with a collision energy profile optimised for various mass ranges of ion precursors. Four ion precursors were allowed to be fragmented at a time. Mass data collected during a nanoLC-MS/MS analysis were processed and then submitted to *de novo* sequencing.

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