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Yinshan Yang[1,*], Vianney Poignavent[2,ǂ,*], François-Xavier Gillet[2,±], Florence Vignols[2,3] and Hélène Déméné[1]

[1] Centre de Biochimie Structurale (CBS), INSERM, CNRS, Univ Montpellier, France.

[2] IRD, CIRAD, Univ Montpellier, Interactions Plantes Microorganismes et Environnement (IPME), Montpellier, France.

[3]Laboratoire BPMP, CNRS, INRA, SupAGRO, Univ de Montpellier, Montpellier-France

[ǂ]Current address: Centre National de la Recherche Scientifique, Institut de Biologie Moléculaire des Plantes, Université de Strasbourg, Strasbourg, France

[±]Current address: Laboratoire de Physiologie Cellulaire et Végétale, Institut de Biosciences et Biotechnologies de Grenoble, CNRS, CEA, INRA, Université Grenoble Alpes, Grenoble, France

[*]These authors contributed equally to the work

Corresponding authors:

Hélène Déméné and Florence Vignols

Helene.demene@cbs.cnrs.fr

+33 (0) 4 67 41 77 01

Author contributions: FV and HD designed the study; VP and FXG prepared protein samples; YY and HD analyzed the data; YY, FV and H. wrote the paper.

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Abstract

RNA silencing describes a pan-eukaryotic pathway of gene regulation where doubled stranded RNA are processed by the RNAse III enzyme Dicer or homologs. In particular, plants use it as a way to defend themselves against pathogen invasions. In turn, to evade the plant immune response, viruses have developed anti-RNA silencing mechanisms. They may indeed code for proteins called “viral suppressor of RNA silencing” which block the degrading of viral genomic or messenger RNA by the plant. The Rice Mottle Virus is an African virus of the sobemovirus family, which attacks the most productive rice varieties cultivated on this continent. It encodes P1, a cysteine-rich protein described as a potential RNA silencing suppressor. P1 is a 157 amino-acid long protein, characterized by a high propensity to aggregate concomitant with a limited stability with time in the conditions used in structural studies. To overcome this problem, shorter fragments were also studied. This strategy enabled the assignment of more than 90% backbone resonances of P1. This assignment should set the base of future NMR investigation of the protein structure and of its interactions with rice cellular partners.

Keywords: RNA silencing; viral suppressor; cysteine rich; RYMV; P1; sobemovirus.

Abbreviation: RYMV, Rice Yellow Mottle Virus; RNA -Induced Silencing Complex, RISC; siRNA, Small Interfering RNA; VSR, virus silencing repressor.

Biological context.

Rice occupies a central place in the diet of many people in Africa and Asia. Rapid population growth and food habit changes result in an increasing demand, while the rice is very susceptible to pathogen attack. Among these is the Rice Yellow Mottle Virus (RYMV) in the African continent, the infection thereof generates crop losses ranging from 20 to 100%. Like many other plants when confronted with pathogens, rice has evolved an immunity response under the form of viral silencing. Viral silencing represents an essential defense mechanism in plants (Ding and Voinnet, 2007). In short, plants can initiate the degradation of viral double stranded RNA, either genomic or messenger, by processing them with Dicer-like RNAses III into Small Interfering RNAs (siRNAS). Those are recruited in the multi subunit RNA-induced Silencing complex (RISC), which targets them to nuclear or cellular viral RNA for destruction. To counteract host RNAi antiviral defense, viruses from almost all plant virus genera (and also from some animal viruses) encode viral suppressors of RNA silencing (VSRs) which inhibit key steps of the cellular siRNA system

(Song et al., 2011). They are often multifunctional and play also important roles in viral replication, coating, movement, and pathogenesis. We and others have proposed that the P1 protein of RYMV belongs to this class based on its importance for viral particle spread in rice (Siré et al., 2008) and on its ability to counteract GFP silencing in a plant reporter system (Voinnet et al., 1999).

The P1 protein is a 157 amino acid long protein, characterized by the presence of numerous cysteines (7) and histidines (6). Biochemical analyses have already revealed important properties of P1, including its capacity to bind two zinc atoms (Gillet et al., 2013). Further structural analyses would help in deciphering how P1 molecular features support its potential functions. However, the protein is insoluble when produced in a culture medium with a zinc concentration below 100 μM (Gillet et al., 2013) and its structure has never been reported. As a first step towards solving the P1 solution structure and investigating its potential interactions with rice cellular components (proteins or nucleic acids), we report here its sequence specific backbone and some side chain assignments.

Methods and experiments

Protein production and purification

Construction of P1 clones has been previously described (Gillet et al., 2013). Full-length P1 (here termed FL P1), and its truncations P1_1-100 and P1_102-157 (comprising the segment from Met1 to Gln100 and Arg102 to Phe157, respectively) were expressed in *E. coli* BL21 (DE3) cells using M9 minimal medium supplemented with 1 g/L $^{15}NH_4Cl$, 2 g/L^{-1} ^{13}C-D-glucose, 1 mg/L thiamine and biotin and 450 μM/L $ZnSO_4$ at 37°C. Expression was induced by addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when an OD_{600} 0.6 was reached. Cells were then subjected to a thermal shock for 2 hours at 6°C, before further induction during five hours at 30 °C for FL P1 and 20°C for P1 P1_1-100 and P1_102-157. With this protocol, P1_1-100 and P1_102-157 were produced as soluble proteins whereas FL P1 accumulated as inclusion bodies. Proteins were then purified as described in (Gillet et al., 2013), except that lysis buffer (LyB 50 mM Tris pH 8, 150 mM NaCl, 1 mM Zn, 1 mM DTT) for FL P1 contained 6 M urea to dissolve the inclusion bodies. After lysis by sonication (and dialysis against 2 L of LysB for FL P1), BL21 supernatant was loaded onto 1 mL of Q-Sepharose Fast Flow pre-equilibrated with buffer A (50 mM Tris–HCl, pH 8.0, 1 mM DTT). P1 constructs were eluted with buffer B (buffer A + 1 M NaCl). The Zn^{2+} content of eluted

fractions was estimated by the photometric monitoring of Zn^{2+} release induced by 4-(2-pyridylazo) resorcinol (PAR) which complexes zinc (Gillet et al., 2013). Fractions where a maximal amount of Zn was present, were pooled, dialyzed against buffer A, and reloaded onto the Q-Sepharose Column. The repeat of this step was essential to obtain a clear separation of the different redox forms. Elution fractions containing P1 bound to the maximal amount of Zn^{2+}, were again pooled and loaded into a Superdex 75 Hiload 26/60 column (GE Healthcare) equilibrated with buffer C (25 mM Tris–HCl, pH 7.5, 200 mM NaCl, 1 mM DTT, and 500 μM $ZnSO_4$). The purified proteins were vaccuum concentrated and kept at 4 °C until use. Just before their use for NMR, the buffer was exchanged by dialysis against 100 mM NaCl, 60 mM Na_2SO_4, pH8, 500 μM $ZnSO_4$, 1 mM DTT as stability studies by thermal shift assay showed that sodium sulfate has a net stabilizing effect.

NMR spectra

All NMR experiments were performed at 300 K on a Bruker Avance III spectrometer (Bruker, Rheinstetten, Germany) operating at ^{1}H frequency of 699.97 MHz, using a 5 mm cryogenic H/ C/N/D probe with Z-axis gradient. Proton chemical shifts were referenced with respect to DSS and the frequency ratios of $^{15N}/^{1}H$ = 0.101329118 and $^{13}C/^{1}H$ = 0.251449530 (Wishart et al. 1995). Assignment of the spectrum of P1 was carried out using a dissection approach. NMR spectra were collected for the full-length P1, but also for the shorter proteins P1_1-101, and P1_102-157. These two fragments were chosen on the basis on our previous study and on ongoing work, which characterized them as likely to contain a zinc finger each (Gillet et al., 2013). Protein concentration was 200 μM (P1_1-100 and FL P1) and 2 mM (P1_102-157). Initial sequential backbone assignments were obtained with data from 3D ^{15}N-edited TOCSY-HSQC, NOESY-HSQC and 2D ^{1}H-^{1}H NOESY experiments for P1_1-100 and P1_102-157. These sequential ^{1}H and ^{15}N assignments were assessed and extended to ^{13}C with 3D HNCA, HNCACB, CBCA(CO) NH, (H)CCH-TOCSY and HNCO for FL P1. Gifa was used for NMR data processing (Pons et al., 1996) and NMRView for subsequent analysis (Johnson and Blevins, 1994)

NMR assignment and deposition

FL P1 contains 157 amino acids encompassing 5 prolines. Its behaviour in solution was characterized by a

poor solubility and a high propensity to aggregate within hours. Furthermore, the best determined solubility and stability conditions required a high ionic force, greater than 200 mM, and a high pH (>= 7.5). These conditions are known to diminish the sensitivity of NMR experiments, in particular those where the amide proton is detected during acquisition. Even in these optimized conditions, we observed an apparent loss of sensitivity of 30%-60% within 3 days, depending on the preparation, presumably because of protein aggregation. Most of the missing resonances belong to contiguous segments of the N-terminal part of P1: 12-13, 15-16, 32-34, 61-68, suggesting that the concerned segments are exposed to solvent or subjected to conformational exchange. For the C-terminal segment, only Ser131 and Gly147 could not be assigned. For Ala16, even if the N and HN resonances could not be retrieved, we were able to assign its Cα and Cβ carbon resonances on the HCCH-TOCSY experiment, after all other alanine spin systems were assigned. The low-signal/over noise ratio precluded unambiguous further assignment. Altogether, these results allowed us to assign 92 % of N, HN, Ha, C, Cα and Cβ resonances (fig. 1). This percentage increases to 94.6% for the HN and N resonances, i.e. 142 backbone cross-peaks could be assigned on the ^{1}H-^{15}N HSQC spectrum, of the 150 expected (considering the presence of 5 prolines and the absence of cross-peak for the first residue). In addition, all amide 15N and HN resonances of the 6 Gln and Asn side chains could be assigned. Chemical shifts were analysed using TALOS-N to identify regions of secondary structure (Shen and Bax, 2013). According to TALOS-N, the protein has 5 beta strands and 1 small helix in the N-terminal part, whereas the C-terminal part harbors a long helix (from residue 96 to 126) (Figure 2). Our NMR study of shifts of P1 will enable the investigation of its interaction with partners, in particular small RNAs. In future, determination of the structure of P1 and characterization of its dynamics will shed a new light on the three-dimensional properties of this domain and its numerous functions.

The chemical shift assignments have been deposited in the BioMagResBank (http://www.bmrb.wisc.edu) under the 27880 accession number.

Figure caption

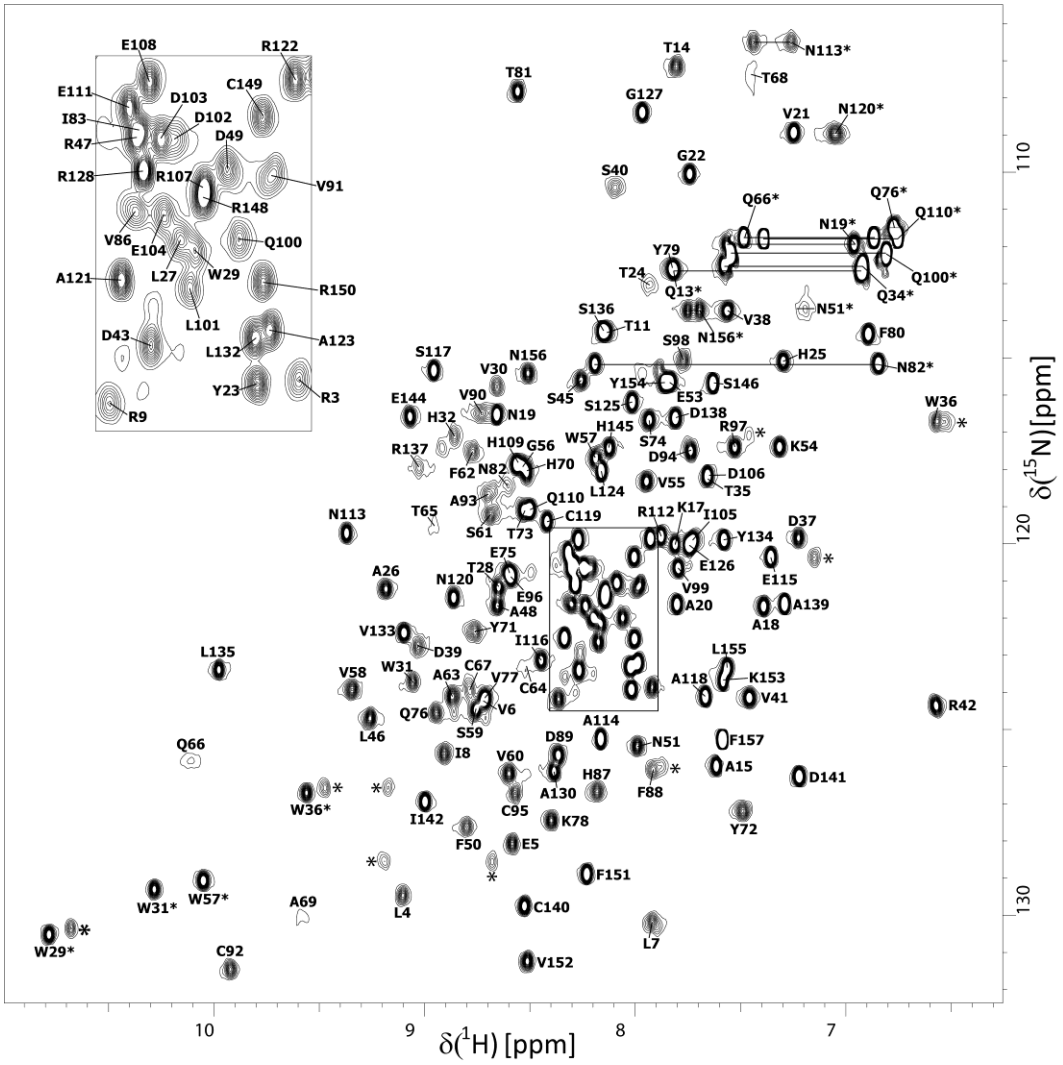

Fig. 1: 2D ^{1}H-^{15N} HSQC spectrum of 200 μM sample of ^{15}N-labeled P1 in 100 mM NaCl, 60 mM Na_2So_4, 50 mM Tris buffer, pH 8 at 300K. Each backbone amide resonance is labeled with the amino acid type (one-letter code) and the number in the sequence. Hε1-Nε1 amino groups from tryptophanes are labeled with an asterisk (*). Also labelled with an asterisk are the assigned amide groups from glutamine and asparagine side chains, which are in addition outlined with horizontal lines.

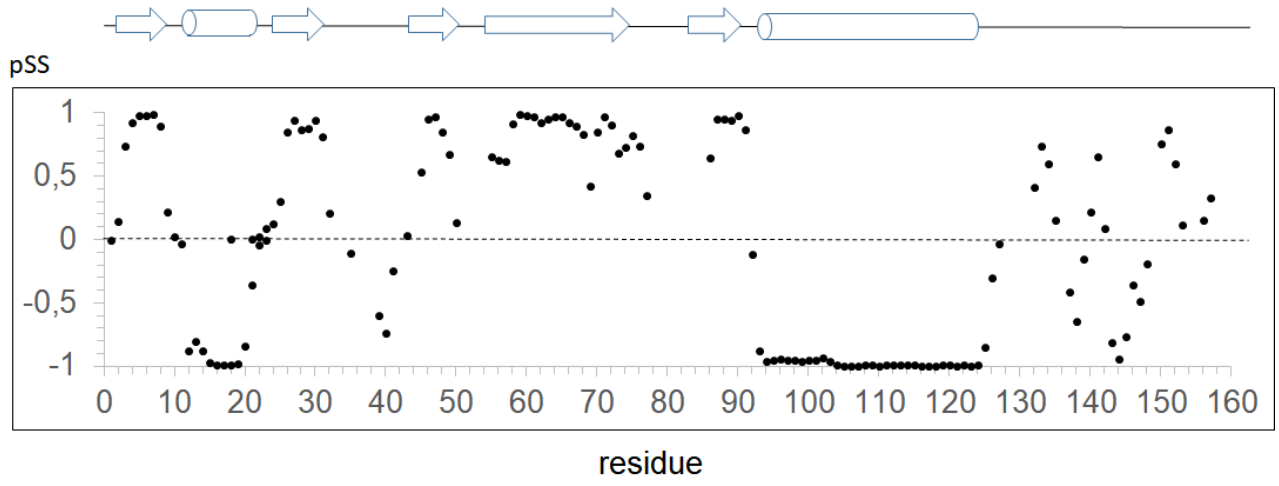

Fig. 2. The secondary structure propensities pSS of the P1 protein derived from TALOS-N on the basis of the chemical shifts of $H\alpha$, ^{15}N, $C\alpha$, $C\beta$ and C' atoms (Shen and Bax, 2013). Positive and negative values stand for β-sheet and α-helice predictions respectively. The predicted secondary structures are schematized above.

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