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# Different pathotypes of the sunflower downy mildew pathogen *Plasmopara halstedii* all contain isometric virions†

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## SUMMARY

Eight pathotypes of *Plasmopara halstedii* were screened to investigate the occurrence of virions and the potential viral influence on the pathogenicity of the sunflower downy mildew pathogen. In 23 of 26 *P. halstedii* isolates derived from eight countries in Europe, North America and South America, virions were detected by transmission electron microscopy. By contrast, there were no ultrastructural indications of virus-like particles in eight other related Oomycetes. The virions of representative *P. halstedii* isolates were morphologically and biochemically characterized and compared among each other. Regardless of their host's pathotypes, the geographical origin of the isolate and the sensitivity towards the fungicide metalaxyl, the viral characters obtained were uniform. The virions were isometric and measured approximately 37 nm in diameter. One polypeptide of *c.* 36 kDa and two segments of single-stranded RNA (3.0 and 1.6 kb) were detected. Both viral RNA segments were detected by capillary electrophoresis in the three remaining *P. halstedii* isolates where virions were undetectable by transmission electron microscopy. Virus-specific primers for the 1.6 kb-segment were synthesized and used to determine and compare a partial sequence of the viral coat protein among virions of different *P. halstedii* pathotypes. In all tested isolates, fragments of 0.7 kb were amplified which were directly sequenced. Sequence variation was insignificant. As both less aggressive and more aggressive *P. halstedii* isolates contained virions, the presence or absence of virions could not explain the diverse aggressiveness of the downy mildew pathogen towards sunflower. Moreover, the results indicated that pathogenicity of *P. halstedii* was not related to variation in morphological or biochemical characters of the virions.

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## INTRODUCTION

Very few species within the Oomycetes are known to host virus-like elements such as virus-like particles (VLPs), double-stranded RNA (dsRNA) or single-stranded RNA (ssRNA). Some isolates of *Albugo candida* contained VLPs measuring 200 nm in diameter (Buck, 1986). In *Phytophthora infestans*, the potato late blight pathogen, bacilli-shaped VLPs were observed (Styer and Corbett, 1978). *Ph. infestans* isolates which were hosts to viral dsRNA showed an increased virulence compared with virus-free isolates (Tooley *et al.*, 1989), indicating a phenomenon named hypervirulence. Later, Newhouse *et al.* (1992) detected several segments of linear dsRNA (11.10–1.35 kbp) in *Ph. infestans*. Intranuclear VLPs were found in an isolate of *Phytophthora drechsleri*, a pathogen of pepper plants (Roos and Shaw, 1985). In *Phytophthora nicotianae*, VLPs measuring 130 nm in width and 170 nm in length were found (Buck, 1986). dsRNA in a so far unnamed *Phytophthora* species, pathogenic to Douglas fir, was recently detected and sequenced (Hacker *et al.*, 2005). An isolate of *Pythium irregulare*, associated with infected Canadian cucumber plants, harboured virions which measured 28 nm in diameter and contained dsRNA (Klassen *et al.*, 1991). Associated with Australian wheat, another isolate of *Pythium irregulare* was found to hold dsRNA but these VLPs measured 45 nm in diameter (Gillings *et al.*, 1993). dsRNA but no VLPs were reported to occur in *Pythium butleri* (Buck, 1986). Although some of these virus-like elements have been known for more than 20 years, no further characteristics have been reported. The only oomycotic viruses which have been studied more intensively are those occurring in *Sclerophthora macrospora*, the downy mildew pathogen of rice and other species within the Poaceae. In this oomycete, two distinguishable types of virions were found which were named SmV A and SmV B. Both virus types were isometric and used ssRNA to encode their viral genomes. Two coat proteins (43 and 39 kDa) and three segments of ssRNA (2928, 1981 and 977 nt) were found to set up SmV A (Honkura *et al.*, 1983; Shirako and Ehara, 1985; Yokoi *et al.*, 2003). SmV B featured one coat protein of 41 kDa and one ssRNA segment of 5533 nt (Honkura *et al.*, 1983; Shirako and Ehara, 1985; Yokoi *et al.*, 1999).

Possibly due to the difficult handling of most Oomycetes, the understanding of the influence of virus-like elements is still insufficient. Even for *S. macrospora* viruses, there has not been any report on observations regarding viral influence on the pathogenicity of the oomycete.

*Plasmopara halstedii* is a biotrophic pathogen of sunflower causing downy mildew infections. It is another oomycete which is known to contain virions. Gulya *et al.* (1990) described isometric virions which were found in a single North American pathotype of *P. halstedii*. The putative coat protein was determined to consist of a 37.5 kDa-polypeptide and RNA was determined to encode the viral genome (Gulya *et al.*, 1992; Mayhew *et al.*, 1992). *P. halstedii* is distributed world-wide and shows a broad spectrum of pathotypes (physiological races) as reported by Gulya (2007). However, no further data have yet been reported on virus occurrence in other *P. halstedii* pathotypes, the geographical distribution or the nucleic acid sequence of the virions or the viral influence on pathogenicity.

Here we report on the presence of virions in representative *P. halstedii* isolates. These isolates differed in their pathotypes, their geographical origin and their fungicide sensitivity towards metalaxyl. To investigate the potential viral influence on the pathogenicity of *P. halstedii*, *P. halstedii* virions were characterized and compared with respect to their morphology and biochemical composition. This also included a 0.7 kb-nucleic acid region of the 1.6 kb-segment which was amplified with virus-specific primers and compared among viral isolates extracted from representative *P. halstedii* isolates. Additionally, this research provided first insight into the relationship between the oomycotic virus of *P. halstedii* and other viruses.

## RESULTS AND DISCUSSION

### Characterization by transmission electron microscopy

Transmission electron microscopy of ultrathin sporangia sections revealed the presence of isometric virions in the cytoplasm of *P. halstedii* regardless of its pathotype (Table 1). The same virions were also found in hyphae and haustoria (Fig. 1a). They mostly occurred in large quantities freely dispersed throughout the cytoplasm as well as in vesicles. The virions often formed a kind of crystalline structure, especially when enclosed by a membrane (Fig. 1b). Similar virions or other VLPs were never observed in sunflower cells of either infected or healthy plants. These results were in accordance with those reported for the North American race 2 isolates of *P. halstedii* (Gulya *et al.*, 1992), which corresponds to pathotype 300 (Gulya, 2007).

The use of a negative staining technique allowed an extended screening of *P. halstedii* isolates varying in pathogenicity, geographical origin, fungicide sensitivity and host (Table 1). Virions were observed in 23 of 26 isolates of *P. halstedii*, representing a total of eight pathotypes from five European (Austria, France, Germany, Hungary and Slovakia) and three American (Argentina, Canada, USA) countries. The virions occurred in less aggressive *P. halstedii* pathotypes (e.g. 100 or 300) as well as in more aggressive ones (e.g. 703 and 730). Among these were isolates

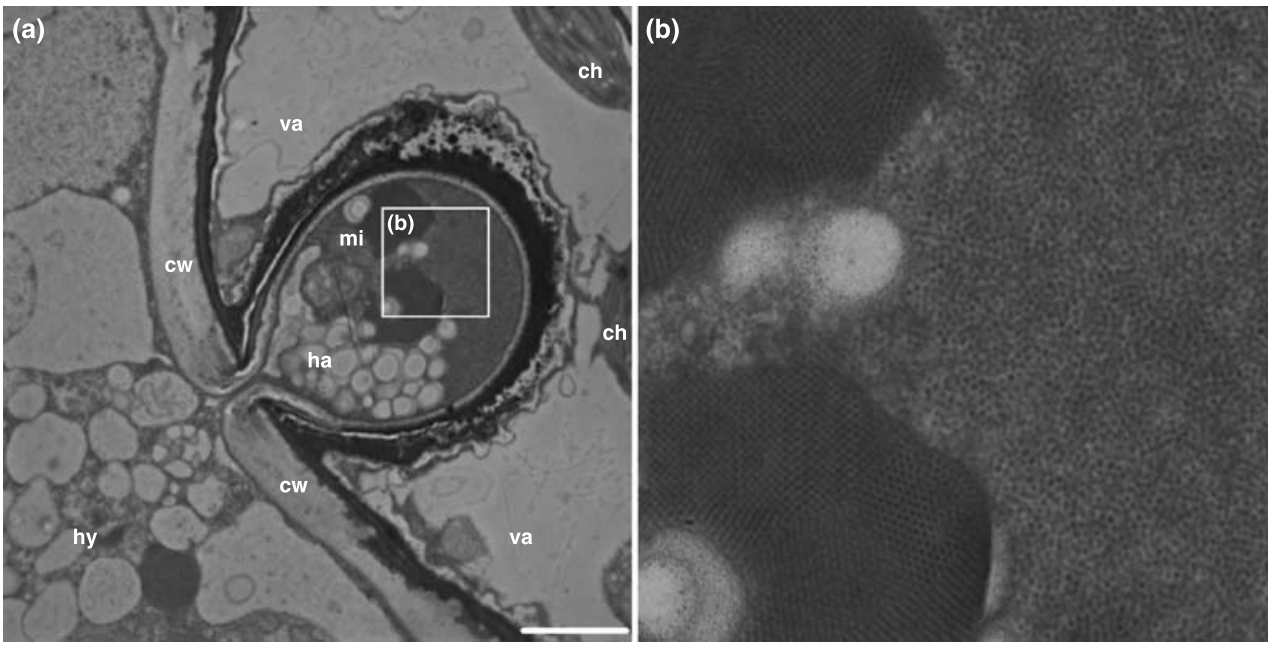


**Fig. 1** Hyphae (hy) of *P. halstedii* projecting a haustorium (ha) in a sunflower parenchyma cell. Insert (b) shows virions dispersed in the haustorium cytoplasm and within vesicles, containing crystalline-like aggregations of the virions. cw: cell wall; mi: mitochondrium; va: sunflower vacuole; ch: chloroplast. Scale bar: 2 μm.

**Table 1** *P. halstedii* isolates and the characteristics of their virions. The common sunflower, *H. annuus*, was host to all but one *P. halstedii* isolate (Ph5-01, host *H.* × *laetiflorus*). Information and results about the isolates and their virions were provided as far as tested.

| | | | Characteristics of *P. halstedii* | | Examination of *P. halstedii* sporangia by TEM | | | Viral protein analysis | | Viral RNA analysis | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *P. halstedii* isolate/viral isolate | Year of collection | Geographical origin | Fungicide* | Pathotype | Samples with virions/sample total† | Virion diameter (nm)† | Virions in ultrathin sections‡ | No. of protein bands§ | Molecular mass (kDa) | No. of segments | Segment sizes (kb) | GenBank accession number |
| Ph4-93 | 1993 | Fargo, ND, USA | | 710 | 1/1 | | | 1 | 36 | 2 | 3.0, 1.6 | EU126922 |
| Ph3-96 | 1996 | Kitzingen, Germany | | 710 | 1/1 | 37 | + | | | | | |
| Ph1-97 | 1997 | Groß Gerau, Germany | | 330 | 1/2 | | | | | | | |
| Ph6-97 | 1997 | Sinzheim, Germany | R | | 1/1 | | | | | | | |
| Ph9-97 | 1997 | Fargo, ND, USA | | 710 | 1/1 | | | | | | | |
| Ph9-98 | 1998 | Leinfelden, Germany | S | 310 | 4/7 | 37 | + | 1 | 36 | 2 | 3.0, 1.6 | EU126923 |
| Ph14-98 | 1998 | Heidelberg, Germany | | | 1/1 | | | | | | | |
| Ph5-99 | 1999 | Slovakia | | 720 | 1/1 | 37 | + | | | | | |
| Ph6-99 | 1999 | Austria | | | 1/1 | | | | | | | |
| Ph7-99 | 1999 | Austria | | | 1/2 | | | | | | | |
| Ph8-99 | 1999 | Bléré, France | R | 730 | 2/2 | 37 | + | 1 | 36 | 2 | 3.0, 1.6 | EU126925 |
| Ph1-00 | 2000 | Clermont-Ferrand, France | S | 703 | 2/2 | | + | 1 | 36 | 2 | 3.0, 1.6 | EU126924 |
| Ph10-00 | 2000 | Poutoux, France | R | 710 | 1/1 | 37 | | | | | | |
| Ph13-00 | 2000 | St. Christoph, France | R | 710 | 2/2 | | | | | | | |
| Ph21-00 | 2000 | Filderstadt-Sielmingen, Germany | | 710 | 1/1 | | | | | | | |
| Ph5-01 | 2001 | Tübingen-Lustnau, Germany | | | 2/5 | | | | | 2 | 3.0, 1.6 | EU126921 |
| Ph8-01 | 2001 | Leinfelden-Steinenbronn, Germany | | 300 | 1/1 | | | | | | | |
| Ph10-01¶ | 2001 | Stuttgart-Plieningen, Germany | S | 730 | 0/1 | | | | | | | |
| Ph14-01 | 2001 | Gödöllö, Hungary | | 730 | 1/1 | 37 | | | | | | |
| Ph19-01 | 2001 | Gödöllö, Hungary | | 100 | 1/1 | | + | 1 | 36 | 2 | 3.0, 1.6 | EU126920 |
| Ph8-03 | 2003 | Herrenberg-Kuppingen, Germany | S | 710 | 1/1 | | | | | | | |
| Ph10-03 | 2003 | Stuttgart-Rotenberg, Germany | S | 710 | 1/2 | | | | | | | |
| #529 | 2003 | Balcarce, Argentina | | | 1/2 | | | | | | | |
| #530¶ | 2003 | Balcarce, Argentina | | | 0/2 | | | | | | | |
| #534 | 2001 | Manitoba, Canada | | | 1/1 | | | | | | | |
| #535¶ | 2002 | Manitoba, Canada | | | 0/1 | | | | | | | |

* *P. halstedii* isolate is either resistant (R) or sensitive (S) to fungicide treatment with metalaxyl (Spring *et al.*, 1997).

†As determined by randomly sampled viral isolates using negative staining (2% uranyl acetate solution).

‡In all the tested ultrathin sections of sporangia (marked with +), isometric virions of very similar sizes were found.

§Number of protein bands detected with Coomassie blue staining.

¶Although virions were undetectable by transmission electron microscopy, these *P. halstedii* isolates contained viral RNA as determined by capillary electrophoresis and/or RT-PCR experiments (data not shown).

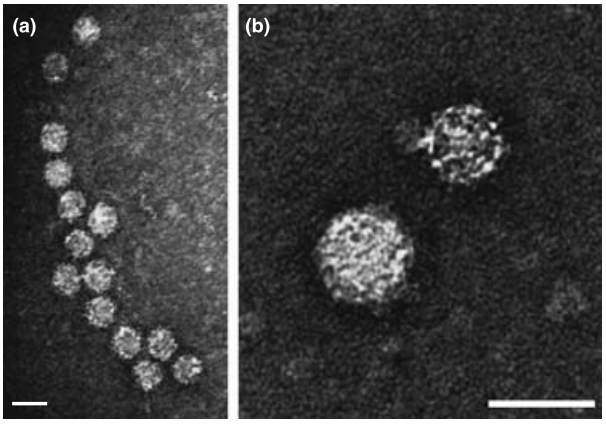


**Fig. 2** Negatively stained virions of *P. halstedii*. (a) Isolate Ph14-01: pathotype 730, Hungary, 2001; (b) isolate Ph10-00: pathotype 710, France, 2000). Scale bar: 40 nm.

(e.g. Ph8-99, Ph10-00) showing resistance to high concentrations of the systemic fungicide metalaxyl (Rozynek and Spring, 2001). Another virion-positive isolate (Ph5-01) was collected from *H.* × *laetiflorus*, a perennial sunflower hybrid which has become established in Germany (Spring *et al.*, 2003). No significant differences in viral morphology were observed among these 23 virion-positive isolates. The negatively stained virions measured approximately 37 nm ($n = 202$; 37 ± 7.3 nm) in diameter and showed a granular surface (Fig. 2) as was previously reported for those of the single pathotype tested by Mayhew *et al.* (1992). The few cases in which the virions could not be observed were more probably due to low virus titres in combination with the insensitivity of transmission electron microscopy screenings rather than to the absence of virions. This assumption was confirmed by capillary electrophoresis in which both viral RNA segments were identified (data not shown). All 26 *P. halstedii* isolates tested were found to contain virions and/or viral RNA segments of identical sizes.

Within eight other taxa of Oomycetes representing related *Plasmopara* species (*P. angustiterminalis*, *P. nivea*, *P. pusila* and *P. viticola*) as well as *Bremia lactucae*, *Peronospora tabacina*, *Ph. infestans* and *Pustula tragopogonis*, no similar or other VLPs were observed by transmission electron microscopy. RT-PCR experiments with virus-specific primers (2-f3 and 2-r4) were conducted to verify these results. Products of 0.7 kb were never obtained with these oomycotic pathogens (data not shown).

## Biochemical characterization

After the extraction and purification procedure, virions of five *P. halstedii* isolates (Ph4-93, Ph9-98, Ph8-99, Ph1-00, Ph19-01; Table 1) were characterized biochemically. As verified by transmission electron microscopy, in all five samples the isometric virions were successfully purified and concentrated, whereas the control (healthy sunflower) lacked virions. Additionally, it was observed that the morphology of the virions was unaffected by chloroform treatment during the purification procedure, probably indicating non-enveloped virions.

After the centrifugation steps, the suspensions of *P. halstedii* virions showed UV absorption spectra typical for viral nucleoproteins (Hull, 1985) and an absorbance ratio $A_{260}/A_{280}$ of 1.34–1.48. The coat protein was detected as a single polypeptide band with an estimated molecular mass of 36 kDa in SDS-PAGE gels of all viral isolates tested (Fig. 3a). Some preparations occasionally contained minimal amounts of additional proteins. These polypeptides represented aggregated and/or partial viral coat proteins as identified by protein mass spectrometry (data not shown). The extracted viral nucleic acid samples showed an average $A_{260}/A_{280}$ ratio of 1.8. The nucleic acids of the viral samples were unaffected by DNase I treatment but were susceptible to RNAse A digestion in both low and high salt conditions, indicating an ssRNA genome (Supporting Information Fig. S1). This was verified by RT-PCR experiments using either primer 2-f3 or 2-r4 for transcription and then both primers for PCR. A product was yielded only when transcribed with primer 2-r4 (data not shown). Purity of viral RNA preparations was tested in RT-PCR experiments using a universal primer set for eukaryotic 18S rDNA. Visible bands were never obtained, when transcribed either with the 18SR primer or with the 18SF primer (data not shown). However, extracted sunflower DNA as well as extracted *P. halstedii* sporangia DNA showed bands of expected sizes (Fig. 4; lanes 9 and 11). The presence of two viral RNA segments consisting of approximately 3.0 kb (RNA 1) and 1.6 kb (RNA 2), respectively (Fig. 3b), was revealed by agarose gel electrophoresis. The two viral segments were separated by agarose gel electrophoresis and individually isolated from the gel (Supporting Information Fig. S2a). As revealed by RT-PCR experiments using virus-specific primers for the coat protein gene, the coat protein was (at least partially) encoded by RNA 2 (Supporting Information Fig. S2b; lanes 4–7). Again, this assumption was verified by protein mass spectrometry (Fig. 5).

Control experiments with samples of healthy sunflower identically treated during the extraction and purification procedure never contained the 36 kDa-coat protein or the two RNA segments (data not shown).

## Sequence data analysis

A sequence fragment coding for a part of the viral coat protein was considered as another characteristic to be compared among representative *P. halstedii* virus isolates. Viral isolates (Ph4-93, Ph9-98, Ph8-99, Ph1-00, Ph5-01, Ph19-01; Table 1) which were partially sequenced were chosen by their host's pathotypes, geographical origin and fungicide sensitivity. After RT-PCR experiments with virus-specific primers, for all six viral isolates a single band of approximately 0.7 kb resulted and this was directly

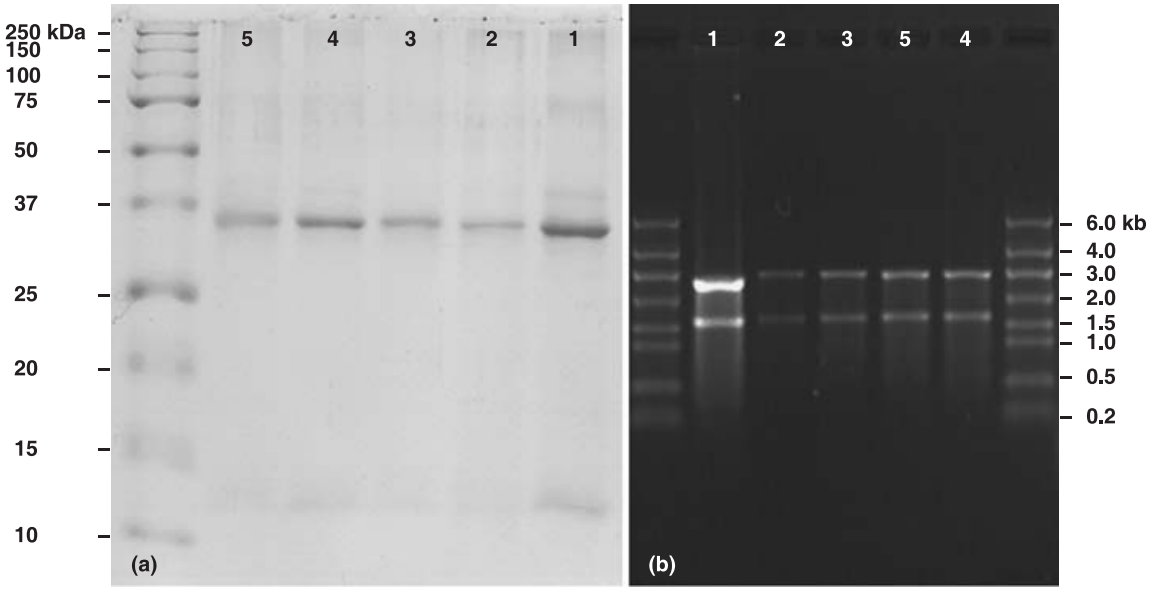


**Fig. 3** Biochemical characters of virions from *P. halstedii* isolates of different pathotypes and geographical origin. (a) SDS-PAGE of the viral polypeptides (Coomassie blue staining); (b) viral RNA on a native agarose gel using an ssRNA marker for sizing. Slight differences in the motility of the two RNA segments in lane 1 in comparison with lanes 2, 3, 5 and 4 are thought to be due to unequal sample quantity. 1: isolate Ph19-01 (pathotype 100, Hungary, 2001); 2: isolate Ph9-98 (pathotype 310, Germany, 1998, metalaxyl-sensitive); 3: isolate Ph1-00 (pathotpye 703, France, 2000, metalaxyl-sensitive); 4, isolate Ph8-99 (pathotype 730, France, 1999, metalaxyl-resistant); 5: isolate Ph4-93 (pathotype 710, USA, 1993). Additional polypeptide bands represented aggregated and/or partial viral coat proteins as analysed by protein mass spectrometry.

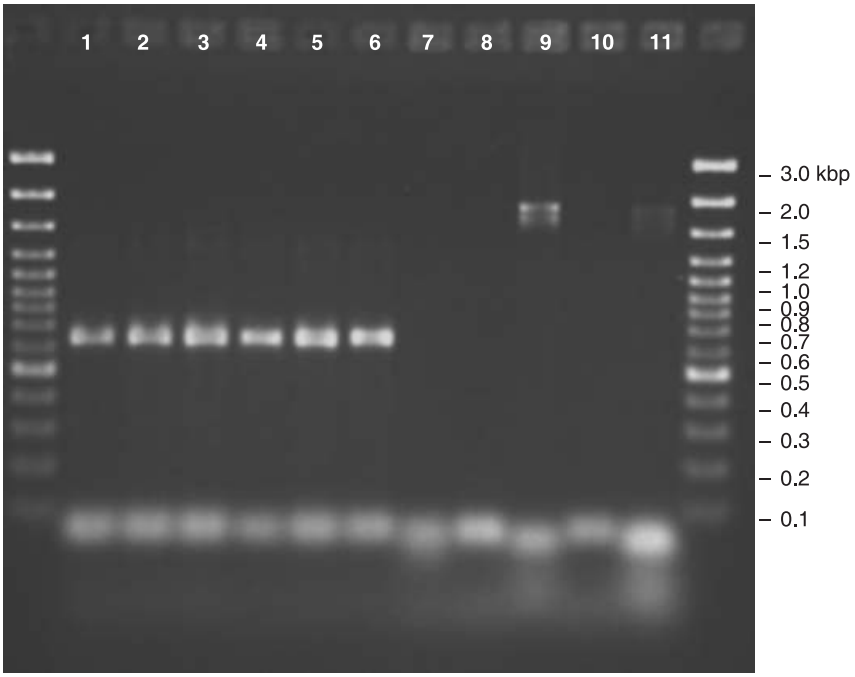


**Fig. 4** DNA gel electrophoresis after PCR. In lanes 1–8 and 10, virus-specific primers (2-r4 and 2-f3) were used. In lanes 9 and 11, universal eukaryotic primers (18SF and 18SR) were used. 1: isolate Ph5-01 (host *H.* × *laetiflorus*, Germany, 2001); 2: isolate Ph19-01 (pathotype 100, Hungary, 2001, metalaxyl-sensitive); 3: isolate Ph9-98 (pathotype 310, Germany, 1998, metalaxyl-sensitive); 4: isolate Ph1-00 (pathotype 703, France, 2000, metalaxyl-sensitive); 5: isolate Ph4-93 (pathotype 710, USA, 1993); 6: isolate Ph8-99 (pathotype 730, France, 1999, metalaxyl-resistant); 7: non-template control (water); 8 and 9: *P. halstedii* DNA; 10 and 11: healthy sunflower (*H. annuus*) DNA.

sequenced (Fig. 4; lanes 1–6). Although the *P. halstedii* isolates covered a broad collection range, the viral nucleic acid as well as the deduced amino acid sequences only varied insignificantly. The viral amino acid sequences of Ph4-93, Ph8-98, Ph1-00 and Ph5-01 did not differ at all. In Ph19-01, an isoleucine was replaced by a methionine and in Ph8-99, an alanine substituted a valine.

As far as yet determined, but regardless of their host's pathotype, geographical origin and fungicide sensitivity, all viral characteristics

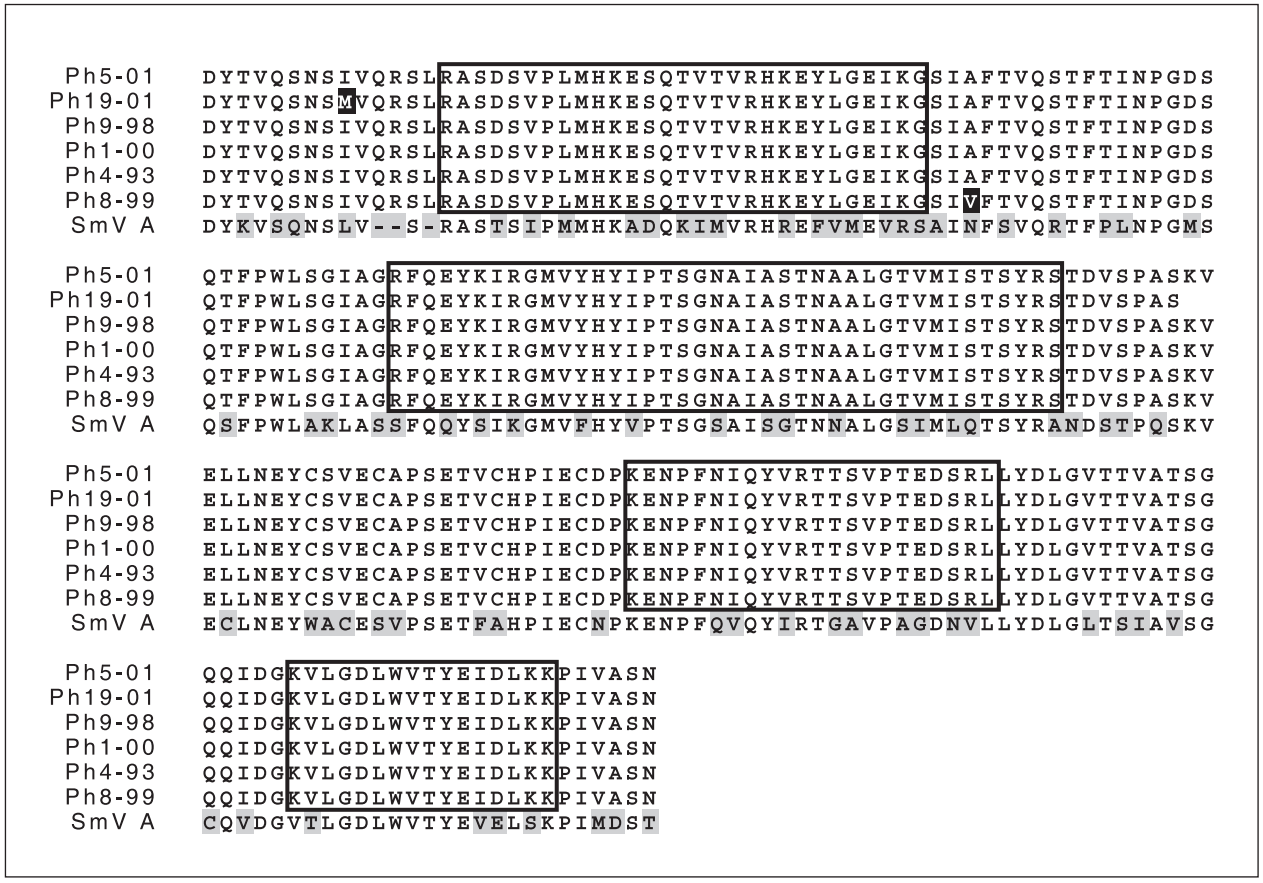


**Fig. 5** Comparison of the deduced amino acid sequences representing partial coat proteins. Six viral isolates of *P. halstedii* (Ph5-01, Ph19-01, Ph9-98, Ph1-00, Ph4-93, Ph8-99) were compared with the amino acid sequence 71–274 of SmV A RNA 2. In black are highlighted the only two amino acids which were different within the *P. halstedii* virus isolates (M instead of I in Ph19-01; V instead of A in Ph8-99). In grey are highlighted the amino acids which were different between the *P. halstedii* virus and SmV A. The framed sequence parts represented fragments of the *c.* 36 kDa-coat protein as found by protein mass spectrometry.

of different *P. halstedii* isolates were identical to one another. Also in agreement with the result of this study were the morphological and biochemical characters which have been previously reported for virions occurring in a single North American pathotype (Gulya *et al.*, 1992; Mayhew *et al.*, 1992). In contrast to *S. macrospora*, in which two isometric viruses showed clear differences in many characters (Honkura *et al.*, 1983; Shirako and Ehara, 1985; Yokoi *et al.*, 1999, 2003), the results of our study suggested the presence of a single virus in *P. halstedii.*

## Relationship of the *P. halstedii* virus to other viruses

Some characteristics between the *P. halstedii* virus and SmV A (Honkura *et al.*, 1983; Shirako and Ehara, 1985; Yokoi *et al.*, 2003), one of the two viruses occurring in *S. macrospora*, were obvious but both virus types are clearly distinguishable. Both viruses were isometric and showed a granular surface. *P. halstedii* virus measured *c.* 37 nm in diameter, whereas SmV A was slightly larger (*c.* 40 nm in diameter) when negatively stained with 2% uranyl acetate solution. Both viral genomes were encoded in ssRNA. In SmV A, three RNA segments were detected, whereas *P. halstedii* virus only contained two segments. RNA 2 of SmV A with a length of 1981 nt coded for the two coat proteins (43 and 39 kDa). In *P. halstedii* virus, a similar sized segment (RNA 2, 1.6 kb) was found to code for the single coat protein (36 kDa). SmV A RNA 1 with 2928 nt coded for the RNA-dependent RNA polymerase (RdRp). In *P. halstedii* virus, RNA 1 with its size of 3 kb might analogously code for the RdRp. The nucleic acid sequence of *P. halstedii* virus, which has been sequenced so far, has not shown any similarity to any known sequences. However, partial amino acid sequences revealed similarities of approximately 58% to a part of the coat protein of SmV A (Fig. 5). To a much lesser extent, *P. halstedii* as well as SmV A showed similarities to amino acid sequences of coat proteins of members within the family Tombusviridae. As only 15% of the *P. halstedii* virus genome has been sequenced so far, more studies are necessary before any concluding remarks can be made regarding the relationship of this virus to others.

### Viral influence on pathogenicity

Little is known on the effects of eumycotic viruses on the infection process of fungal plant pathogens (Ghabrial, 1980; McCabe *et al.*, 1999; Nuss, 2005; Varga *et al.*, 2003) and even less is known about the influence of oomycotic viruses on virulence and infectivity. Only dsRNA of *Ph. infestans* is known to have an influence on its host by enhancing the growth of the oomycete (Tooley *et al.*, 1989). As both less aggressive and more aggressive *P. halstedii* isolates contained virions, the presence or absence of virions could not explain the diverse aggressiveness of the downy mildew pathogen towards sunflower. It was also impossible to correlate a certain virion composition or nucleotide sequence or deduced amino acid sequence to a more aggressive or less aggressive pathotype of *P. halstedii*. Based on data obtained in this study, the uniformity of virions found in different *P. halstedii* pathotypes led to the assumption that pathogenicity of *P. halstedii* was unrelated to characteristics of viral components.

## EXPERIMENTAL PROCEDURES

### Origin and culturing of fungal isolates

As *P. halstedii* is a biotrophic oomycete, it cannot be cultivated on synthetic media. Therefore, a generally susceptible cultivar (*Helianthus annuus* cv. Giganteus) was used to culture all *P. halstedii* isolates (Spring *et al.*, 1997). Virulence studies for *P. halstedii* pathotype characterization were performed using a set of sunflower differential lines (Tourvieille *et al.*, 2000), with a whole seedling inoculation technique (Spring *et al.*, 2003). Downy mildew symptoms were assessed 12–14 days after inoculation and the pathogen was harvested as previously described (Rozynek and Spring, 2001) for subsequent ultrastructural examination and biochemical analysis or for intermediate storage at −18 °C. Isolates of *P. halstedii* used in this study are listed in Table 1. Sporangium samples are deposited in the Oomycetes collection of O. Spring, University of Hohenheim, and stored at −70 °C. Healthy sunflower leaf and hypocotyl tissue of the same *H. annuus* cultivar served as controls in numerous experiments.

Several other oomycotic isolates were screened for VLP occurrence using the negative staining technique. These were *P. angustiterminalis* (host *Xanthium strumarium*; voucher #508: Austria, 2000; #509: Austria, 2001), *P. nivea* (host *Aegopodium podagraria*; #463: Germany, 2002), *P. pusila* (host *Geranium pratense*; #409: Germany, 2001), *P. viticola* (host *Vitis vinifera*; #456: Germany, 2002), *Bremia lactucae* (host *Lactuca sativa*; Haas #20: Germany, 2001), *Peronospora tabacina* (host *Nicotiana tabacum*; #489: Germany, 2002), *Ph. infestans* (host *Solanum tuberosum*; #522 and #524: Germany, 2002) and *Pustula tragopogonis* (syn. *Albugo tragopogonis*, host: *Helianthus annuus*; #567: Germany, 2003). Vouchers of infected plant material are deposited in the Hohenheim herbarium (HUH).

### Ultrathin sections for TEM

*P. halstedii* sporangia were suspended in 0.1 M phosphate buffer, pH 7.2. A double volume of a 2% (w/v) agarose solution was then added to the sporangia suspension. This mixture was cooled on ice. After solidification of the agarose, samples were cut into small cubes, fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, for 1 h at room temperature, and then postfixed for 1 h in 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.2. Samples were then dehydrated in ethanol and finally embedded in LR-White for ultrathin sectioning. Sections were stained with 2% uranyl acetate solution (pH 4) for 10 min and subsequently transferred to lead citrate solution for 1 min (Reynolds, 1963). Healthy sunflower material, which served as a control, and *P. halstedii*-infected sunflower leaf and hypocotyl tissue was cut into small pieces and fixed, dehydrated and stained in the same way.

### Negative staining for TEM

Sporangia of *P. halstedii* and other Oomycetes were ground at full speed in a tissue homogenizer (Ultra-Turrax, IKA, Staufen, Germany) in 0.1 M phosphate buffer, pH 7.2, for 1 min. A drop of the resulting solution was placed on Parafilm M (Pechiney, VWE, Darmstadt, Germany) and covered with a grid. After 30 min of adsorption the grids were stained with 2% uranyl acetate solution (pH 4) for 40 min. Leaf tissue of healthy sunflower plants was treated in the same way and served as a control.

### Transmission electron microscopy

Ultrathin sections and negatively stained samples were examined at 80 kV (EM 10, Zeiss, Oberkochen, Germany).

### Virus extraction and purification

In total, 120–175 g of primary foliage leaves as well as hypocotyl and cotyledon tissue infected with different pathotypes of *P. halstedii* were ground in 0.05 M sodium phosphate buffer, pH 7.0, containing 0.1% (w/v) sodium sulfite and then filtered through cheesecloth. The homogenate was centrifuged at low speed (4000 ***g***, 10 min, 4 °C). The aqueous supernatant was then subjected to ultracentrifugation (75 000 ***g***, 3 h, 4 °C). The resulting pellet was suspended in 0.05 M phosphate buffer with 0.1% (w/v) sulfite and extracted twice with chloroform (1 mL chloroform per 4 mL buffer) to remove contaminants such as RuBisCO and ribosomes (Hull, 1985). The aqueous phase was layered on a 40–10% (w/v) linear sucrose density gradient and centrifuged at 103 900 ***g*** for 3 h (4 °C). The virion-containing fraction was extracted with a syringe,

added to 0.05 M phosphate buffer with 0.1% (w/v) sulfite and again centrifuged (75 000 ***g***, 3 h, 4 °C). The pellet was re-suspended in 0.025 M sodium phosphate buffer, pH 7.0, and the suspension was stored at 4 °C until further analyses. Leaf and hypocotyl tissue of healthy sunflower plants was treated in the same way and used as control. Sample quality was checked spectroscopically ($A_{260}/A_{280}$ ratio) and by RT-PCR experiments using a universal primer set (18SF, 5′-TACCTGGTTGATCCTGCCAGT-3′; 18SR, 5′-TTGATCCTTCTGCAGGTTCACCTAC-3′; White *et al.*, 1990) for eukaryotic organisms to exclude ribosomal RNA contamination. Healthy sunflower DNA, *P. halstedii* DNA and total RNA extracted from *P. halstedii* sporangia served as controls. Control DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA).

### Protein extraction and characterization

The purified virions were dissociated by heating the samples twice to 95 °C for 5 min in 25 mM Tris buffer, pH 7.5, with 10 mM DTT and 2.5 μL PAGE loading buffer (Roti-Load, Carl Roth, Karlsruhe, Germany) per 25 μL sample volume. Protein electrophoresis was performed on 13% polyacrylamide gels (60 V, 2.5 h, aqueous running buffer: 50 mM Tris, 384 mM glycine and 0.1% SDS, pH 8.3; Coomassie blue staining). Molecular markers (Precision Plus Protein Standards All Blue, Bio-Rad, Hercules, CA) were used for sizing. In order to examine the identity and origin of the proteins, the resulting polypeptide bands were analysed after tryptic in-gel digestion by MALDI-TOF/TOF (Autoflex III, Bruker Daltonics) and UPLC-ESI-MS/MS (nano Acquity Ultra Performance Liquid Chromatography, Waters; coupled to an LTQ Orbitrap XL Fourier Transform Mass Spectrometer, Thermo Fisher Scientific) at the Core Facility of the Life Science Center at the University of Hohenheim.

### Nucleic acid extraction, electrophoresis and characterization

Nucleic acid was extracted from purified virions using the Aurum Total RNA Mini Kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. RNA sample quality was checked spectroscopically ($A_{260}/A_{280}$ ratio). The size of the extracted RNA was determined by electrophoresis on native 1% agarose gels (1× TBE buffer; ethidium bromide staining). Before loading onto the gels, the samples were kept for 10 min at 70 °C with the loading dye (2× RNA Loading Dye; Fermentas, St. Leon-Rot, Germany). ssRNA molecular markers (RNA ladder, high range, ready-to-use, Fermentas) were used as size standards and heated in the same way as the samples. The nucleic acid type was determined enzymatically (Hull, 1985) within 15 min at room temperature using 5 μg/mL RNase A (Ribonuclease A, Fermentas, Hanover, MO) in 2× SSC and 0.1× SSC, respectively (2× SSC: 0.3 M sodium chloride and 0.03 M sodium citrate in DEPC-treated water, GIBCO, Invitrogen, Grand Island, NY; pH 7.0). Prior to gel electrophoresis, the RNA samples were desalted using the Aurum Total RNA Mini Kit. Except for the DNA digestion, which was omitted, all steps were carried out according to the manufacturer's instructions. Additionally, RT-PCR experiments using virus-specific primers (2-r4 and 2-f3) were conducted to confirm the nucleic acid type. Capillary electrophoresis was performed on an Agilent 2100 Bioanalyzer using the RNA 6000 Pico LabChip Kit (Agilent Technologies, Böblingen, Germany).

### Primers, cDNA synthesis, PCR and sequencing

A ligation procedure was conducted which had the objective to elongate the viral RNA with known sequences. Adaptors (LiPB, 5′-ACTAGGATCCAAGCTTGGAATTCGTACGTCTAGAGATATC-3′ with a fluorescein blocked 3′ end; LiPC, 5′-CTATAGAGATCTGCATGCT-TAAGGTTCGAACCTAGGATCA-3′ with an amino blocked 5′ end) as well as complementary primers were designed and synthesized. The experiment was carried out according to Weng and Xiong (1995). cDNA synthesis was conducted using the RevertAid First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instructions and n1LiPB primer (5′-GATATCTCTAGACGTACGAATTC-3′) and n1LiPC primer (5′-ATAGAGATCTGCATGCTTAAG-3′). cDNA was amplified by PCR (first denaturing: 94 °C, 3 min; 35 cycles of denaturing: 94 °C, 30 s, annealing: 56 °C for 45 s, elongation: 72 °C for 3.30 min; finale elongation: 72 °C for 6 min) using the primers n1LiPB and n1LiPC. As the first amplification did not result in visible bands on agarose gels (1% agarose gel; 1× TBE buffer; ethidium bromide staining), a nested PCR was conducted using the primers n2LiPB (5′-CGTACGAATTCCAAGCTT-3′) and n2LiPC (5′-CTTAAGGTTC-GAACCTAGGA-3′). A single fragment, most likely a one-primer product, was obtained which was cut out, cloned and amplified in *Escherichia coli* and sequenced in both directions using standard M13 primers. The deduced amino acid sequence from this PCR fragment was identical with amino acid sequences found by protein mass spectrometry of the purified viral coat protein (Fig. 5, framed parts). Virus-specific primers (2-r4 and 2-f3) were designed based on this nucleic acid sequence. Viral RNA of six isolates was transcribed into cDNA using the primer 2-r4 (5′-CGAGACAGTTGCGTTGGA-3′) and a First Strand cDNA Synthesis Kit (Fermentas, Hanover, MO). PCR was conducted using primer 2-f3 (5′-CTGGGTAGTGGAGACTACACA-3′) and primer 2-r4 (first denaturing: 94 °C, 1 min; 30 cycles of denaturing: 94 °C, 30 s, annealing: 50 °C for 1 min, elongation: 72 °C for 1 min; finale elongation: 72 °C for 10 min). After submitting the PCR product to agarose gel electrophoresis, a single fragment of 0.7 kb resulted which was cut off and directly sequenced using the primers 2-f3 and 2-r4, respectively. Thus, both strands were sequenced.

### Sequence analysis

Sequence data were subjected to BLAST analysis and aligned using the software BioEdit (version 7.0.5.3; Hall, 1999). The GenBank accession number for SmV A RNA 2 is AB083061.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Fig. S1** RNA gel electrophoresis of enzymatic determination of RNA strandedness. An ssRNA marker (6000–200 nt) was used. 1:

RNA in 2× SSC solution; 2: RNA in 2× SSC solution with RNase A; 3: RNA in 0.1× SSC solution; 4: RNA in 0.1× SSC solution with RNase A.

**Fig. S2** (a) RNA gel electrophoresis after separation of RNA 1 and the RNA 2. An ssRNA marker (6000–200 nt) was used. 1: viral RNA 2; 2: viral RNA 1. (b) Reverse transcription followed by PCR using the primers RNA 2-r4 and RNA 2-f3. 3: non-template control (water); 4: DNA product of RNA 2; 5: DNA product of RNA 2 (diluted 1 : 2); 6: DNA product of RNA 2 (diluted 1 : 4); 7: DNA product of RNA 2 (diluted 1 : 10); 8: DNA product of RNA 2; 9: DNA product of RNA 1 (diluted 1 : 2); 10: DNA product of RNA 1 (diluted 1 : 4); 11: DNA product of RNA 1 (diluted 1 : 10).

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