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PCR-based detection of sunflower white blister rust (*Pustula helianthicola* C. Rost & Thines) in soil samples and asymptomatic host tissue

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Abstract Sequencing of partial *cox*2 (part of the mitochondrial cytochrome-c-oxydase (COX) gene) was performed with samples from the oomycete genus *Pustula,* the white blister rusts of Asteraceae and related families. Sequence comparison uncovered several single nucleotide polymorphisms (SNPs) between *P. spinulosa* and host specific strains of *Pustula* isolated from *Senecio vulgaris*, *Tragopogon pratensis* and cultivated sunflower, *Helianthus annuus*. Based on these differences, specific primers were designed for PCR-based detection of white blister rust strains pathogenic to sunflower. The specificity of the primers was confirmed by cross testing with DNA from various oomycetes occurring in the same locality. The limit of detection for DNA of *P. helianthicola* was 10 pg. This allowed detection with DNA from single sporangia and single oospores. The PCR-based experiments allowed detection of the presence of sunflower white blister rust in soil samples from fields on which infected plants had been cultivated several months before. Moreover, the molecular tools were successfully applied to trace the pathogen in asymptomatic tissue of infected plants, demonstrating the systemic nature of *Pustula* on sunflower.

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Introduction

Molecular techniques have gained growing importance in the diagnosis of plant pathogens, but have not yet been applied broadly for oomycetes (Spring and Thines 2010). While the exploration for suitable tools and genetic markers in the plant pathogenic Peronosporomycetidae and Albuginomycetidae has primarily focused on phylogenetic and taxonomic aspects, reports on the use of molecular techniques for the solution of epidemiological questions are still sparse. Successful employment of DNA-based markers has been reported for the detection of pathogen contamination in soil (e.g. Wang et al. 2006; Pavon et al. 2008) or in asymptomatic host tissue (e.g. Aegerter et al. 2002; Hukkanan et al. 2006). A particular field of interest is the identification of seed contamination with pathogenic oomycetes for which successful approaches have been published for the downy mildew pathogens of basil (Belbahri et al. 2005) and sunflower (Thines et al. 2004; Ioos et al. 2007). Molecular diagnostic tools were also revealed to be useful in isolate differentiation of sunflower downy mildew (Intelman and Spring 2002; Spring et al. 2006; Delmotte et al. 2008) and oomycete populations of *Plasmopara viticola* (Furuya et al. 2009) and

Peronospora tabacina (Zipper et al. 2009) differing in fungicide sensitivity.

The white blister rust (WBR) of sunflower, caused by *Pustula helianthicola* C.Rost & Thines is a newly described species (Rost and Thines 2011) which was separated from the former species complex of *P. tragopogonis* (syn. *Albugo tragopogonis*) due to molecular phylogenetic reasons and distinctive morphology in the oospore ornamentation. Moreover, in the course of their study, the authors found reasons to abandon the incorrect epitheton "tragopogonis" and proposed the name *P. obtusata* for the WBR pathogenic to species of *Tragopogon*. The genus *Pustula,* which houses pathogens of the so-called asterids, had previously been separated from *Albugo* by Thines and Spring (2005) in a revision of the Albuginaceae. A recent study has shown a significant diversity in the genus *Pustula* (Ploch et al. 2011), and has revealed that *P. obtusata* (as *P. tragopogonis*) is restricted to *Tragopogon*.

Sunflower WBR has become an emerging problem for the crop production over the past 20 years. After it was first observed on sunflower in South Africa (Verwoerd 1929) the pathogen occurred rather sporadically in that area and subsequently reached other continents with the intensification of sunflower production (for references see Thines et al. 2006b). Meanwhile its epidemiological status has changed so that it is regarded as one of the major diseases in sunflower production in South Africa (Van Wyk et al. 1995), South America (Delhey and Kiehr-Delhey 1985) and other areas especially under hot and dry weather conditions. Since suitable molecular tools for molecular detection of *Pustula* are lacking, it was the aim of this study to search for genetic markers that could be used for the detection and diagnosis of sunflower WBR. In this study we have identified the highly variable cox2 gene as a potential candidate for developing diagnostic tools and report on the development of specific primers for a PCR-based identification and monitoring of the pathogen in soil and plant tissue.

Materials and methods

Host plants and *Pustula* isolates

Isolates of *Pustula* from cultivated sunflower (*Helianthus annuus*), *Senecio vulgaris* and *Tragopogon pratensis* as well as isolates of *Pustula spinulosa* (de Bary) Thines from *Cirsium arvense* were gained from field accessions. Collection data are listed in Table 1 and vouchers of the infected plants were deposited in the Herbarium of the University of Hohenheim (HOH).

Pathogen isolation and maintenance

Infected leaf samples from field collections were used for harvesting sporangia for DNA extraction and infection experiments. Sporangia were scratched or rinsed out from pustules after disrupting the epidermis. They were counted in water suspension and diluted to the desired inoculum density. Pathogen cultivation was performed transferring sporangia to detached leaves (5,000 per leaf) of suitable host plants. Inoculated leaves were incubated in Petri dishes on wet filter paper and kept in a climate chamber at 16°C with a 14 h photoperiod. In addition, whole seedling infection of *Helianthus annuus* cv. "Giganteus" was used for the cultivation of sunflower WBR. For these experiments, seedlings were raised for 4 to 5 days on wet filter paper, potted in soil and inoculated with 5,000–10,000 sporangia per plant by placing 50 µl inoculum on the apical bud between the cotyledons. Infection was monitored by pathogen sporulation on the leaf surface, which usually occurred after 10 to 12 days. Fresh sporangia were used for further experiments or stored at −70°C.

DNA isolation

DNA of pathogen and plant material was extracted using the innuPREP Plant DNA extraction kit (AnalyticJena, Jena, Germany) according to the manufacturer's instructions. Sporangiospores of the pathogen samples were disrupted in a mixer mill (9 Hz, 3 min) prior to DNA extraction. For the detection of the pathogen in host tissue, leaf disks of ca 1 cm^2 were excised and treated in the same manner. For the experiments with asymptomatic tissue of infected plants, the material was thoroughly rinsed with water to prevent external contamination with sporangia of the pathogen.

The purified DNA was eluated in 100 µl sterile water and used directly for PCR or stored at −70°C. As controls in PCR experiments, DNA from uninfected host leaf tissue (plants kept under the same culture conditions as infected plants) and from

Table 1 Oomycete isolates used for this study

Sample no HOH [a]…	Taxon	Host	Origin	Collection date
OS 492	*Pseudoperonospora cubensis*	*Cucurbita sativus*	Lichtenwald, Germany	05.08.2002
OS 505	*Albugo candida*	*Capsella bursa-pastoris*	Scharnhausen, Germany	18.09.2002
OS 528	*Pustula helianthicola*	*Helianthus annuus*	Bethlehem, South Africa	15.02.2002
OS 537	*Pseudoperonospora humuli*	*Humulus lupulus*	Tettnang, Germany	01.05.2003
OS 548	*Bremia lactucae*	*Lactuca sativa*	Hohenheim, Germany	01.10.2001
OS 631	*Plasmopara halstedii*	*Helianthus annuus*	Jülich, Germany	11.09.2005
OS 676	*Plasmopara angustiterminalis*	*Xanthium strumarium*	Bicserd, Hungary	18.10.2002
OS 721	*Pustula helianthicola*	*Helianthus annuus*	Scharnhausen, Germany	08.06.2005
OS 726	*Pustula obtusata*	*Tragopogon dubius*	Schwechat, Austria	20.07.2005
OS 730	*Wilsoniana portulacae*	*Portulaca oleracea*	Ludwigshafen, Germany	26.07.2005
OS 732	*Pustula spinulosa*	*Cirsium arvense*	Tübingen, Germany	28.07.2005
OS 765	*Plasmopara viticola*	*Vitis vinifera*	Wien, Austria	20.09.2005
OS 968	*Peronospora tabacina*	*Nicotiana tabacum*	Golta, Germany	20.09.2007
OS 976	*Pustula helianthicola*	*Helianthus annuus*	Plieningen, Germany	11.10.2007
OS 995	*Pustula tragopogonis*.	*Tragopogon pratensis*	Esslingen, Germany	03.07.2008
OS 999	*Pustula helianthicola*	*Helianthus annuus*	Sielmimgen, Germany	20.07.2008
OS 1000	*Pustula helianthicola*	*Helianthus annuus*	Plieningen, Germany	02.07.2008
OS 1001	*Pustula obtusata*	*Tragopogon pratensis*	Hohenheim, Germany	03.07.2008
OS 1012	*Pustula helianthicola*	*Helianthus annuus*	Esslingen, Germany	22.07.2008
OS 1014	*Pustula helianthicola*	*Helianthus annuus*	Sielmimgen, Germany	20.07.2008
OS 1015	*Pustula helianthicola*	*Helianthus annuus*	Echterdingen, Germany	20.07.2008
OS 1029	*Pustula spinulosa*	*Cirsium arvense*	Neuffen, Germany	02.08.2008
OS 1033	*Pustula helianthicola*	*Helianthus annuus*	Scharnhausen, Germany	18.08.2008
OS 1034	*Pustula helianthicola*	*Helianthus annuus*	Scharnhausen2, Germany	18.08.2008
OS 1038	*Pustula helianthicola*	*Helianthus annuus*	Neuhausen, Germany	25.08.2008
OS 1041	*Pustula sp.*	*Senecio vulgaris*	Plieningen, Germany	27.08.2008
OS 1042	*Pustula helianthicola*	*Helianthus annuus*	Bernhausen, Germany	28.08.2008
OS 1043	*Pustula helianthicola*	*Helianthus annuus*	Jebenhausen, Germany	18.07.2008
OS 1048	*Pustula helianthicola*	*Helianthus annuus*	Uhingen, Germany	18.09.2008
OS 1049	*Pustula helianthicola*	*Helianthus annuus*	Holzhausen, Germany	22.09.2008
OS 1055	*Pustula helianthicola*	*Helianthus annuus*	Oberaichen, Germany	09.10.2008
OS 1070	*Pustula helianthicola*	*Helianthus annuus*	Kassel, Germany	10.10.2008
OS 1123	*Hyaloperonospora arabidopsidis*	*Arabidoposis thaliana*	Baisingen, Germany	08.05.2009

[a] HOH, Herbarium of the University of Hohenheim

sporangia of other oomycetes (e.g. *Plasmopara halstedii*) was used.

DNA amplification and sequencing

If not stated otherwise, PCR experiments were carried out in a total volume of 25 µl containing 30 ng of DNA, 0.8 U Mango Taq (Bioline, Luckenwalde, Germany), 20 µg Bovine Serum Albumin Fraction V (Carl Roth GmbH, Karlsruhe, Germany), 50 mM $MgCl_2$, 2 mM dNTPs and 25 mM Primer.

The *cox2* region was amplified using the oomycete specific primers described by Hudspeth et al. (2000) (Table 2). PCR was carried out in an Eppendorf Mastercycler (Eppendorf, Germany) using the following conditions: initial denaturation at 94°C for 4 min followed by denaturation at 94°C for 40 s, annealing 40 s at 52°C, elongation for 40 s at 72°C, 36 cycles

Table 2 Primers used in this study

Primer name	Sequence 5' ➔ 3'	Amplicon size	Ref.
Peter_RubisCO_down	CCAAACGTGAATACCCCCCGAAGC	ca. 760 bp	Chandler and Plunkett 2004
Peter_RubisCO_up	GCTCTACGTCTGGAAGATTTGCGA		
COX2_Hud F	GGCAAATGGGTTTTCAAGATCC	ca. 640 bp	Hudspeth et al. 2000
COX2_Hud R	CCATGATTAATACCACAAATTTCACTAC		
COX2_Hel F3	TTAGAAACTTTTGTACAC	ca. 190 bp	this study
COX2_Hel R2	AAATATCAGAATATTCATAT		this study

repetition for the last three steps. A final extension step was carried out for 4 min at 72°C. The amplification products were analysed on 1.5% agarose gels (19.5× 10 cm; 125 V for 90 min; ethidium bromide staining; documentation under UV 302 nm using the Easy Store system of INTAS, Germany). Alternatively, sample analysis was performed on a microchip capillary electrophoresis (MultiNA, Shimadzu, Duisburg, Germany) according to the protocol of the manufacturer. *Cox*2 amplicons were excised from the agarose gel and extracted using the PerfectPrep Gel Cleanup Kit (Eppendorf, Germany) according to the manufacturer's protocol. The isolated DNA fragments were sequenced by a commercial sequencing provider (GATC biotech, Konstanz, Germany) using the primers applied for PCR. All amplicons were of the expected sequence.

Based on SNPs present in the *cox*2 region shared by sunflower-infecting strains of *Pustula*, as revealed by Ploch et al. (2011), but deviating from all other oomycetes tested, primers for the specific amplification of *Pustula* from sunflower were designed manually (Table 2). Sequences of the *cox*2 region of *Pustula helianthicola* have been deposited in GenBank (GU292165, GU292166, JF793557) by Ploch et al. (2011) and Rost and Thines (2011).

For selective amplification of the partial *cox2* region from sunflower white blister rust, the sequence-specific primers COX2-HelF3 and COX2-HelR2 were used under the same conditions as given above. Nested PCR applification, if necessary to increase sensitivity, can be achieved using 1:10 diluted PCR reactions from the *cox2* amplifications with COX2-Hud F/R for PCR with COX2-Hel F3/R2.

PCR experiments with primers for ribulose-1,5-bisphosphate carboxylase were carried out according to Chandler and Plunkett (2004) and served as a positive control in samples which contained DNA from host and pathogen.

Specificity and sensitivity of the primers

Primer sensitivity was tested using specific amounts of sonication-treated spores of sunflower WBR directly as a template. For these experiments, dry sporangia and oospores were spread on a micoscope slide and were picked manually one by one through adhesion to the tip of a thin needle. Subsequently, they were placed into 12.5 µl of sterile water, sonicated for 2 min (Bransonic B-1200 E2, Branson Ultrasonics, Danbury, USA) and used directly for PCR.

Further experiments were carried out using DNA of sunflower WBR in decreasing amounts from 30 ng to 0.5 pg per sample. The influence of host DNA on the specific amplification of sunflower WBR was tested by using 30 ng DNA of the pathogen in mixtures with increasing amounts of host DNA up to a ratio of 1:1000.

DNA preparation from soil samples

Soil samples were collected in December 2008 from two fields near Stuttgart-Plieningen, Germany, on which sunflower had been cultivated in 2007 and 2008 and where infection with *Pustula* had been detected on leaves. Four samples per plot were randomly taken at different sites from 10 cm deep. Soil from a field in the vicinity, on which no sunflower had been cultivated before, served as a control. The soil was dried and subsequently sieved through metal sieves of 1 mm, 0.5 mm and 0.25 mm mesh. From the final fraction of each sample, 0.5 g of soil was homogenized in a mixer mill (9 Hz; 3 min). The DNA was extracted according to the method described by Michiels et al. (2003). Soil samples (0.5 g) of the control field to which sporangia (1.6 mg) and oospores (~2000), respectively, of sunflower white blister rust had been added prior to homogenization served as additional controls.

Results

Specificity of the PCR-test

The specificity of the primers was tested against DNA from the oomycetes most prevalent in the region. Twelve species belonging to eight genera of the Albuginomycetidae and Peronosporomycetidae were tested (Fig. 1). While the COX2_Hud F/R primers (Hudspeth et al. 2000) gave the expected amplification product with all oomycetes samples (Fig. 1a), the primers COX2 Hel F3/R2 resulted in the amplification of a 190b fragment only in samples from *Pustula* isolated from sunflower (Fig. 1b, lane Pt1–Pt4). This fragment was uniformly present in PCR from 14 additional samples of *Pustula*-infected sunflower collected at different sites in Southern Germany (isolates listed in Table 1; PCR data not shown). *Pustula* isolates from *Senecio vulgaris, Tragopogon dubius* (Fig. 1b, lane Pt5–Pt6) and *T. pratensis* (isolates listed in Table 1; data not shown) gave no amplification products with the COX2 Hel F3/R2 primers. Slight cross reactions

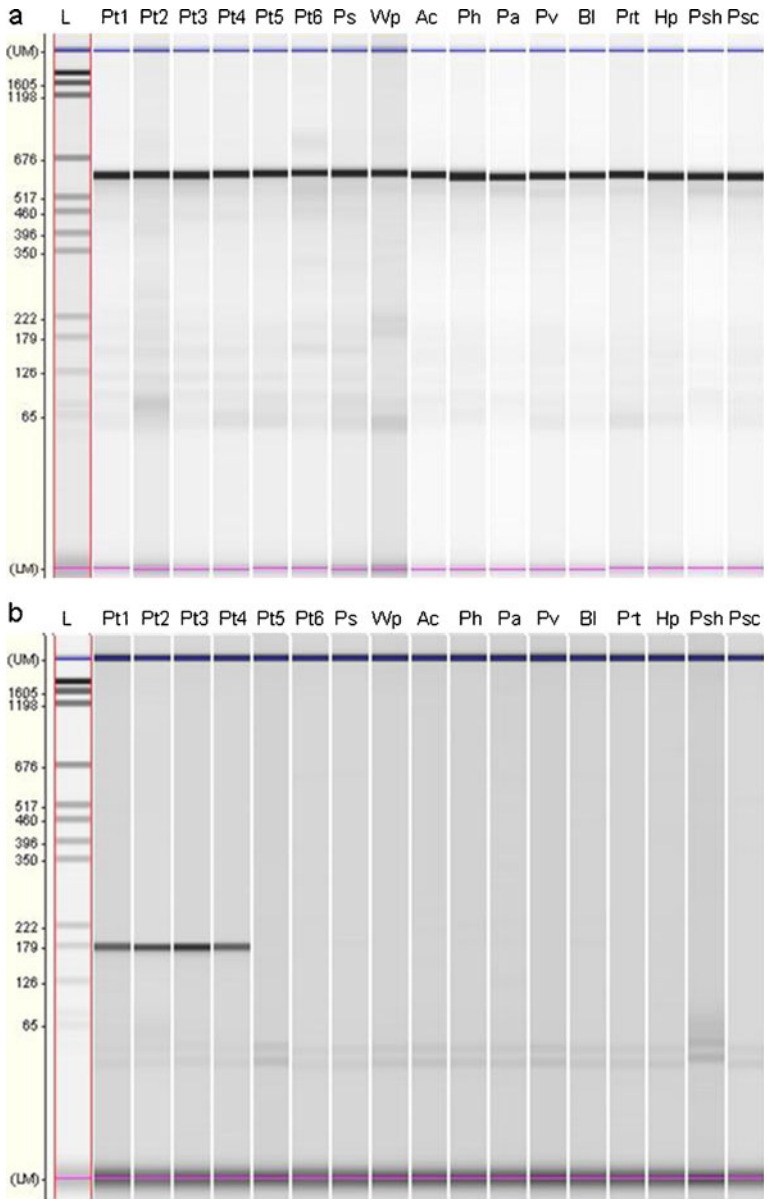

Fig. 1 Amplification products obtained with the primers (**a**) COX2_Hud F/R and (**b**) COX2 HelF3/R2 in PCRs with DNA of different plant pathogenic oomycetes. Sample analysis by microchip capillary electrophoresis. Lanes: Ladder (L; size marker MultiNA Shimadzu); *Pustula helianthicola* from *H. annuus* (Pt1–Pt4, sample HOH 528, 721, 1033, 1070); *P. obtusata* from *T. pratensis* (Pt5, sample HOH 726); *Pustula sp.* from *S. vulgaris* (Pt6, sample HOH 1041); *P. spinulosa* from *C. arvense* (Ps, sample HOH 732); *W. portulacae* from *P. oleracea* (Wp, sample HOH 730); *A. candida* from *C. bursa-pastoris* (Ac, sample HOH 505); *P. halstedii* from *H. annuus* (Ph, sample HOH 631); *P. angustiterminalis* from *X. strumarium* (Pa, sample HOH 676); *P. viticola* from *V. vinifera* (Pv, sample HOH HUH 765); *B. lactucae* from *L. sativa* (Bl, sample HOH HUH 548); *P. tabacina* from *N. tabacum* (Prt, sample HOH 968); *H. arabidopsidis* from *A. thaliana* (Hp, sample HOH 1123); *P. humuli* from *H. lupulus* (Psh, sample HOH 537); *P. cubensis* from *C. sativus* (Psc, sample HOH 492)

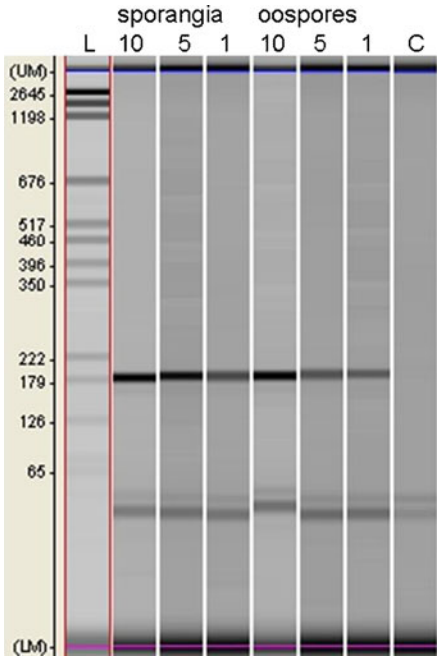

Fig. 2 Direct PCR with COX2 HelF3/R2 on decreasing numbers (10, 5, 1) of sporangia and oospores. L, ladder; C, water control

with DNA of *P. spinulosa* and *B. lactucae* at low annealing temperatures (below 50°C) disappeared when the PCR was carried out above 51°C.

In order to assess putative cross reaction with DNA of the host plants, samples from uninfected *H. annuus*, *T. pratensis, Senecio vulgaris* and *Cirsium arvense* were tested as well, but gave no amplification products (data not shown).

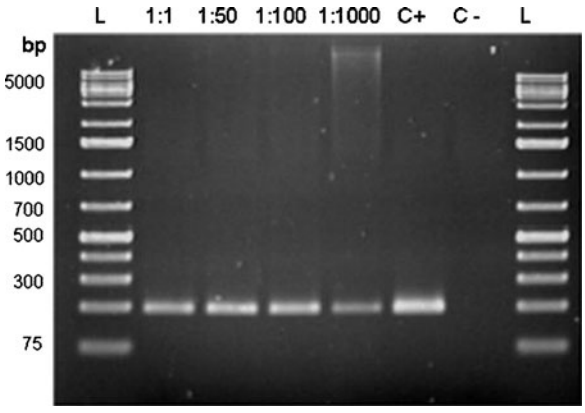

Fig. 3 Influence of non-target host DNA on the sensitivity of *Pustula* detection with COX2 HelF3/R2. Pathogen DNA (30 ng) was mixed with increasing amounts of host DNA. Lane1-4: 1:1; 1:50, 1:100 and 1:1000. C+, 30 ng of pure *Pustula* DNA; C-, water control, L, DNA ladder

Sensitivity of the PCR-test

The sensitivity of the PCR with COX2 Hel F3/R2 was tested with decreasing amounts of DNA from sample HOH OS1042 in the range of 30 ng to 0.5 pg total DNA.

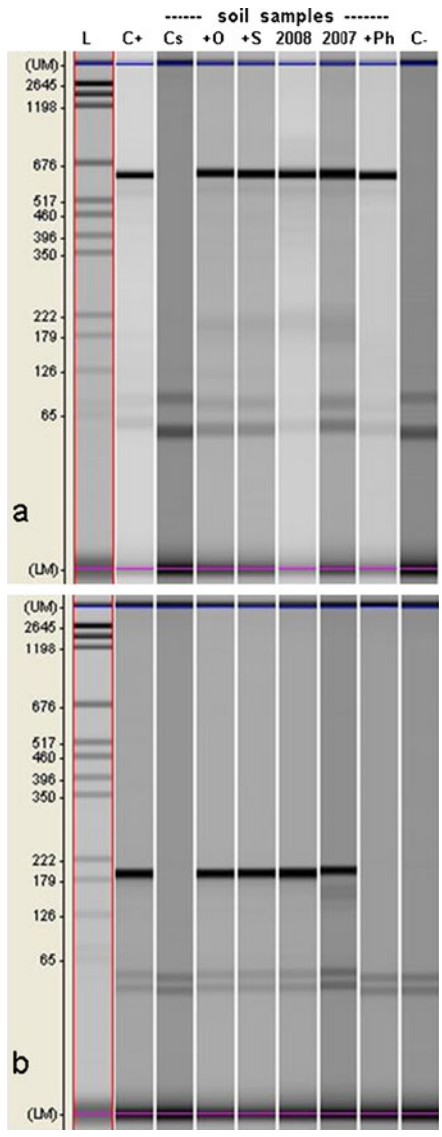

Fig. 4 Pathogen detection in PCR of soil samples. Lanes: L, ladder; C+, sporangia of HOH 1070; (Cs), soil from control field without previous sunflower cultivation; (+O) control soil contaminated with oospores (2,000 per 0.5 g of soil); (+S), control soil contaminated with sporangia 1.6 mg per 0.5 g of soil; (2008), soil from field with sunflower cultivation in 2008; (2007), soil from field with sunflower cultivation in 2007, (+Ph), control soil contaminated with sporangia of *P. halstedii;* (C-); water control. Upper part (**a**) shows amplicons obtained with COX2, lower part (**b**) shows amplicons with COX2 HelF3/R2

The expected amplification product of 190 bp in length was detected in all samples which contained at least 10 pg DNA of sunflower WBR (data not shown).

Moreover, when sporangia and oospores were directly used in PCR (for details see Materials and Methods), the 190b fragment was detected in samples which contained the DNA of a single spore (Fig. 2). This equals the DNA content of ca. 10 to 15 nuclei for sporangia (the number of nuclei in mature oospores is not known).

The influence of high amounts of host DNA on the sensitivity of the PCR-based detection of *Pustula* with COX2 Hel F3/R2 was tested by mixing 30 ng of sporangial DNA with increasing amounts of sunflower DNA (Fig. 3). The pathogen specific fragment was still detected at a ratio of 1:1000 of target to non-target DNA

Identification of oospores in soil samples

Soil samples from two field plots on which *Pustula*-infected sunflower had been observed in 2007 and 2008, respectively, were taken in December of 2008 for tracing contamination with oospores of the pathogen which could lead to primary infection in the following season. PCR gave amplification products for oomycetes in seven out of eight samples when the COX2 primers were used (one sample out of four from the 2007 field was negative). All four samples of 2008

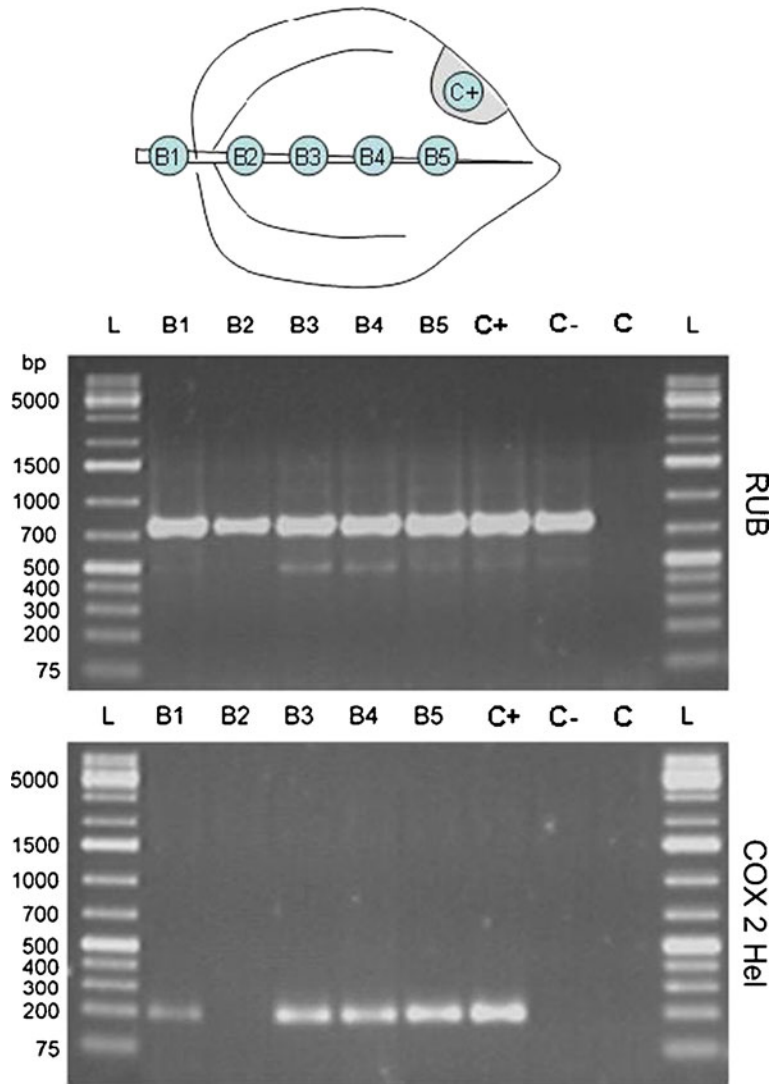

Fig. 5 Pathogen detection in asymptomatic host tissue in PCR with COX2 HelF3/R2. DNA samples: C+, leaf area with pustules, B1 to B5, symptomless areas of an infected leaf (positions as indicated in the scheme above the gel); C- leaf sample of uninfected plant; C, water control; L, DNA ladder. PCR with primers for Rubisco (RUB) served as control for the functionality of the DNA samples

and two of the samples of 2007 gave amplification products specific for sunflower WBR with COX2 Hel F3/R2 primers. Among the two negative samples, one had been tested positive with COX2 primers, thus indicating the presence of oomycetes, but different from sunflower WBR. A similar effect was achieved, when sporangia of the sunflower downy mildew pathogen *Plasmopara halstedii* was added to uninfested soil as shown in lane "+Ph" of Fig. 4. Soil from a field in which no sunflower had previously been cultivated served as a control and gave no amplification products (Fig. 4, lane Cs). The detection of sporangia and oospores from soil of controlled contamination experiments showed that the PCR-based pathogen test was not inhibited by the soil (Fig. 4; lane + S and + O).

Pathogen detection in asymptomatic host tissue

PCR experiments with COX2 Hel F3/R2 on DNA samples of infected and uninfected sunflower tissue were carried out in order to assess the presence or absence of the pathogen in asymptomatic plant parts. In a naturally infected leaf, which showed pustules on the distal margin, tissue samples of ca 1 cm^2 were taken along the central vein down to the petiole for DNA extraction and subsequent PCR. In four of the five asymptomatic samples the pathogen was traced by PCR with COX2 Hel F3/R2 (Fig. 5). Microscopic studies of the midrib close to the petiole (area close to B1 and B2 in the scheme of Fig. 5) identified hyphae with oospores of *Pustula,* thus confirming the presence of the pathogen in plant tissue which does not show infection symptoms such as pustules, chlorosis or necrosis.

Discussion

White blister rust of sunflower, which previously was known as a major disease of this crop only in areas with predominantly hot and dry seasons (Van Wyk et al. 1995), has started to spread in Germany over several years (Thines et al. 2006a). The origin of the pathogen in Germany is unknown, but the introduction with contaminated seed is most likely since Viljoen et al. (1999) found oospores in seeds of the crop, which can serve as inoculum for the primary infection. This hypothesis is supported by morphological, chemical and molecular genetic characteristics which revealed high similarity among isolates of the pathogen on sunflower from three different continents (Thines et al. 2006b). On the other hand, several taxa of *Pustula* are prevalent in Central Europe on common hosts of *Cirsium*, *Senecio* or *Tragopogon* species. Their ability to infect sunflower under favourable conditions has not yet been tested. Hence, molecular diagnostic tools to distinguish sunflower white blister rust from its closest relatives and to identify contamination of seed samples, plants or soil would be desirable.

Hudspeth et al. (2000, 2003) had shown the usefulness of *cox2* sequence variation for the identification of phylogenetic traits in the Peronosporomycetes and for the delineation of Albuginaceae. The current results have shown that SNPs in the *cox2* region specific for *Pustula helianthicola* infecting sunflower can be used for the development of a PCR-based detection method of the pathogen. The use of a mitochondrial gene as a template is advantageous, because the number of copies per cell is high, thus increasing the sensitivity of the diagnostic test. In addition, the sensitivity can be enhanced by using the specific primers COX 2 Hel F3/R2 for nested PCR with samples which had been amplified with oomycete-specific primers for the *cox2* gene beforehand.

The use of these primers on soil samples of sunflower fields has shown the presence of oomycete DNA in seven out of eight samples, whereas DNA of sunflower WBR was found in only six of these. Besides possible soil contamination with other oomycetes such as *Pustula spinulosa* from infected *Cirsium arvense* or *Bremia lactucae* from *Sonchus* sp., *Plasmopara halstedii* may have contributed to the COX2 amplicons. The latter pathogen often co-occurs with white blister rust in sunflower fields and frequently infects the same host individual simultaneously (Spring 2009). But also soil-borne *Phytophthora* or *Pythium* species are a potential source of the *cox2* amplicons.

The amplicons derived from PCR with COX2_Hel_F3/R2 on DNA from asymptomatic plant tissue, as expected, revealed that *Pustula* infection of sunflower is not restricted to the sites where mitotic sporulation occurs, forming the characteristic subepidermal blisters. Unlike sunflower downy mildew, which causes growth inhibition and chlorosis in all infested plant parts, *Pustula* infection of sunflower appears to expand systemically without such macroscopically visible symptoms. Oospores are frequently formed in the vascular system of seemingly healthy parts of the leaf,

demonstrating the ability of the pathogen to asymptomatically persist and reproduce in sunflower leaves. We often observed huge amounts of oospores in petioles of leaves on which only small blisters at the far distant tip visually indicated the presence of the pathogen.

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