European Journal of Plant Pathology (2005) 112:395–398
DOI 10.1007/s10658-005-6606-6 © Springer 2005

Short communication

Exceptional length of ITS in *Plasmopara halstedii* is due to multiple repetitions in the ITS-2 region

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Accepted 25 April 2005

Key words: downy mildew, ITS variability, oomycete, sunflower pathogen

Abstract

Plasmopara halstedii, cause of downy mildew of sunflower, is a pathogen of worldwide economic importance. Efforts to amplify the ITS-region from this organism revealed an unexpected fragment length of about 2600 bp, in contrast to about 900 bp, reported for other members of the Peronosporaceae. First attempts to obtain the complete sequence of the *P. halstedii* fragment were unsuccessful, due to repeated elements in ITS, which were uncovered later on. The presence of a single *Eco*RI-site allowed us to apply a restriction-ligation procedure to amplify parts of the ITS fragment separately. Sequencing of these fragments revealed the presence of four copies of a tandemly arranged repetitive element in the ITS-2 region. The complete sequence was obtained by using a sequencing primer which annealed shortly before the repetitions so covering the gap in the sequence around the restriction site. The ITS sequence in *P. halstedii* (AY773346) consisted of 2587 bp in total, with ITS-2 accounting for 2212 bp alone. This is the longest ITS-2 sequence reported so far for any examined species.

Plasmopara halstedii, cause of downy mildew of sunflower is a devastating pathogen, which causes severe economic loss in sunflower production worldwide (Sackston, 1981; Gulya et al., 1997). Although it is diverse in virulence phenotypes, molecular variability by random amplification techniques proved to be low and uncorrelated with pathotype differentiation (Roeckel-Drevet et al., 1997, 2003; Intelmann and Spring, 2002). Another possibility to screen molecular diversity within species is to amplify elements from the multi copy rDNA genes (White et al., 1990). For the understanding of evolutional changes and species differentiation below the generic level, the 28S-rDNA, which lately received attention by several researchers (Petersen and Rosendahl, 2000; Riethmüller et al., 2002; Göker et al., 2003; Spring et al., 2003; Voglmayr, 2003), does not exhibit sufficient variability. The ITS-region is reported to be much more polymorphic and therefore valuable for the differentiation of taxa, especially on and below the generic level (White et al., 1990). For part of the Peronosporomycetidae an ITS phylogeny has been evaluated by Voglmayr (2003), covering the genera *Hyaloperonospora*, *Perofascia*, *Peronospora*, *Peronophytophthora*, *Phytophthora*, *Pseudoperonospora* and *Pythium*, but not *Plasmopara*. In most species included in Voglmayr's (2003) survey, ITS amplification resulted in fragment lengths of about 900 bp. Only in a few cases were short insertions observed in either the ITS-1 or ITS-2 region, resulting in a maximum length of 1247bp for the entire ITS-region in *Peronospora trifolii-alpestris*. For *P. halstedii*, however, amplification of ITS proved to be difficult, until specific primers were developed by Bachofer (2004). Amplification with these oomycete specific primers gave a ca. 2600 bp fragment, exceeding the length of most species by a factor two or more. While partial sequence of the ITS was established for

phylogenetic studies, efforts to obtain the whole sequence of the ITS region by cloning and primer-walking failed, due to the repetitive elements, which were uncovered later. The aim of the current study was to clarify the ITS-structure of *P. halstedii* and to obtain its complete sequence, using a restriction-ligation procedure. Here we report the amplification and sequencing of the ITS-region of *P. halstedii*, highlighting its multiple insertion feature within ITS-2. It is, to our knowledge, the longest ITS region known so far within all investigated straminipilous species.

For the investigations, a laboratory strain from *P. halstedii* (pathotype 703) was used, which originated from France and has been recurrently propagated in our laboratory. Sporangia were collected from the surface of cotyledons, using a vacuum manifold and then transferred to 2 ml tubes. Sporangia were disrupted dry at room temperature using a Mixer Mill (Retsch, Germany) at 10 Hz for 3 min, adding one magnetic ball. DNA extraction from 5 mg of sporangia was carried out using a DNA purification kit (MBI Fermentas, Germany). The ITS-region was amplified using the oomycete specific primer ITS1-O 5′-CGG AAG GAT CAT TAC CAC-3′ (Bachofer, 2004), which contains the conserved starting motive of ITS-1, 18S_rc 5′-GTAGGT GAACCTGCAGAAGGATCAA-3′ (reverse complementary to 18SR, Medlin et al., 1988) and LR-0 5′-GCT TAA GTT CAG CGG GT-3′ (reverse complementary to LR-0R, Moncalvo et al., 1995). PCR was carried out in an Eppendorf Mastercycler (Eppendorf, Germany) using the following conditions: denaturation at 94 °C for 42 s, annealing at 56 °C for 42 s, elongation for 1 min 42 s at 72 °C. Cycles were repeated 30 times and succeeded by a final elongation step of 4 min 20 s. Amplification resulted in a bright fragment of approximately 2600 bp and two faint side-bands of smaller size. The 2600-bp fragment was cut from the gel and cleaned, using the QiaQuick PCR-Purification Kit (Qiagen, Germany). In order to receive fragments with favourable sizes for direct sequencing, the product was digested with *Hin*dIII, *Hpa*II, *Ava*III and *Eco*RI restriction enzymes. *Eco*R1 (Fermentas, Germany) digestion resulted in two fragments of ca. 1400 bp and ca. 1200 bp in length. The two fragments were cut separately from the gel and cleaned again as described above. *Eco*R1 adapter (Vos et al., 1995) was ligated to the fragments, according to the manufacturer's instructions, using the T4-Ligase from Abgene (France). Successful ligation was checked by PCR amplification, using selectively the adapter primers with the ITS1-O or LR-0 primers, respectively. Amplification revealed that the longer fragment was located downstream of the shorter fragment. Both fragments were cut from the gel and were cleaned as described above. After cleaning, they were used for direct sequencing using the appropriate combinations of adapter- and ITS-primers. To unravel the sequence around the cutting site of the enzyme, a third primer was designed, specific to the sequence, which annealed shortly before the beginning of the repeated elements on positions 711–730 of the ITS, named ITS-FFL 5′-ACT TGT AGC CAG ACG GCG AC-3′. Sequencing of the whole ITS-fragment with this primer enabled us to compile the amplified ITS-sequence for *P. halstedii*, revealing a total length of the ITS-2 region of 2212 bp (AY773346).

The sequence data were compared to *Phytophthora infestans* AF228084, *Phytophthora megasperma* AF266794 and *Peronospora rumicis* AF465758 sequences from GenBank. The ITS-region in *P. halstedii* can be described as having different regions based on the level of similarity to the corresponding sequence data from the accessions mentioned above (Figure 1). In *P. halstedii* the ITS-1 region consists of 216 bp, which can be divided into two parts (a, b). In the part (a) the variation is higher than in part (b). This region is succeeded by the conserved part of the 5.8S rDNA (158 bp) and the ITS-region 2 (c–f, 2212 bp), which can be divided into several parts. The first 146 bp of ITS-2 (c, d) show similarity to *Peronospora* and *Phytophthora* species, especially in part (c). These are followed by an insertion of 1822 bp (e), not present in any ITS sequences in public databases. This region contains the repeated elements r1–r4 and the spacers s1-s3 (1400 bp in total). The tandemly repeated elements r1–r4 were 322 bp in length each. They were separated by variable elements, which were not present before the first or after the last of the repetitions and are referred to as spacers 1–3 here (s1–s3). The first of these elements (s1) was 38 bp, the other two (s2 and s3) were 34 bp in length. The four-time repeated insertion proved to have no significant similarities to any sequence within public data-

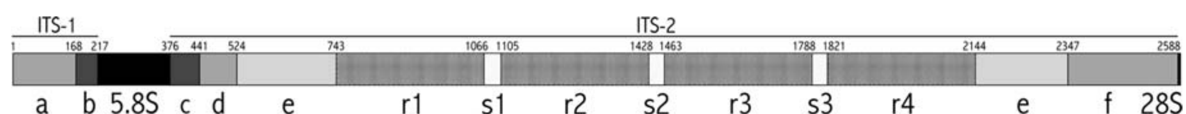

Figure 1. Structure of the ITS-region in *Plasmopara halstedii.* (a and b) ITS-1; 5.8S, region coding for the 5.8S ribosomal subunit; (c–f) ITS-2; (e) part of ITS-2, reported for neither *Phytophthora* nor *Peronospora*, Figure 1: including the repetitions, r1–r4, and the spacers in between the repetitive units, s1–s3; 28S, part of the large ribosomal subunit (LSU). Figures above the drawing relate to base pairs, indicating the beginning of each of the parts described. Greyscales indicate the level of similarity to *Phytophthora* and *Peronospora*; black (5.8S, 28S): similarity higher than 90%, dark grey (b, c): similarity between 80% and 90%, grey (a, d, f): similarity less than 80%, other colours: regions reported for neither *Phytophthora* nor *Peronospora*.

bases. The elements showed a high degree of similarity to each other, with several base-pair differences in some variable regions (Table 1). This similarity generally decreased from r1 to r4. The recognition site of *Eco*R1 was situated in the second repeated element (positions 1244–1249 of the ITS). In the last part of ITS-2, region (f) (240 bp), the sequence again shared similarity to other oomycetes, leading to LSU (positions 2588–2594).

The ITS-region of *P. halstedii* could help in understanding the process of how such insertions develop, also in other species, due to their repetitive occurrence and their variation in comparison to each other. Closely related species should be screened to elucidate whether similar insertions are present, and to which extent they are similar to each other in sequence and number of the repeated elements. This could help in the understanding of the evolution of the *P. halstedii* complex on Compositae and thereby elucidate the divergence of these pathogens. The latter aspect might be important for evaluating the possible epidemiological development of *P. halstedii* in the future. Furthermore, the high degree of variation of the repetitions might allow the specific detection and diagnosis of different *P. halstedii* strains, which is crucial for understanding the epidemics of and for taking defensive measures against this devastating pathogen.

Table 1. Similarities of the repetitions. The repetitive units show about 90% similarity to each other, with generally decreasing similarities from r1 to r4

	r1	r2	r3	r4
r1	–			
r2	90.2	–		
r3	91.3	89.4	–	
r4	88.3	87.4	88.3	–

Acknowledgements

This study was partly financed by the Ministry of Science, Research and Arts of Baden-Württemberg and DAAD (A/03/30179). We are indebted to C. Lanz, Tübingen, Germany, for sequencing support. We wish to thank M. Göker, A. Riethmüller and especially M. Bachofer for providing sequence information on the ITS1-O primer, before the publication of his dissertation.

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