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Structural characterization of dioicin 1 from *Phytolacca dioica* L. gains novel insights into phylogenetic relationships of Phytolaccaceae type 1 RIPs

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ABSTRACT

Ribosome-inactivating proteins are plant cytotoxic enzymes, also present in fungi, algae and bacteria, mainly known for their ability to inhibit protein synthesis. We previously purified and structurally characterized three type 1 RIPs (PD-S_{1-3}) from *Phytolacca dioica* seeds and four type 1 RIPs (PD-L_{1-4}) from adult plant leaves. Two additional RIPs, named dioicin 1 and dioicin 2, were isolated from leaves of young plants and developing leaves of adult plants. The evidence that *P. dioica* synthesizes and accumulates these RIPs isoforms suggests that these proteins have been conserved during evolution. Though several aspects of *P. dioica* type 1 RIP characterization have been studied, some important questions remain to be answered especially with respect to Phytolaccaceae RIP evolution. One of the major problems encountered in approaching RIPs phylogeny concerns the availability of their sequences.

In this study, we report the characterization of biological and structural properties of dioicin 1, including the determination of its primary structure by using a combined approach based on Edman degradation, *de novo* sequencing by ESI-Q-TOF-MS/MS and peptide mapping by MALDI-TOF MS. Knowledge of dioicin 1 primary structure provide us a mean to deepen Phytolaccaceae's RIPs phylogeny. We speculate that both dioicins 1 and 2 share common ancestors with PAP-II and PAP icos-II and that dioicin 1 is not closely related to other members of this clade, thus shedding lights on evolutionary relationships among type 1 RIPs from Phytolaccaceae.

1. Introduction

Ribosome-inactivating proteins (RIPs; rRNA N-β-glycosidases; EC 3.2.2.22) are cytotoxic enzymes which selectively remove a single adenine from the conserved sarcin/ricin domain (S/R domain) of the largest rRNA [1], thus inhibiting protein synthesis. RIPs also remove adenine from DNA and other polynucleotides with variable efficiency, and consequently the denomination of adenine polynucleotide glycosylases (APGs) was proposed [2].

RIPs are classified into groups based on their structures: type 1 RIPs are single-chain, basic proteins with molecular weights of ~30 kDa and type 2 RIPs have an A chain with N-glycosidase activity, linked to a lectin-like B chain by a disulphide bond. In addition, some non-canonical type 1 RIPs, previously reported as type 3 RIPs, are synthesized as pro-RIPs and require proteolytic cleavages to form active proteins [3].

RIPs are present in several plants and have also been found in fungi, algae and bacteria [4]. Although the biological role of RIPs has not been completely unveiled, it was proposed that they may be involved in plant defence [1,5], since their expression is related to the plant response to a variety of biotic stress, such as viruses, microorganisms, insects and fungi [3,5]. The interest for these enzymes is also due to their potential use for the treatment of several important human diseases such as cancer [6] or in plant biotechnology applications to obtain resistance against pathogens [7–9].

Several plants commonly express various RIP isoforms encoded by multi-gene families [10]. The highest number of RIPs was found in Caryophyllaceae, Sambucaceae, Cucurbitaceae, Euphorbiaceae, and Poaceae [4,11]. In addition, several isoforms of type 1 RIPs were purified from Phytolaccaceae [4,12,13] and in particular from *Phytolacca dioica* L., a woody plant introduced in Italy from South America.

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We previously purified and structurally characterized three type 1 RIPs (PD-S_{1-3}) from *P. dioica* seeds [12] and four type 1 RIPs (PD-L_{1-4}) from adult plants leaves [14]. Furthermore, two type 1 RIPs were isolated from leaves of young plants (up to three years) and developing leaves of adult plants, named dioicin 1 and dioicin 2 [15]. Dioicin 2 was found to be always present, whereas dioicin 1 was expressed up to day seventeen [12,15]. The evidence that *P. dioica* synthesizes and accumulates these RIPs isoforms suggests that these proteins have been conserved during evolution due to their beneficial effects for plant fitness.

Though several aspects of the *P. dioica* type 1 RIP characterization have been studied [11,12,16], some important questions remain to be answered especially with respect to Phytolaccaceae RIP evolution. One of the major problems encountered in approaching RIPs phylogeny concerns the availability of their sequences, either derived from protein or nucleotide sequencing.

In this study, we report the characterization of biological and structural properties of dioicin 1, including the determination of its primary structure, whose knowledge allowed us to deepen phylogenetic relationships among type 1 RIPs from Phytolaccaceae.

2. Materials and methods

2.1. Enzymes and chemicals

Endoproteinases Glu-C (V8 Protease) and trypsin (sequencing grade) were purchased from Sigma–Aldrich (Milan, Italy). HPLC-grade solvents and reagents were obtained from Carlo Erba (Milan, Italy). Cyanogen bromide was obtained from Fluka (Milan, Italy). For peptide separation by RP-HPLC, the C-4 (0.46 × 25 cm; 5 μm particle size) or C-18 columns (0.46 × 15 cm; 5 μm particle size) were obtained from Phenomenex (Castel Maggiore, BO, Italy) and Waters S.p.A (Milan, Italy), respectively. The following solvents were used: solvent A, 0.1% TFA; solvent B, acetonitrile containing 0.1% TFA.

2.2. Dioicin 1 purification and primary structure determination

Analytical methods for the purification and primary structure determination of dioicin 1 are reported in Supplementary material [15,17].

2.3. Mass spectrometry and de novo sequencing

The relative molecular masses (Mr) of native or reduced (see paragraph 2.4) dioicin 1 was determined on a Q-TOF Micro mass spectrometer (Waters, Milford, MA USA), as previously described [17]. The sample was solubilised in acetonitrile: 0.1% formic acid in water (50:50; v:v) at a concentration of 1 pmol/μL and infused into the system at a flow rate of 5 μL/min. The acquisition and deconvolution of data were performed by using the Mass Lynx 4.1 software (Waters). Tandem MS/MS data on the tryptic peptide mixture were acquired on the Q-TOF mass spectrometer in the data directed analysis (DDA) MS/MS mode following separation on the CapLC chromatographic system as described elsewhere [18]. Mass spectra of peptides obtained by chemical or enzymatic digestions, were analysed with a MALDI-TOF mass spectrometer (Waters Micromass Co., Manchester, UK) externally calibrated using a tryptic alcohol dehydrogenase digest (Waters) in reflectron mode [19,20]. Briefly, RP-HPLC eluted peptides were concentrated and analysed. Prior to the acquisition of spectra, 1 μL of each peptide solution was mixed with 1 μL of saturated α-cyano-4-hydroxycinnamic acid matrix solution [10 mg/mL in ethanol:water (1:1; v:v), containing 0.1% trifluoroacetic acid] and a droplet of the resulting mixture (1 μL) was spotted on the instrument's sample target and analysed as previously reported [18].

2.4. Reduction of methionine sulfoxide

To revert any methionyl sulfoxide back to methionyl residues, native dioicin 1 (30 μg) was treated with 2 M DTT in 0.1 M Tris•Cl, 6 M guanidine•Cl, 5 mM EDTA, pH 8.8, at 37 °C. After 12 h treatment, protein was quickly desalted by RP-HPLC as reported in paragraph 1.1 of the Supplementary material and subjected to ESI Q-TOF mass spectrometry analysis as reported above.

2.5. Homology and bioinformatic studies

Amino acid sequences of RIPs from Phytolaccaceae were obtained from Uniprot database (http://www.uniprot.org/). The accession numbers and lengths of non-redundant amino acid sequences of RIPs from Phytolaccaceae were reported in the paragraph 1.6. of Supplementary material.

Physical and chemical parameters for type 1 RIPs from *P. dioica* were obtained using the Protparam tool (http://web.expasy.org/protparam/). Sequence alignment was performed using the ClustalW tool included in the MEGA6 suite [21]. The similarity/identity matrix was obtained using the BOXSHADE tool (http://mobyle.pasteur.fr/cgi-bin/MobylePortal/portal.py?form=boxshade).

The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model [22]. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analysed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was reported next to the branches in Fig. 3 [23]. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA6 [21].

2.6. Biological activity assay

Adenine polynucleotide glycosylase (APG) activity was determined for dioicin 1, dioicin 2 and PD-L4 on hsDNA substrate as described elsewhere [24]. Values are means of quadruplicate analyses ±SD.

2.7. Circular dichroism spectroscopy

CD spectra were obtained at room temperature on a Jasco J-815 dichrograph (Jasco Europe, Cremella, Lecco, Italy) [25,26]. For spectra in the near- and far-UV, measures were performed on a protein solution at a concentration of 0.05 mg/mL in 100 mM Na-phosphate, pH 7.2, using a 0.1 cm path-length quartz cuvette [27].

3. Results and discussion

3.1. Dioicin 1 structural characterization

Dioicin 1 was purified from fully expanded leaves of *P. dioica* (Fig. 1A) and subjected to enzymatic and chemical hydrolysis for primary structure determination as previously published [15,17].

The primary structure of dioicin 1 was determined by a combined strategy based on automated Edman degradation and mass spectrometry as previously reported [17,28]. At first, we determined the N-terminal sequence of pyridylethylated dioicin 1. Then, pyridylethylated peptides following chemical fragmentation with CNBr was separated by RP-HPLC (Fig. 1B) and sequenced by Edman degradation (Table 1). CNBr sequence were used to perform an homology search with the BLAST software. The highest identity

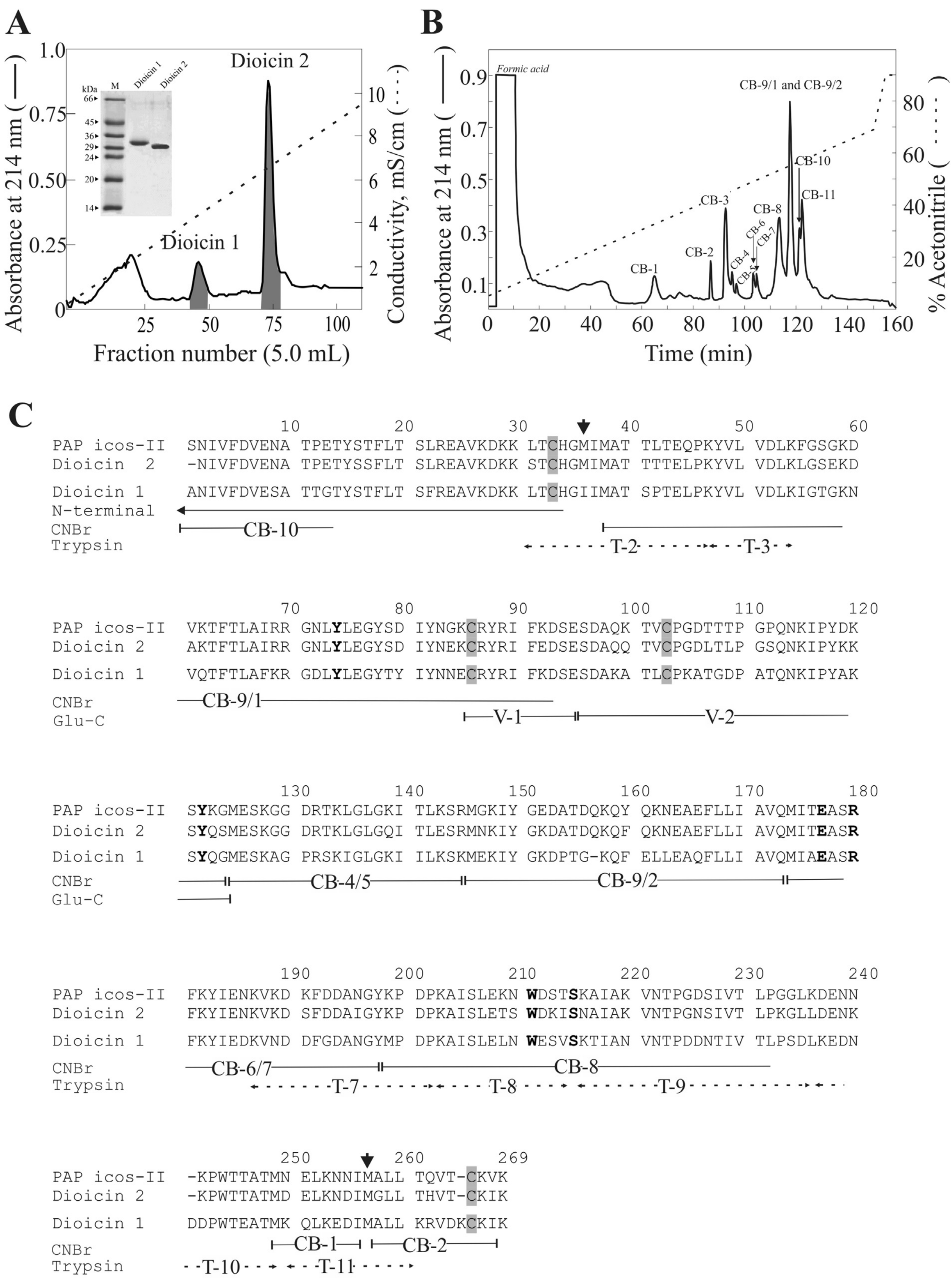
A
Absorbance at 214 nm
Fraction number (5.0 mL)
Conductivity, mS/cm
Dioicin 1
Dioicin 2
kDa
M
66
45
36
29
24
20
14
B
Absorbance at 214 nm
Time (min)
% Acetonitrile
Formic acid
CB-9/1 and CB-9/2
CB-1
CB-2
CB-3
CB-4
CB-5
CB-6
CB-7
CB-8
CB-10
CB-11
C
PAP icos-II SNIVFDVENA TPETYSTFLT SLREAVKDKK LTCHGMIMAT TLTEQPKYVL VDLKFGSGKD
Dioicin 2 -NIVFDVENA TPETYSSFLT SLREAVKDKK STCHGMIMAT TTTELPKYVL VDLKLGSEKD
Dioicin 1 ANIVFDVESA TTGTYSTFLT SFREAVKDKK LTCHGIIMAT SPTELPKYVL VDLKIGTGKN
N-terminal
CNBr CB-10
Trypsin T-2 T-3
PAP icos-II VKTFTLAIRR GNLYLEGYSD IYNGKCRYRI FKDSESDAQK TVCPGDTTTP GPQNKIPYDK
Dioicin 2 AKTFTLAIRR GNLYLEGYSD IYNEKCRYRI FEDSESDAQQ TVCPGDLTLP GSQNKIPYKK
Dioicin 1 VQTFTLAFKR GDLYLEGYTY IYNNECRYRI FKDSESDAKA TLCPKATGDP ATQNKIPYAK
CNBr CB-9/1
Glu-C V-1 V-2
PAP icos-II SYKGMESKGG DRTKLGLGKI TLKSRMGKIY GEDATDQKQY QKNEAEFLLI AVQMITEASR
Dioicin 2 SYQSMESKGG DRTKLGLGQI TLESRMNKIY GKDATDQKQF QKNEAEFLLI AVQMITEASR
Dioicin 1 SYQGMESKAG PRSKIGLGKI ILKSKMEKIY GKDPTG-KQF ELLEAQFLLI AVQMIAEASR
CNBr CB-4/5 CB-9/2
Glu-C
PAP icos-II FKYIENKVKD KFDDANGYKP DPKAISLEKN WDSTSKAIAK VNTPGDSIVT LPGGLKDENN
Dioicin 2 FKYIENKVKD SFDDAIGYKP DPKAISLETS WDKISNAIAK VNTPGNSIVT LPKGLLDENK
Dioicin 1 FKYIEDKVND DFGDANGYMP DPKAISLELN WESVSKTIAN VNTPDDNTIV TLPSDLKEDN
CNBr CB-6/7 CB-8
Trypsin T-7 T-8 T-9
PAP icos-II -KPWTTATMN ELKNNIMALL TQVT-CKVK
Dioicin 2 -KPWTTATMD ELKNDIMGLL THVT-CKIK
Dioicin 1 DDPWTEATMK QLKEDIMALL KRVDKCKIK
CNBr CB-1 CB-2
Trypsin T-10 T-11

Fig. 1. A) Dioicins purification from developing leaves of *P. dioica*. In the elution profile from the CM-52 chromatography, fractions containing dioicin 1 and dioicin 2 are shaded. The SDS-PAGE analysis of the purified dioicins (3 μg) is reported in the inset. (M, protein markers). B) RP-HPLC separation of peptides obtained by chemical fragmentation of dioicin 1 with CNBr. C) amino acid sequence of dioicin 1. The overlapping peptides used for assembling the protein sequence are reported. Conserved residues of the active site are highlighted in bold. The cysteinyl residues and oxidized methionyl residues are highlighted in green and with arrows, respectively. Abbreviations: CB, cyanogen bromide; V, endoproteinase Glu-C and T, tryptic peptides. Dioicin 2 (AC: P85208) and PAP icos-II (AC: Q4A524) sequences are reported as reference sequence.

Table 1
CNBr (CB), Glu-C (V) and N-terminal sequence of *S*-modified protein used for dioicin 1 primary structure determination. Only the sequenced amino acid residues have been reported. Experimental relative molecular masses (Mr), reported as $[M+H]^+$, were obtained by MALDI-TOF MS. Theoretical Mr values have been calculated on full length peptides following completion of primary structure determination by considering average Mr for molecular weight values above 3 k.

Protein/peptide	Sequence[a]	Experimental M_r	Theoretical M_r	Position	Notes[b]
Dioicin 1	ANIVFDVESATTGTYSTFLTSFREAVKDKKLTCHG	–	–	1–35	*N*-terminal
CB-1	KQLKEDIM	956.65	956.54	249–256	
CB-2	ALLKRVDKCKIK	1519.82	1519.95	257–268	*C*-terminal
CB-3	KQLKEDIXAL LKRVDKCKIK	2476.29	2507.16	249–268	[c]
CB-4/CB-5	ESKAGPRSKIGLGKIILKSKM	2193.16	2193.34	126–146	
CB-6/CB-7	IAEASRFKYIEDKVNDDFGDANGYM	2820.16	2820.31	175–199	
CB-8	PDPKAISLELNWESVSKTIANVNTPDDNTIVTLP	5493.13	5494.94	200–233	→248
CB-9	*Sequence 1:* ATSPTELPKYVLVDLKIGTGKNVQTFTLAF KRGDLYLEGY TYIYNNECRY RIFKD	–	–	39–93	→125
	Sequence 2: EKIYGKDPTGXQFELLEAXFXLI			147–170	→174
CB-9/1	ATSPTELPKYVLVDLKIGTGKNVQTFTLAF KRGDLYLEGYTYIYNNECRYRIFKDS	9947.49	9946.32	39–94	→125
CB-9/2	EKIYGKDPTGKQFELLEAQFLLIAVQM	3060.60	3061.66	147–174	
V-1	CRYRIFKDSE	1421.76	1421.69	86–95	
V-2	SDAKATLCPK ATGDPATQNK IPYAKSYQGM	3214.96	3214.62	96–125	

[a] X, unidentified residues; methionine residues were considered as homoserine lactone (Hsl).
[b] Numbers following arrows indicate positions of full length fragments.
[c] X residue at position 256, assigned to Met by tandem MS, was found to be modified to homoserine ($\Delta = 30.87$ Da).

percentages were found with the sequences of PAP icos-II, isolated from *Phytolacca octandra* (AC: Q4A524) and dioicin 2 (AC: P85208; [15]) that were used as reference proteins. The alignment of the CNBr (CB)-peptides provided about 85% of dioicin 1 amino acid sequence. All CNBr peptides were analysed by MALDI-TOF MS (Table 1) mostly confirming the agreement of experimental with theoretical molecular weights values, with the only exception of CB-3 (see later). MALDI-TOF analysis of peptides CB-10 and CB-11 also revealed that they both contained high molecular weight forms corresponding to the un-fragmented protein, likely due to the presence of oxidized methionines [29].

Two co-eluting peptides, present with different amounts, were found within CB-9, whose sequences were not completely determined by Edman degradation. We therefore decided to further separate these peptides by RP-HPLC using nearly isocratic elution conditions. By this approach, two peptides (CB9-1 and CB9-2) were obtained that were subjected to Edman degradation. Beside confirming the previously obtained sequence (39–93), additional sequence information were obtained. In particular, an additional Ser residue was assigned at the C-terminal of CB9-1. In addition, the previously unidentified positions Lys158, Gln166 and Leu169 were determined on CB9-2. Due to the still incomplete sequence determination of the CB9-1 peptide, we decide to perform a sub-digestion with endoproteinase Glu-C. This strategy allowed to obtain the missing sequence at the CB9-1 C-terminal (Table 1).

In order to complete the dioicin 1 primary structure, we performed a tryptic hydrolysis on *S*-pyridylethylated dioicin 1 and the resulting peptides were directly analysed by LC–ESI/Q-TOF mass spectrometry by recording the MS/MS spectra on the three most intense mass peaks generated in each scan. The MS/MS data were processed automatically and *de novo* sequencing was performed by using the Biolynx application of MassLynx 4.0 software. All MS/MS spectra leading to protein identification were manually double checked to verify sequence assignments. Tryptic peptide sequences, obtained by tandem MS, along with the molecular masses of precursor ions and those of deduced sequences, are reported in Table 2. An amino acid sequence coverage of about 40% was obtained *de novo* sequencing. In particular, the sequencing by MS/MS of the T-2 peptide allowed the assignment of the sequence positions 36–38, not previously sequenced. In addition, T-9 and T-10 peptide sequence provided the missing region 234–248. Notably, T-11 revealed that position 256 was a methionyl residue. This information allowed to explain the missed identification of Met256 by CNBr fragmentation. Indeed, also considering the 30 Da difference of the experimental *versus* theoretical molecular mass values observed on CB-3 by MALDI-TOF MS, we hypothesize that following CNBr treatment, Met256 was modified to homoserine, without peptide bond cleavage. It has been previously reported that this phenomenon can be observed due to specific conformational restrictions of the peptide backbone [30,31].

The amino acid sequence[1] of dioicin 1, derived from the sequence data obtained from the native protein, the tryptic, Glu-C and CNBr peptides is reported in Fig. 1C along with the homologous sequences of dioicin 2 and PAP icos-II used for the alignment. As expected from the high similarity between dioicins 1 and 2 (75.1%), the comparison of their far UV circular dichroic spectra revealed that also a similar periodic secondary structure for the two proteins (Fig. 2A).

The calculated M*r* of purified dioicin 1 was 30035.40 (30031.45, considering the native protein with two disulphide bonds). The Mr values determined by ESI/Q-TOF mass spectrometry were 30047.11 ($\Delta = 0.3$ Da, in agreement with that previously reported for dioicin 1 [15]) and 30064.27 ($\Delta = 0.8$ Da; Fig. 2B), which differed of about +16 and + 32 Da, respectively from the calculated M*r* value of the native protein. Based on previous literature data [32–34] and considering the incomplete cleavages by CNBr at Met38 and Met256 (cfr. CB-3 of Table 1), we hypothesised the occurrence of oxidized methionyl residues [Δ molecular masses of +16 and + 32 Da for single (Met-sulphoxide) and double (Met-sulphone) oxidized methionyl residues, respectively]. This hypothesis was confirmed by treating dioicin 1 with 2 M DTT to reduce Met-sulphoxide to Met [35]. Following extensive reduction with DTT, a peak with a molecular mass of 30033.96 was clearly observed, in good agreement with the theoretical mass calculated for the reduced dioicin 1 ($\Delta = 1.4$ Da, Fig. 2C). The reversion to Met was not complete as oxidized forms of dioicin 1 (M*r* 30051.43 and 30066.74, $\Delta = 0.1$ Da and 0.8 Da), were still detected (Fig. 2C).

3.2. Physical and chemical parameters and enzymatic activity on hsDNA of dioicin 1

The physical and chemical parameters of dioicin 1 are reported in Table 3 in comparison with those of other type 1 RIPs purified from *P. dioica*. As observed for all the *P. dioica* RIPs, dioicin 1 is stable (II < 40) and thermostable (AI > 70), with four conserved cysteinyl

[1] Submitted to the UniProt Knowledgebase under the accession number P86144.

Table 2
Amino acid sequences of tryptic peptides from dioicin 1, obtained by tandem mass spectrometry. Sequence position, experimental masses of precursor ions, charge state and molecular weight of MS/MS sequences deduced from y series, together with mass accuracy, are reported.

Peptide	Sequence	Position	Precursor ion	Charge state	MW from *de novo* sequence	Δ(Da)
T-2[a]	LTCHGIIMATSPTELPK	31–47	1915.99	3	1915.89	−0.10
T-3	YVLVDLK	48–54	848.45	2	848.50	0.05
T-7	VNDDFGDANGYMPDPK	188–203	1753.73	2	1753.70	−0.03
T-8	AISLELNWESVSK	204–216	1474.76	2	1474.66	−0.10
T-9	TIANVNTPDDNTIVTLPSDLK	217–237	2240.15	2	2240.14	−0.01
T-10	EDNDDPWTEATMK	238–249	1550.56	2	1550.61	0.05
T-11	QLKEDIMALLK	250–260	1300.71	2	1300.74	0.03
T-12[a]	EDIMALLK	253–260	931.50	2	931.49	−0.01

[a] Ion signals corresponding to oxidized peptide forms (+16) were also detected within T-2 and T-12 spectra.

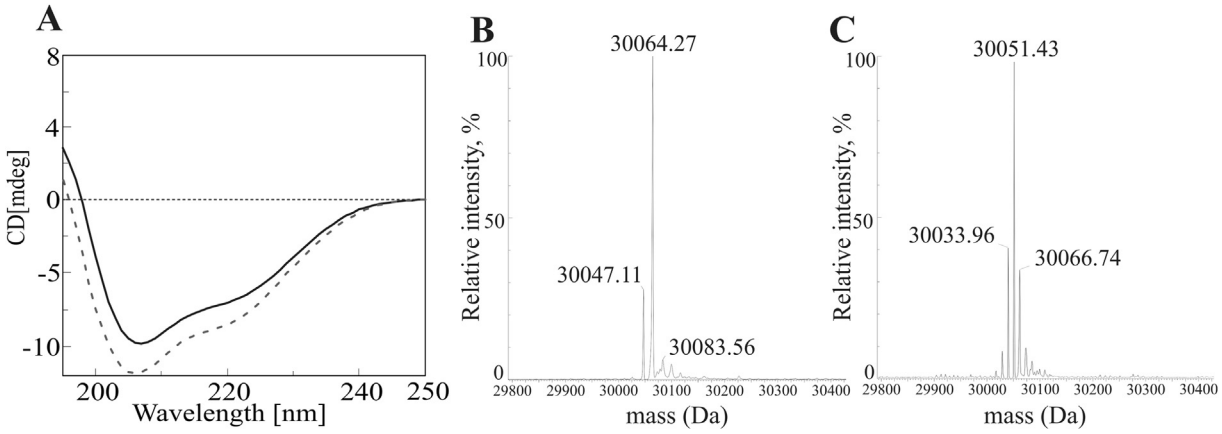

Fig. 2. A) CD spectra of dioicin 1 (solid line) and diocin 2 (dotted line) in the far UV region. B) Deconvoluted ESI/Q-TOF mass spectra of purified dioicin 1 under native conditions and C) after reduction with 2 M DTT, respectively.

residues, likely engaged in two S–S bridges, as found for dioicin 2 [15]. It has an overall low hydrophobicity (low, negative grand *av*erage of hydropathicity values- GRAVY) and a molar extinction coefficient value of 29130 in agreement with the highest number of aromatic amino acid residues (2W, 12Y and 10F). It is composed of 268 amino acid residues, the highest number among type 1 RIPs isolated from *P. dioica* and in general from Phytolaccaceae. Accordingly, the molecular weight of its polypeptide chain is higher than that of the Phytolaccaceae RIPs. The ratio of basic (Lys + Arg) *versus* acid (Asp + Glu) residues for dioicin 1 was 1, determining, as for other *P. dioica* RIPs, a theoretical pI 6.86 lower than the experimental pI value (8.74) previously determined by capillary electrophoresis on the native protein [15]. These discrepancies may depend on the specific algorithm used for calculation that has not yet been tested for prediction of basic proteins [36].

We previously reported that dioicin 1 was endowed with a lower capability of inhibiting protein synthesis in a rabbit reticulocyte lysate (IC_{50}: ~20 ng/mL) with respect to dioicin 2 (IC_{50}: 7 ng/mL) and PD-L4 (IC_{50}: 4 ng/mL) [14,15]. Therefore, we decided to investigate quantitative differences in enzymatic activities of these proteins by evaluating the adenine polynucleotide glycosylase (APG) activity of dioicin 1 on hsDNA with respect to dioicin 2 and

Table 3
Physico-chemical properties of type 1 RIPs isolated from *P. dioica*. Cys containing polypeptide chains. Dioicin 1, dioicin 2 and PD-L4 were not glycosylated.

RIP	Mr (reduced protein)		Amino acid residues	pI		$\frac{Lys + Arg}{Asp + Glu}$	ε[a]	Abs[b] (0.1%)	Phe + Tyr + Trp	II[c]	Half-life[d] (h)	AI[e]	GRAVY[f]
	Exp.	Theor.		Exp.	Theor.								
Dioicin 1	30,033.96	30,035.40	268	8.74	6.86	1.00	29130	0.962	24	25.14 (A)	4,4	80.11	−0.441
Dioicin 2	29,914.12	29,914.1 0	266	9.37	7.73	1.03	27640	0.924	21	26.73 (N)	1.4	77,74	−0.559
PD-L1	32,719 ± 1	29,222.0 0	261	≥9.50 ≤9.70	8.26	1.07	27640	0.946	21	28.86 (I)	20.0	83.33	−0.444
PD-L2	31,546 ± 1	29,222.0 0	261	≥9.50 ≤9.70	8.26	1.07	27640	0.946	21	28.86 (I)	20.0	83.33	−0.444
PD-L3	30,360 ± 1	29,190.10	261	≥9.50 ≤9.70	8.54	1.12	27640	0.947	21	29.80 (V)	100.0	86.28	−0.373
PD-L4	29,189 ± 1	29,190.10	261	≥9.50 ≤9.70	8.54	1.12	27640	0.947	21	29.80 (V)	100.0	86.28	−0.373
PD-S2	30,757.80	29,586.8 0[g]	265	≥9.50 ≤9.70	9.18	1.31	29130	0.985	23	34.15 (V)	100.0	85.36	−0.343

[a] Extinction coefficient units of M^{-1} cm^{-1}, at 280 nm measured in water.
[b] (1 g/L), assuming all Cys residues are reduced.
[c] Instability Index. In parenthesis the amino terminal residue.
[d] Half-life in mammalian reticulocytes, *in vitro*. Dioicin 1: >10 h in *E. coli*, *in vivo* and >20 h in yeast, *in vivo*. Dioicin 2: >10 h in *E. coli*, *in vivo* and 3 min in yeast, *in vivo*. PD-L_{1-2}, >10 h in *E. coli*, *in vivo* and 30 min in yeast, *in vivo*. PD-S2: >20 h in *E. coli*, *in vivo* and >20 h (yeast, *in vivo*).
[e] AI, Aliphatic Index.
[f] Grand average of hydropathicity.
[g] Major component. The values obtained for the native PD-S1 and PD-S3 were 30,957.10 and 29,785.10, respectively.

PD-L4 (Fig. 3A). At the higher concentration assayed (1.75 and 3.00 μg), dioicin 1 showed a higher APG activity than PD-L4 and a reduced activity with respect to dioicin 2.

3.3. Sequence alignment and phylogenetic relationships among type 1 RIPs from Phytolaccaceae

A multiple sequence alignment analysis of dioicin 1 and type 1 RIPs sequences from Phytolaccaceae revealed the occurrence of a consensus sequence of 282 amino acids (Fig. S1A). The distinctive pattern of four cysteinyl residues (at positions 36, 91, 113 ant 278), engaged in two disulphide bridges, which contributes to the stability of these proteins, was conserved together with the active site region at positions 176–197 containing 15 out of 21 identical amino acid residues (70%). The amino acid residues known to be involved in the catalytic process [i.e. Tyr74(78), Tyr122(132), Glu177(189), Arg180(192), W211(223), numbering in parenthesis is referred to multiple alignment reported in Fig. S1A] as well as additional invariant amino acid residues [i.e. Ser215(227) and Phe181(193)] were found to be conserved in dioicin 1 sequence [11,37]. As evidenced by the identity/similarity matrix (Fig. S1B), dioicin 1 showed the highest percentages of sequence identity with dioicin 2 (67.7%), PAP-II (69.5%) and PAP icos-II (70.6%). On the contrary, the lowest percentage of identity (33.3%) was calculated for PD-L_{1-2} and other RIPs isolated from Phytolaccaceae, with identity values comprised between 34 and 38%.

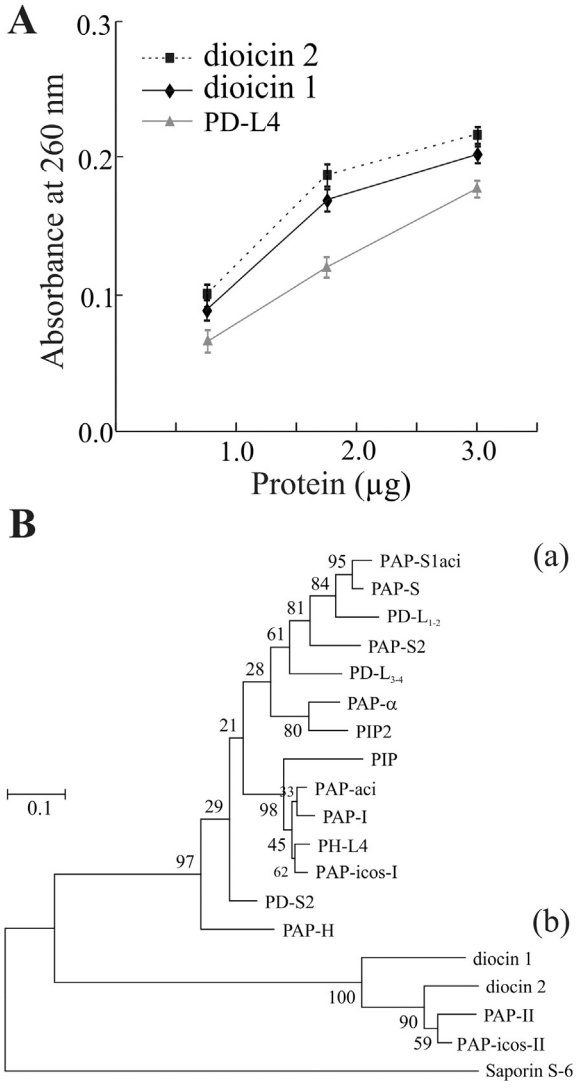

Fig. 3. A) adenine polynucleotide glycosylase activity of native dioicin 1 (solid line), dioicin 2 (dotted line) and PD-L4 (gray line) assayed on hsDNA substrate. B) Unrooted phylogenetic tree of type 1 ribosome-inactivating proteins from Phytolaccaceae.

The availability of primary structures of different type 1 RIP sequences from Phytolaccaceae provide us a mean to investigate phylogenetic relationships among them. The evolutionary relationships among the 18 type 1 RIPs sequences from Phytolacaceae were analysed by using the Maximum Likelihood method. The sequence of saporin S-6, a ribosome inactivating protein from *Saponaria officinalis* L. belonging to Caryophyllaceae, was used as outgroup. As previously reported [12,15], the occurrence of two main groups was evidenced by the analysis of the unrooted phylogenetic tree including the Phytolaccaceae's RIPs (Fig. 3B). The first group (a) included dioicins 1 and 2, PAP-II and PAP icos-II while all other Phytolaccaceae RIPs were located on a separate branch group (b). These findings suggest that both dioicins 1 and 2 share common ancestors with PAP-II and PAP icos-II. Interestingly, dioicin 1 stems from the base of the group b, thus appearing as an outgroup and therefore not closely related to other members of this clade.

In conclusion, in this work, the amino acid sequence of dioicin 1, type 1 ribosome-inactivating protein isolated from fully expanded young *P. dioica* leaves, was determined by a combined approach based on Edman degradation and mass spectrometry. Furthermore, dioicin 1 structural and enzymatic characterization was performed. Knowledge of dioicin 1 primary structure allowed to investigate phylogenetic relationships among type 1 RIPs from Phytolaccaceae. Overall, presented results provide the basis to better understand differential seasonal and age expression of dioicin 1.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.06.003.

Transparency document

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