Development of Pathotype-Specific SCAR Markers for Detection of *Verticillium albo-atrum* Isolates from Hop

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ABSTRACT

Radišek, S., Jakše, J., and Javornik, B. 2004. Development of pathotype-specific SCAR markers for detection of *Verticillium albo-atrum* isolates from hop. Plant Dis. 88:1115-1122.

Rapid polymerase chain reaction (PCR) assays were developed for the identification and detection of *Verticillium albo-atrum* hop pathotypes PG1 and PG2 from Slovenia. Of 17 pathotype-linked amplified fragment length polymorphism (AFLP) markers, 11 were cloned successfully and sequenced. To convert polymorphic AFLP markers into pathotype-specific sequence-characterized amplified region (SCAR) markers, 22 PG2- and 10 PG1-specific primer pairs were designed from 16 sequences. When primer specificity was tested on a wide range of *Verticillium* isolates, 10 PG2- and 6 PG1-specific primer pairs retained amplification specificity for *V. albo-atrum* Slovene hop isolates, but also amplified sequences in *V. albo-atrum* and *V. dahliae* hop isolates from different hop production areas in Europe, as well as in some isolates from other hosts. Primer combinations obtained from the AFLP-9-1 marker were specific only for *V. albo-atrum* PG2 isolates. The highly specific primers were used in multiplex PCR and a nested PCR to detect the *V. albo-atrum* PG2 pathotype in xylem tissue of hop plants. These new SCAR markers provide a valuable tool for rapid identification of *V. albo-atrum* PG1 and PG2 hop pathotypes.

Additional keywords: hop wilt, *Humulus lupulus*

Verticillium albo-atrum Reinke & Berthier and *V. dahliae* Kleb. are important plant-pathogenic fungi causing vascular wilts in many crop species (9). In Europe, these two soilborne fungi have caused considerable economic damage on hop (*Humulus lupulus* L.), with *V. albo-atrum* being the principal pathogen in most hop-growing areas (21). Hop is a perennial plant that needs an extensive support system for cultivation; therefore, contamination of hop gardens with *Verticillium* spp. is a major limiting factor in hop production. Due to the lack of effective chemical control, crop rotation, planting resistant or tolerant cultivars, and carrying out phytosanitary measures are the only successful means of preventing *Verticillium* wilt.

Verticillium wilt on hops has appeared in fluctuating (mild) or progressive (lethal) forms, depending on the pathogen virulence, the sensitivity of cultivars, and ecological factors (13,26,29). The main differ-

ence between the forms is that the lethal one causes hop to wither, whereas plants continue to grow with the mild form. In England, three types of lethal *V. albo-atrum* isolates (PV1, PV2, and PV3) have been reported, which can be distinguished on the basis of pathogenicity tests on different sets of hop cultivars (27).

Slovenia has three main hop-production areas: Savinja Valley (1,000 ha), the Koroska area (400 ha), and the Podravje area (300 ha). The first record of *Verticillium* wilt in Slovenia was in 1974, when the mild form was detected as a cause of infection by *V. albo-atrum* and *V. dahliae* (8). Hop wilt appeared only sporadically in some hop gardens until 1997, when an outbreak of the lethal form was reported in the western part of the Savinja valley and *V. albo-atrum* was identified as the casual agent. Over the next 6 years, new outbreaks of the lethal form were detected in a radius of 7 km from the first outbreak and, to date, more than 180 ha of hop gardens have been affected (Annual Report for 2003: Monitoring Survey of Hop Wilt [*Verticillium albo-atrum* and *V. dahliae*] in 2003. MAFF; Republic of Slovenia, unpublished). On the basis of pathogenicity tests and molecular analysis, *V. albo-atrum* hop isolates from Slovenia have been classified as PG1 (mild) and PG2 (lethal) pathotypes (21). The spread of the PG2 pathotype is a serious threat to Slovene hop production, in view of the close prox-

imity of hop gardens to each other in all production areas and the prevalence of susceptible hop cultivars.

The identification and rapid detection of pathotypes is very important for adequate disease management and for resistance breeding. *V. albo-atrum* hop isolates have been best characterized by pathogenicity tests (5,27). These bioassays are laborious and time-consuming; therefore, the development of a more rapid method is required. In the last decade, various molecular genetic techniques have been applied for the characterization and detection of *Verticillium* spp. Differences in internal transcribed spacer (ITS) regions ITS1 and ITS2 of nuclear rDNA enable the development of polymerase chain reaction (PCR) primers specific for the identification of *V. albo-atrum*, *V. dahliae*, and *V. tricorpus* (17,19,24). Specific primers have been used for the detection and quantification of *Verticillium* biomass in different host plants and in soil samples (11,12,16,18,23). However, specific primers for the detection of *V. albo-atrum* hop isolates have not yet been developed. In our study on the molecular variability of *V. albo-atrum* isolates, 17 amplified fragment length polymorphism (AFLP) markers linked to the virulence of hop isolates from Slovenia were identified (22), providing potential markers to be converted into specific, single-locus diagnostic markers (20).

Our objective in this study was to develop specific sequence-characterized amplified region (SCAR) markers for the determination of *V. albo-atrum* PG1 and PG2 hop pathotypes. We also report here on the use of specific SCAR primer pairs in multiplex PCR for distinguishing the two pathotypes, and in nested PCR assays for the detection of these pathotypes in hop plants.

MATERIALS AND METHODS

Fungal isolates. In all, 92 *Verticillium* isolates were obtained from different geographical locations in Europe and North America (Table 1). Of the *V. albo-atrum* isolates, 55 were from infected hop plants from important hop production areas in Europe (34 from Slovenia, 15 from the United Kingdom, 5 from Germany, and 1 from Poland). The other isolates represented different host plants, geographic locations, or *Verticillium* spp. (*dahliae*,

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Table 1. Fungal isolates used in this study and the amplification specificity of sequence-characterized amplified region (SCAR) markers^a

Species	Isolate designation	Host or substrate ^b	PG ^c	Geographical origin	Source ^d	S-1		S-2		S-5-1		S-9-1		S-9-2		S-10-1		S-10-2a		S-11	
						EXT	INT	EXT	INT	EXT	INT	EXT	INT	EXT	INT	EXT	INT	EXT	INT	EXT	INT
Verticillium spp.																					
V. albo-atrum	Or00	Hop	PG2	Slovenia	IHPS	-	-	-	-	+	+	+	+	+	+	-	-	+	+	+	+
V. albo-atrum	Prev	Hop	PG2	Slovenia	IHPS	-	-	-	-	+	+	+	+	+	+	-	-	+	+	+	+
V. albo-atrum	Or99	Hop	PG2	Slovenia	IHPS	-	-	-	-	+	+	+	+	+	+	-	-	+	+	+	+
V. albo-atrum	Pust	Hop	PG2	Slovenia	IHPS	-	-	-	-	+	+	+	+	+	+	-	-	+	+	+	+
V. albo-atrum	Vran	Hop	PG2	Slovenia	IHPS	-	-	-	-	+	+	+	+	+	+	-	-	+	+	+	+
V. albo-atrum	Laz	Hop	Lethal (n.t.) ^e	Slovenia	IHPS	-	-	-	-	+	+	+	+	+	+	-	-	+	+	+	+
V. albo-atrum	Tr98	Hop	PG2	Slovenia	IHPS	-	-	-	-	+	+	+	+	+	+	-	-	+	+	+	+
V. albo-atrum	Roz	Hop	PG2	Slovenia	IHPS	-	-	-	-	+	+	+	+	+	+	-	-	+	+	+	+
V. albo-atrum	Ciz	Hop	PG2	Slovenia	IHPS	-	-	-	-	+	+	+	+	+	+	-	-	+	+	+	+
V. albo-atrum	Ranc	Hop	PG2	Slovenia	IHPS	-	-	-	-	+	+	+	+	+	+	-	-	+	+	+	+
V. albo-atrum	Pov	Hop	PG2	Slovenia	IHPS	-	-	-	-	+	+	+	+	+	+	-	-	+	+	+	+
V. albo-atrum	Tr99	Hop	PG2	Slovenia	IHPS	-	-	-	-	+	+	+	+	+	+	-	-	+	+	+	+
V. albo-atrum	Jer	Hop	PG2	Slovenia	IHPS	-	-	-	-	+	+	+	+	+	+	-	-	+	+	+	+
V. albo-atrum	Rot	Hop	PG2	Slovenia	IHPS	-	-	-	-	+	+	+	+	+	+	-	-	+	+	+	+
V. albo-atrum	T2	Hop	PG2	Slovenia	IHPS	-	-	-	-	+	+	+	+	+	+	-	-	+	+	+	+
V. albo-atrum	T6	Hop	Lethal (n.t.) ^e	Slovenia	IHPS	-	-	-	-	+	+	+	+	+	+	-	-	+	+	+	+
V. albo-atrum	Vran01	Hop	PG2	Slovenia	IHPS	-	-	-	-	+	+	+	+	+	+	-	-	+	+	+	+
V. albo-atrum	Led	Hop	PG2	Slovenia	IHPS	-	-	-	-	+	+	+	+	+	+	-	-	+	+	+	+
V. albo-atrum	Pov02	Hop	Lethal (n.t.) ^e	Slovenia	IHPS	-	-	-	-	+	+	+	+	+	+	-	-	+	+	+	+
V. albo-atrum	Pec	Hop	Lethal (n.t.) ^e	Slovenia	IHPS	-	-	-	-	+	+	+	+	+	+	-	-	+	+	+	+
V. albo-atrum	Rov	Hop	PG2	Slovenia	IHPS	-	-	-	-	+	+	+	+	+	+	-	-	+	+	+	+
V. albo-atrum	Roj	Hop	Lethal (n.t.) ^e	Slovenia	IHPS	-	-	-	-	+	+	+	+	+	+	-	-	+	+	+	+
V. albo-atrum	Nat	Hop	PG2	Slovenia	IHPS	-	-	-	-	+	+	+	+	+	+	-	-	+	+	+	+
V. albo-atrum	Slan	Hop	Lethal (n.t.) ^e	Slovenia	IHPS	-	-	-	-	+	+	+	+	+	+	-	-	+	+	+	+
V. albo-atrum	Drc	Hop	PG2	Slovenia	IHPS	-	-	-	-	+	+	+	+	+	+	-	-	+	+	+	+
V. albo-atrum	Urs	Hop	Lethal (n.t.) ^e	Slovenia	IHPS	-	-	-	-	+	+	+	+	+	+	-	-	+	+	+	+
V. albo-atrum	Jel	Hop	PG2	Slovenia	IHPS	-	-	-	-	+	+	+	+	+	+	-	-	+	+	+	+
V. albo-atrum	Ocer	Hop	PG1	Slovenia	IHPS	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
V. albo-atrum	Kres99	Hop	PG1	Slovenia	IHPS	+	+	+	+	-	-	-	-	-	-	+	+	-	-	-	-
V. albo-atrum	CasA	Hop	PG1	Slovenia	IHPS	+	+	+	+	-	-	-	-	-	-	+	+	-	-	-	-
V. albo-atrum	Zup	Hop	PG1	Slovenia	IHPS	+	+	+	+	-	-	-	-	-	-	+	+	-	-	-	-
V. albo-atrum	Rec	Hop	PG1	Slovenia	IHPS	+	+	+	+	-	-	-	-	-	-	+	+	-	-	-	-
V. albo-atrum	Mo3	Hop	PG1	Slovenia	IHPS	+	+	+	+	-	-	-	-	-	-	+	+	-	-	-	-
V. albo-atrum	KresCer	Hop	PG1	Slovenia	IHPS	+	+	+	+	-	-	-	-	-	-	+	+	-	-	-	-
V. albo-atrum	11041	Hop	n.t.	Great Britain	1	-	-	-	-	+	+	-	-	+	-	-	+	+	-	-	-
V. albo-atrum	11047	Hop	n.t.	Great Britain	1	-	-	-	-	+	+	-	-	+	-	-	+	+	-	-	-
V. albo-atrum	11052	Hop	n.t.	Great Britain	1	-	-	-	-	+	+	-	-	+	-	-	+	+	-	-	-
V. albo-atrum	11055	Hop	n.t.	Great Britain	1	-	-	-	-	+	+	-	-	+	-	-	+	+	-	-	-
V. albo-atrum	11056	Hop	n.t.	Great Britain	1	-	-	-	-	+	+	-	-	+	-	-	+	+	-	-	-
V. albo-atrum	11097	Hop	n.t.	Great Britain	1	-	-	+	-	+	+	-	-	+	-	-	+	+	-	-	-
V. albo-atrum	298092	Hop	Fluctuating	Great Britain	CABI	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
V. albo-atrum	298095	Hop	Fluctuating	Great Britain	CABI	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
V. albo-atrum	298099	Hop	Lethal	Great Britain	CABI	-	-	-	-	+	+	-	-	+	-	-	-	+	+	-	-
V. albo-atrum	298100	Hop	Lethal	Great Britain	CABI	-	-	-	-	+	+	-	-	+	-	-	-	+	+	-	-
V. albo-atrum	298101	Hop	Lethal; supvir	Great Britain	CABI	-	-	-	-	+	+	-	-	+	-	-	-	+	+	-	-
V. albo-atrum	298102	Hop	Lethal; supvir	Great Britain	CABI	-	-	-	-	+	+	-	-	+	-	-	-	+	+	-	-
V. albo-atrum	1974	Hop	Lethal	Great Britain	2	-	-	-	-	+	+	-	-	+	-	-	-	+	+	-	-
V. albo-atrum	1985	Hop	Lethal	Great Britain	2	-	-	-	-	+	+	-	-	+	-	-	-	+	+	-	-
V. albo-atrum	1953	Hop	Fluctuating	Great Britain	2	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
V. albo-atrum	Sol	Hop	n.t.	Poland	3	+	+	+	+	-	-	-	-	-	-	+	+	-	-	-	-
V. albo-atrum	1140	Hop	n.t.	Germany	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
V. albo-atrum	16/00	Hop	n.t.	Germany	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
V. albo-atrum	14/93	Hop	n.t.	Germany	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
V. albo-atrum	6/99	Hop	n.t.	Germany	4	+	+	+	+	-	-	-	-	-	-	+	+	-	-	-	-
V. albo-atrum	15/98	Hop	n.t.	Germany	4	+	+	+	+	-	-	-	-	-	-	+	+	-	-	-	-
V. albo-atrum	11	Alfalfa	n.t.	Canada	5	-	-	-	-	+	+	-	-	+	-	-	-	-	-	+	+
V. albo-atrum	41	Alfalfa	n.t.	Canada	5	-	-	-	-	+	+	-	-	+	-	-	-	-	-	+	+
V. albo-atrum	107	Alfalfa	n.t.	United States	5	-	-	-	-	+	+	-	-	+	-	-	-	-	-	+	+
V. albo-atrum	140	Alfalfa	n.t.	United States	5	-	-	-	-	+	+	-	-	+	-	-	-	-	-	+	+

(continued on next page)

^a EXT and INT: SCAR markers generated from the external and internal part, respectively, of the AFLP marker; SCAR marker amplified (+) or not amplified (-).^b Alfalfa (*Medicago sativa*), Cave cricket (*C. cricket*; *Troglophilus neglectus*), Chrysanthemum (*Chrys*; *Chrysanthemum* sp.), Cucumber (*Cucumis sativus*), Fat-hen (*Chenopodium album*), Green pepper (G. pepper; *Capsicum annuum*), Hop (*Humulus lupulus*), Japanese maple (J. maple; *Acer palmatum*), Linden (*Tilia* sp.), Petunia (*Petunia hybrida*), Potato (*Solanum tuberosum*), Southern catalpa (*S. catalpa*; *Catalpa bignonioides*), Tomato (*Lycopersicon esculentum*), and ... = unknown.^c PG = pathogenicity group; Supvir = supervirulent, Vir = virulent, Avr = avirulent, and R tom = on resistant tomato.^d IHPS = Slovenian Institute for Hop Research and Brewing, Žalec, Slovenia; CABI = CABI Bioscience, Genetic Resources Collection, Bakeham Lane, Surrey, UK; 1 = Dr. G. Down, Disease Management, Horticulture Research International, West Malling, UK; 2 = Dr. D. J. Barbara, Horticulture Research International, Wellesbourne, Warwick, UK; 3 = Dr. E. Solarska, Institute of Soil Science and Plant Cultivation, Pulawy, Poland; 4 = Dr. E. Seigner, Bayer Landesanstalt für Bodenkultur und Pflanzenbau, Freising, Germany; 5 = Dr. J. Robb, University of Guelph, Molecular Biology and Genetics, Canada; 6 = Dr. R. Cooper, University of Bath, Department of Biology and Biochemistry, Bath, UK; 7 = Dr. J. K. Goud, Biological Farming Systems, Wageningen, Holland; MZKI = Microbiological Culture Collection of the National Institute of Chemistry, Ljubljana, Slovenia.^e Isolates from plants with lethal form of Verticillium wilt, not tested (n.t.) by pathogenicity test.

tricornis, *lecani*, *luteoalbum*, and *theobromae*). In addition, some common saprotrophic soil fungi also were included (Table 1).

Of the 34 Slovene hop isolates, 27 had been characterized as PG1 or PG2 pathotypes by pathogenicity tests based on the disease reaction of four hop cultivars (22). The remaining seven hop isolates were considered to be lethal in view of the symptoms on the plants from which they were isolated. Pathogenicity characterization of other isolates (Table 1) was specified by the supplier.

All isolates used in this study were maintained in the culture collection of the Slovenian Institute for Hop Research and Brewing, Slovenia, as monospore cultures on potato dextrose agar (PDA; Fluka, Buchs, Switzerland) at 4°C, or stored as

cultures in general fungal medium (31) in 20% glycerol at -80°C. The species identification of isolates was checked by examining them using light microscopy.

Plant inoculation. *V. albo-atrum* hop isolates Tr99 (pathotype PG2) and KresCer (pathotype PG1) were used to inoculate 6-week-old plants of susceptible hop cvs. Fuggle and Celeia, moderately resistant Wye Challenger, and resistant cv. Wye Target. Five plants of each cultivar grown as a single bine were inoculated by stem-puncture inoculation in the basal part of the bine. Control plants were inoculated similarly with sterile distilled water. Inoculum preparation and symptom assessment were carried out according to Radišek et al. (22). For re-isolation, the xylem tissue 10 to 20 cm above the inoculation point

was plated on PDA. The remaining part of the bine was stored at -80°C for DNA extraction. Plants from the above experiment were used for in planta PCR detection of the *V. albo-atrum* PG2 hop pathotype.

Fungal and plant DNA extraction. To obtain mycelia for DNA extraction, cultures were grown by agitation on a rotary shaker (50 rpm) in 50 ml of general fungal medium (31) at room temperature for 5 days in the dark. Fungal genomic DNA was extracted by the sodium dodecyl sulfate (SDS) method of Lee and Taylor (15), with some modifications as described previously (22). Total genomic DNA from infected and noninfected hop plants was extracted by the cetyltrimethylammonium bromide (CTAB) method of Kump and

Table 1. (continued from preceding page)

Species	Isolate designation	Host or substrate ^b	PG ^c	Geographical origin	Source ^d	S-1		S-2		S-5-1		S-9-1		S-9-2		S-10-1		S-10-2a		S-11	
						EXT	INT	EXT	INT	EXT	INT	EXT	INT	EXT	INT	EXT	INT	EXT	INT	EXT	INT
<i>V. albo-atrum</i>	Luc	Alfalfa	Vir	Great Britain	6	-	-	-	-	+	+	-	-	+	-	-	-	-	-	+	+
<i>V. albo-atrum</i>	Surf	Petunia	n.t.	Slovenia	IHPS	+	+	+	+	-	-	-	-	-	-	+	+	-	-	-	-
<i>V. albo-atrum</i>	Kum	Cucumber	n.t.	Slovenia	IHPS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>V. albo-atrum</i>	11066	Potato	n.t.	Great Britain	1	-	-	-	-	+	+	-	-	+	-	-	+	+	-	-	-
<i>V. albo-atrum</i>	11077	Fat-hen	n.t.	Great Britain	1	-	-	-	-	+	+	-	-	+	-	-	+	+	-	-	-
<i>V. albo-atrum</i>	11081	Chrys	n.t.	Great Britain	1	-	-	-	-	+	+	-	-	+	-	-	+	+	-	-	-
<i>V. albo-atrum</i>	AR01/067	Tomato	Vir, R tom	Great Britain	2	-	-	-	-	+	+	-	-	+	+	-	+	+	+	+	+
<i>V. albo-atrum</i>	AR0/140	Tomato	n.t.	Great Britain	2	-	-	-	-	+	+	-	-	+	+	-	+	+	+	+	+
<i>V. albo-atrum</i>	AR01/JS1	Tomato	n.t.	Great Britain	2	-	-	-	-	+	+	-	-	+	-	+	+	+	+	-	-
<i>V. albo-atrum</i>	T179	Tomato	Avr, R tom	Great Britain	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>V. albo-atrum</i>	PD2000/4186a	Tomato	n.t.	Holland	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>V. albo-atrum</i>	PD83/53a	Tomato	n.t.	Holland	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>V. dahliae</i>	CasD	Hop	n.t.	Slovenia	IHPS	+	+	-	-	+	+	-	-	-	-	+	+	-	-	-	-
<i>V. dahliae</i>	MoD	Hop	n.t.	Slovenia	IHPS	+	+	-	-	+	+	-	-	-	-	+	+	-	-	-	-
<i>V. dahliae</i>	KresD	Hop	n.t.	Slovenia	IHPS	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
<i>V. dahliae</i>	12042	Hop	n.t.	Great Britain	1	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
<i>V. dahliae</i>	12099	Hop	n.t.	Great Britain	1	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
<i>V. dahliae</i>	2390	Hop	n.t.	Germany	4	+	+	-	-	+	+	-	-	-	-	+	+	+	+	+	+
<i>V. dahliae</i>	Pap	G. pepper	n.t.	Slovenia	IHPS	+	+	-	-	-	-	-	-	-	-	+	+	-	-	-	-
<i>V. dahliae</i>	Pap02	G. pepper	n.t.	Slovenia	IHPS	+	+	-	-	+	+	-	-	-	-	+	+	-	-	-	-
<i>V. dahliae</i>	JKG1	Potato	n.t.	Holland	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>V. dahliae</i>	JKG8	Potato	n.t.	Holland	7	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
<i>V. dahliae</i>	JKG10	Potato	n.t.	Holland	7	+	+	+	+	+	+	-	-	+	+	+	+	+	+	-	-
<i>V. dahliae</i>	DJK	Chrys	n.t.	Holland	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>V. dahliae</i>	MH	Chrys	n.t.	Holland	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>V. dahliae</i>	JKG2	S. catalpa	n.t.	Holland	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>V. dahliae</i>	JKG7	S. catalpa	n.t.	Holland	7	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
<i>V. dahliae</i>	JKG3	J. maple	n.t.	Holland	7	+	+	+	+	+	+	-	-	+	+	+	+	-	-	+	+
<i>V. tricornis</i>	EXF8	Soil	n.t.	Holland	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>V. tricornis</i>	JKG20	Linden	n.t.	Holland	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>V. lecanii</i>	B-560	C. cricket	n.t.	Slovenia	MZKI	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>V. luteoalbum</i>	B-308	Soil	n.t.	Slovenia	MZKI	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>V. theobromae</i>	B-23	...	n.t.	...	MZKI	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Alternaria</i>																					
<i>radicina</i>	B269	...	n.t.	France	MZKI	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Armillaria mellea</i>	G56	...	n.t.	Slovenia	MZKI	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Aspergillus flavus</i>	A168	...	n.t.	France	MZKI	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Cladosporium</i>																					
<i>oxysporum</i>	B245	...	n.t.	Slovenia	MZKI	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Fusarium</i>																					
<i>oxysporum</i> f. sp.																					
<i>lycopersicum</i>	B37	...	n.t.	...	MZKI	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>F. oxysporum</i>	B36	...	n.t.	...	MZKI	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>F. solani</i>	B27	...	n.t.	...	MZKI	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Monillia</i> sp	K3	...	n.t.	...	MZKI	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Penicillium</i>																					
<i>chrysogenum</i>	P90	...	n.t.	Slovenia	MZKI	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Trichoderma</i>																					
<i>harzianum</i>	B244	...	n.t.	Slovenia	MZKI	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Trichoderma</i>																					
<i>viridae</i>	B118	...	n.t.	United States	MZKI	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Javornik (14). For DNA extraction, plant xylem was removed from the bines with a sterile scalpel, finely cut, ground to a coarse powder in the presence of liquid nitrogen, and suspended in extraction buffer (2% wt/vol CTAB, 1.4 M NaCl, 20 mM EDTA, 0.1 M Tris-HCl, pH 8.0, 0.2% β -mercaptoethanol). Samples were extracted with equal volumes of chloroform:isoamyl-alcohol (24:1). DNA was precipitated with a 1/10 volume of 3 M Na-acetate (pH 5.2) and 1 volume of isopropanol, washed in 70% ethanol, and resuspended in 30 μ l of Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

Isolation, cloning, and sequencing of AFLP fragments. Nine AFLP primer combinations generating *V. albo-atrum* hop pathotype-specific fragments in our previous study (22) and two additional AFLP primer combinations (*EcoRI*-GA/*MspI*-CG and *EcoRI*-GT/*MspI*-CG) also giving pathotype-specific fragments (Table 2) were used to generate corresponding markers by AFLP analysis. These AFLP fragments were excised from the polyacrylamide gels and reamplified in 50 μ l of reaction mixture using the same primer pairs that identified the polymorphic AFLP fragment. The amplification products were electrophoresed on 1.2% agarose gel in 1 $\times$ Tris-acetate EDTA buffer. Following visualization under UV light, the fragments were excised from the gel and purified using a silica suspension (2). The purified AFLP fragments were blunt-end ligated into bacterial plasmid PCR-Script Amp SK (+), transformed into Epicurian Coli XL10-Gold Ultracompetent Cells, and selected using the blue/white method according to the manufacturer's (Stratagene, La Jolla, CA) instructions. After cloning, 20 positive colonies from each transformation were selected and each colony was cultured overnight in Luria-Bertani liquid medium containing ampicillin at 100

mg/liter. The size of the cloned AFLP fragments was verified by PCR analysis using the corresponding AFLP primers or universal primers T3 and T7. The recombinant plasmids were extracted by using a NucleoSpin Plasmid Kit (Macherey-Nagel GmbH & Co., Duren, Germany) and fragments were sequenced by the dideoxy chain terminator method using a Thermo Sequenase Cy5 Dye Terminator Kit and an automated sequencer ALFexpressII (Amersham Biosciences, Freiburg, Germany). Sequences were assembled by an ALFwin Sequence Analyzer 2.10 and have been deposited in the GenBank of the National Center for Biotechnology Information (NCBI) under accession numbers AY518585, AY534125, AY534126, AY534127, AY534128, AY534129, AY534130, AY534131, AY534132, AY534133, AY534134, AY534135, AY534136, AY534137, AY534138, and AY534139. Sequence homologies were analyzed with the BLASTN and BLASTX programs (1) at the NCBI network service.

Design of SCAR primers and PCR. Primers for candidate SCAR markers were designed using Primer 3 (25) and Net-Primer (Premier Biosoft International, Palo Alto, CA) software to test their specificity. For each cloned AFLP fragment, two pairs of primers were made from each sequence. The first pair, designated "external," was designed as described by Xu et al. (33), and included recognition sites for *EcoRI*/*MspI* restriction enzymes, two selective base pairs, and the internal sequences from both ends of the AFLP fragment to a common length of 18 to 23 bp. The second primer pair, "internal," was designed from the internal part of the sequences. For both sets of primers, care was taken to avoid secondary structures, primer dimer generation, and cross hybridization. The primers were synthesized by MWG Biotech (Ebersberg, Germany).

PCR was carried out in 20- μ l reaction volume containing 0.5 μ M of each primer, 0.6 U of *Taq* polymerase (Promega Corp., Madison, WI), 10 $\times$ PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM dNTP, and 20 ng of fungal DNA. Amplification was performed in a PCR System 9700 thermocycler (Perkin-Elmer Corp., Norwalk, CT), programmed for one initial step of 4 min at 94°C followed by 30 cycles with the temperature profile 45 s at 94°C, 30 s at the annealing temperatures (Table 3), and 60 s at 72°C. Reaction products were resolved by electrophoresis on 1.6% agarose gels in 0.5 $\times$ Tris-borate EDTA buffer stained with ethidium bromide. All PCR reactions with SCAR primers were repeated at least three times, and included the following controls: negative (water), negative (hop DNA), and positive (1 ng of plasmid DNA with corresponding AFLP fragment).

Multiplex PCR. For multiplex PCR amplification, 20 μ l of PCR solution was prepared using three different primer pairs (Table 3): 0.25 μ M (9-2I-For/9-2I-Rev), 0.5 μ M (1I-For/1I-Rev), and 0.5 μ M (9-1E-Rev/9-1I-For), 0.6 U of *Taq* polymerase (Promega Corp.), 10 $\times$ PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 2.75 mM $MgCl_2$, 0.2 mM dNTP, and 20 ng of template DNA. Reactions were performed in a PCR System 9700 thermocycler (Perkin-Elmer Corp.), programmed for the following temperature profile: 4 min of denaturation at 94°C; and 30 cycles of 45 s at 94°C, 30 s at 60°C, and 1.10 s at 72°C. PCR reactions were resolved by electrophoresis in 2% agarose gels.

Detection of the PG2 pathotype in hop plants by nested PCR. A two-step procedure (nested PCR) was conducted to detect in planta *V. albo-atrum* isolates belonging to the hop pathotype PG2. The first round of amplification was performed in 20 μ l of a solution containing 0.25 μ M external primers 9-1E-For and 9-1E-Rev, 0.6 U of *Taq* polymerase (Promega Corp.), 10 $\times$ PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM dNTP, and 20 ng of DNA from infected plants. The PCR temperature profile was as described for SCAR amplifications with an annealing temperature of 60°C, except that the number of temperature cycles was reduced to 20. Template from the first amplification was diluted 1:10, and 1 μ l was transferred into a fresh test tube containing the same PCR mixture described above, with 0.5 μ M internal primers 9-1I-For and 9-1I-Rev. For the second amplification, the number of temperature cycles was 30, with a primer annealing temperature of 65°C. To check against false negatives or positives, the set of controls described above was included.

RESULTS

DNA cloning and sequencing. Of 17 *V. albo-atrum* hop pathotype-linked AFLP

Table 2. Primer combinations of *EcoRI* and *MspI* used to generate *Verticillium albo-atrum* hop-pathotype specific amplified fragment length polymorphism (AFLP) markers

Primer combination	AFLP marker designation	Specific for ^a
<i>EcoRI</i> -GA/ <i>MspI</i> -AT	AFLP-1 ^b	PG1
<i>EcoRI</i> -GA/ <i>MspI</i> -TA	AFLP-2 ^b	PG1
<i>EcoRI</i> -GA/ <i>MspI</i> -AG	AFLP-3 ^b	PG1
<i>EcoRI</i> -GA/ <i>MspI</i> -CG	AFLP-4 ^b	PG2
<i>EcoRI</i> -GA/ <i>MspI</i> -GT	AFLP-5-1 ^b	PG2
	AFLP-5-2 ^b	PG2
<i>EcoRI</i> -GT/ <i>MspI</i> -GA	AFLP-6-1	PG1
	AFLP-6-2	PG2
<i>EcoRI</i> -GT/ <i>MspI</i> -CG	AFLP-7-1	PG2
	AFLP-7-2	PG2
<i>EcoRI</i> -AC/ <i>MspI</i> -TC	AFLP-8-1	PG2
	AFLP-8-2	PG1
<i>EcoRI</i> -AC/ <i>MspI</i> -TA	AFLP-9-1 ^b	PG2
	AFLP-9-2 ^b	PG2
<i>EcoRI</i> -AC/ <i>MspI</i> -GT	AFLP-10-1 ^b	PG1
	AFLP-10-2 ^b	PG2
<i>EcoRI</i> -TC/ <i>MspI</i> -TA	AFLP-11-1 ^b	PG2

^a PG1: isolates causing mild form of *Verticillium* wilt; PG2: isolates causing lethal form of *Verticillium* wilt.

^b Successfully cloned AFLP fragments.

markers generated by all primer combinations used (Table 2), 11 were reamplified successfully, purified by silica suspension, and cloned into plasmid vectors. The resulting recombinant clones were screened prior to sequencing for the presence of a band similar in size to the cloned AFLP fragment. Four colonies were selected and sequenced for each AFLP marker. All sequences of 11 AFLP markers showed the presence of the initial AFLP primers and 8 AFLP markers revealed a single sequence. In the case of the AFLP-3, AFLP-4, and AFLP-10-2 markers, two to four different sequences were obtained for each marker, within a size difference range of 0 to 2 bp. This indicated contamination by adjacent bands during polyacrylamide gel isolation of AFLP markers (33) or the presence of different sequences in the same band as a consequence of the AFLP methodology (30). Sequences were analyzed for sequence homology in GenBank, and the AFLP-11 *V. albo-atrum* PG2 hop pathotype-specific marker showed high homology (90% identity over a length of 101 bp) with a DNA fragment (NCBI accession no. AB095266) isolated from *V. dahliae* tomato pathotype and tomato or pepper pathotype (race 1) (T. Usami and Y. Amemiya, unpublished).

SCAR primer design and amplification specificity. Based on sequence data

from cloned AFLP markers, candidate SCAR primers were designed to detect *V. albo-atrum* isolates belonging to hop pathotypes PG1 or PG2. To retain primer specificity, two pairs of primers, external and internal, were designed from each sequence. In summary, 16 sequences were obtained from 11 AFLP cloned fragments, and 32 primer pairs were designed. Genomic DNA from four isolates each of *V. albo-atrum* PG1 (KresCer, CasA, Zup, and Rec) and PG2 (Tr99, Vran, Ciz, and Drc) hop pathotypes were used as template DNA for PCR optimization of the primer annealing temperature (Table 3) and initial screening for primer amplification specificity.

Of the 22 candidate SCAR primer pairs for the specific detection of *V. albo-atrum* PG2 hop pathotype designed, 10 primer pairs originating from AFLP-5-1, AFLP-9-1, AFLP-9-2, AFLP-10-2, and AFLP-11 amplified a single band of predicted size only in isolates of the PG2 pathotype (Table 3). Eight primer pairs from four AFLP-4 sequences, two primer pairs from the AFLP-5-2 marker, and two primer pairs from one AFLP-10-2 sequence showed no amplification in isolates of both pathotypes.

The amplification specificity of 10 candidate SCAR primer pairs for the detection of *V. albo-atrum* PG1 hop pathotype re-

vealed successful PG1-specific amplification with six primer pairs derived from AFLP-1, AFLP-2, and AFLP-10-1 markers (Table 3). Four primer pairs originating from two AFLP-3 sequences showed amplification only with plasmid DNA transformed by the corresponding AFLP fragment.

The developed pathotype-specific SCAR markers from the initial screening were tested further on a broad range of *Verticillium* isolates of different species and from different hop-production regions, host plants, geographical locations, and some other soil fungi (Table 1). The results of amplification specificity tests of all developed SCAR markers are presented in Table 1.

Within PG2-specific SCAR markers, S-9-1EXT and S-9-1INT markers (Table 3), which were developed from a sequence of the AFLP-9-1 marker, yielded 198- and 248-bp fragments, respectively (Fig. 1), only in the PG2 hop pathotype and lethal *V. albo-atrum* hop isolates from Slovenia. All possible combinations of primers from these two SCAR markers gave the same detection results.

SCAR marker S-9-2INT also showed high specificity, amplifying a 163-bp fragment in the PG2 and lethal Slovene hop isolates, but also in highly virulent tomato isolates from England (Aro/140; Aro/067)

Table 3. Length, nucleotide sequences, and annealing temperatures (T_a) of sequence-characterized amplified region (SCAR) markers derived from amplified fragment length polymorphism (AFLP) markers

AFLP marker	Specific for ^a	SCAR marker designation ^b	SCAR primer designation and sequence (5-3)	Size of product	T_a (°C)
AFLP-1	PG1	S-1EXT S-1INT	1E-For ATTCGAGGCTCCGAGCATCC	241 bp	59
			1E-Rev GGATAGGCCGACCTTGAGAA		
AFLP-2	PG1	S-2EXT S-2INT	1I-For CGAGCATCCGATGACACAG	175 bp	60
			1I-Rev AAGAGATCAATAGTCGTTTGCC		
			2E-For ATTCGACGGACTATTCGTAAC	180 bp	60
			2E-Rev GGTAGGTAGCACGGCACTCG		
AFLP-5-1	PG2	S-5-1EXT S-5-1INT	2I-For CGGACTATTCGTAACGGTCGG	167 bp	60
			2I-Rev AGCACGGCACTCGTTAGGT		
			5-1E-For GGGTCGATACGATTACAGAA	174 bp	52
			5-1E-Rev ATTCGAGGCCAAGATACAGT		
AFLP-9-1	PG2	S-9-1EXT S-9-1INT	5-1I-For GATTACGAACTGGCCTTC	157 bp	59
			5-1I-Rev GCCAAGATACAGTCAAGGTAAG		
			9-1E-For GGTAAGACTCCTTACCGATGCTG	248 bp	59
			9-1E-Rev ATTCACACGCTACATATCAAACA		
AFLP-9-2	PG2	S-9-2EXT S-9-2INT	9-1I-For CGATGCTGGATCTGACCAATTAC	198 bp	65
			9-1I-Rev ATAAGGGCAGGCAAGGCAC		
			9-2E-For ATTCACCATCAGCCTGTGC	190 bp	60
			9-2E-Rev GGTAATCCTTGACAACAATATTC		
AFLP-10-1	PG1	S-10-1EXT S-10-1INT	9-2I-For CAGCGATGCACCTTCAGTG	163 bp	60
			9-2I-Rev TGACAACAATATTCTCGAAGCCA		
			10-1E-For ATTCACCCACCAGAGAGGGGCAT	622 bp	62
			10-1E-Rev GGGTCATTGCTGCATGACCA		
AFLP-10-2	PG2	S-10-2aEXT S-10-2aINT	10-1I-For GTCAACTGAAATGGCACCTG	341 bp	59
			10-1I-Rev TTCCTGGTTCCGAAGCAAC		
			10-2aE-For ATTCACGGATCCAAGGATGGCGA	367 bp	60
			10-2aE-Rev GGGTGTGTCTCTCATAGGTA		
AFLP-11	PG2	S-11EXT S-11INT	10-2aI-For GCCAGATACACCAATGTCAAC	293 bp	60
			10-2aI-Rev TACGTGCCAGTAGATGCTG		
			11E-For ATTCTCGCCGATCGTTACTC	216 bp	63
			11E-Rev GGTACAGCAGCAGTTCTCTG		
			11I-For GCCATCGCCAAAGTACCAT	169 bp	63
			11I-Rev GCCAGATCATATACCCATTGTC		

^a PG1: isolates causing mild form of *Verticillium* wilt; PG2: isolates causing lethal form of *Verticillium* wilt.

^b EXT: SCAR markers generated from the external part of the corresponding AFLP marker; INT: SCAR markers generated from the internal part of the corresponding AFLP marker.

and in *V. dahliae* from isolate JKG3 of *Acer palmatum* from Holland. SCAR primers developed for PG1 isolates were less specific, showing amplification products in *V. albo-atrum* isolates from different host plants and isolates of *V. dahliae* in addition to PG1 hop pathotypes (Table 1).

Multiplex PCR by SCAR primers. To develop a more specific test for the diagnosis of *V. albo-atrum* hop PG1 and PG2 pathotypes, multiplex PCR reactions were performed using three pairs of SCAR primers: 9-2I-For/9-2I-Rev, 1I-For/1I-Rev, and 9-1E-Rev/9-1I-For (Table 3). The last primer pair was used as a combination of primers from two SCAR markers, S-9-1EXT and S-9-1INT, in order to achieve a uniform annealing temperature of 60°C for all primers. The multiplex reaction was optimized by adjusting the amount of primers and increasing the concentration of MgCl₂. The amplified PCR products corresponded to the SCAR markers; therefore, the specificity of the primers was not affected by the multiplex reaction. The first two primer pairs from the multiplex reaction amplified a new fragment of 233 bp and the expected 163-bp band, respec-

tively, in all lethal *V. albo-atrum* hop isolates from Slovenia. The *V. albo-atrum* PG1 isolates yielded the predicted 175-bp fragment amplified by the 1I-For/1I-Rev primer pair (Fig. 2).

PCR detection of *V. albo-atrum* PG2 pathotype in hop plants. SCAR primer pair 9-1E-Rev/9-1E-For, which was specific for the detection of the PG2 pathotype, was used for testing artificially inoculated plants of hop cvs. Wye Target, Wye Challenger, Fuggle, and Celeia. The PCR product was detectable as a faint band (Fig. 3) and the results were inconsistent. In order to improve the sensitivity of detection, a nested PCR procedure was applied by using a two-step protocol. Primers of SCAR marker S-9-1EXT were used for the first amplification step in a reduced number of PCR temperature cycles. For the second step, 1 µl of 1:10 diluted solution from the first PCR was used as a template for amplification using SCAR S-9-1INT primers. Nested PCR using genomic DNA obtained from the xylem of plants infected by the PG2 isolate amplified the predicted 198-bp specific diagnostic S-9-1INT marker from all tested plants. Plants

inoculated with sterile distilled water or PG1 isolate did not produce any amplification products (Fig. 3). The quality of isolated total DNA seemed to be important for the specific amplification, because DNA isolated by the CTAB protocol gave much better results than DNA isolated by the SDS protocol. The results of the nested PCR were validated by culturing plant samples on PDA, followed by microscopic identification of the fungi obtained and SCAR amplification with DNA extracted from the cultured isolates.

DISCUSSION

Since 1997, Verticillium wilt has been a serious limiting factor in hop production in Slovenia. The importance of rapid detection of hop pathotypes has been recognized in order to improve the efficiency of disease management, accelerate resistance breeding, and contribute to epidemiological studies. Various techniques have been employed to develop PCR assays for genetic identification and diagnosis of *Verticillium* spp.. Specific primers based on ITS rDNA sequences previously have been designed to identify and distinguish *V. albo-atrum*, *V. dahliae*, and *V. tricorpus* (18,19,24). Sets of primers also are available for the detection of *V. albo-atrum* lucerne (L), nonlucerne (NL), and potato subgroups (4,24).

Our previous study of Slovene *V. albo-atrum* isolates by pathogenicity tests and molecular analysis revealed two hop pathotypes, PG1 and PG2, which express differences in virulence and cause either the mild or the lethal form, respectively, of Verticillium wilt (22). That study identified 17 AFLP markers linked to *V. albo-atrum* hop pathotype specificity. Successful conversion of AFLP fragments into SCAR markers enables the extension of their applicability to less laborious, rapid, locus-specific diversity assessment (3,33). Such a conversion has been attempted for many AFLP markers, but only a few retain their sequence specificity or ability to amplify genomic DNA (10,28). A possible reason for the lack of efficient conversion may lie in the nature of AFLP. The specificity may be lost when primers are generated from the internal parts of the AFLP sequences and nucleotide differences specific for AFLP primers are excluded (28,32). Therefore, we designed two primer pairs for each AFLP fragment sequence, one of which was based on the internal part of the sequence, and the second (extended) which included the AFLP primer sites. In summary, from 17 polymorphic AFLP fragments, 11 were successfully cloned and 16 sequences were obtained, which served for the design of 32 candidate SCAR primer pairs. Testing of primer pairs on representative isolates of PG1 and PG2 resulted in 6 and 10 primer pairs specific for the PG1 and PG2 pathotypes, respectively. All PG2-specific primers also amplified diag-

Fig. 1. Polymerase chain reaction amplification products of genomic DNA of *Verticillium albo-atrum* hop isolates using primers for sequence-characterized amplified region marker S-9-1EXT, which is specific for PG2 isolates. Lane M: 50-bp DNA ladder; lanes 1–4: PG2 isolates (T99, Vran, Ciz, and Drc); lanes 5–8: PG1 isolates (KresCer, CasA, Zup, and Rec); lane P: positive control (plasmid cloned AFLP-9-1 marker); lane H: hop genomic DNA; lane W: water control.

Fig. 2. Identification of *Verticillium albo-atrum* PG1 and PG2 hop pathotypes by multiplex polymerase chain reactions. Lane M: 100-bp DNA ladder; lanes 1–4: PG2 isolates (T99, Vran, Ciz, and Drc); lanes 5–8: PG1 isolates (KresCer, CasA, Zup, and Rec); P₁: positive control (plasmid cloned AFLP-9-2 marker); P₂: positive control (plasmid cloned AFLP-1 marker); P₃: positive control (plasmid cloned AFLP-9-1 marker); lane H: hop genomic DNA; lane W: water control.

nostic SCAR markers in Slovene hop isolates uncharacterized by the pathogenicity test, but obtained from plants showing lethal hop wilt symptoms; such isolates were thus, considered to be the PG2 pathotype.

When the specificity of the primers derived from SCAR markers was tested on a broad range of isolates, only SCAR primers from the AFLP 9-1 fragment retained their specificity for PG2. All other SCAR markers also were amplified in either *V. albo-atrum* hop isolates and *V. dahliae* isolate JKG3 or JKG10 from Holland and in some hop isolates from Slovenia, England, and Germany. SCARs specific for PG1 also were amplified, in addition to mild Slovene hop isolates, in some German and Polish hop isolates, implying similarities among those hop isolates causing mild wilt. Five PG2-specific SCARs obtained from AFLP-5-1, AFLP-9-2, and AFLP 10-2a markers also were amplified in all English isolates, except isolates 1953, 298092, and 298095, which are characterized as mild hop isolates. Furthermore, three PG2 SCARs from AFLP-5-1, AFLP-9-2, and AFLP-11 also were found in lucerne isolates (Table 1). These results suggest specific similarities among PG2, lucerne, and isolates from England, but the

degree of genetic relationship among isolates analyzed needs to be assessed by a larger number of markers.

In addition, very high sequence identity was found between the hop PG2 pathotype AFLP-11 fragment and a DNA fragment isolated from the tomato pathotype and tomato or pepper pathotype of *V. dahliae* (T. Usami and Y. Amemiya, 2002; *unpublished*). Such analysis may provide the means for better understanding the pathogenicity of *Verticillium* isolates in the future.

Identification of Slovene *V. albo-atrum* PG1 and PG2 hop pathotypes by SCAR markers was further improved by the development of multiplex PCR. Such a test increases specificity in the diagnosis of these pathotypes by means of simultaneous amplification of two specific loci for PG2 and one for PG1 (Fig. 2) and can make pathotype screening assays more reliable.

In addition to pathotype identification from fungal genomic DNA, PCR assays for the detection of pathogens in plants allow direct detection in wilted plants and quantification of the pathogen biomass in plants. Such an approach, applying different markers to the detection and quantification of plant-pathogenic *Verticillium* spp., already has been used in alfalfa, sun-

flower, tomato, potato, and olive (6,11,12,16,18). In our study, SCAR primers from the AFLP-9-1 fragment were used for the detection of the *V. albo-atrum* PG2 hop pathotype in artificially infected hop plants of resistant cvs. Wye Target and Wye Challenger and susceptible Fuggle and Celeia. The detection of PG2 in total genomic DNA by single PCR using S-9-1I-For/ S-9-1I-Rev or S-9-1E-For/ S-9-1E-Rev primer pairs gave faint bands and inconsistent results (Fig. 3), probably due to a significantly lower concentration of fungal plant DNA and the presence of PCR plant-derived inhibitory compounds (7,23). In order to increase detection sensitivity, a nested PCR was employed, in which templates first were subjected to amplification with external primers S-9-1E-For/S-9-1E-Rev, followed by S-9-1I-For/S-9-1I-Rev internal primer pair obtained from the AFLP-9-1 sequence. The nested PCR demonstrated consistent detection of the PG2 pathotype in all tested plants, irrespective of cultivar resistance (Fig. 3).

In conclusion, we report here on the conversion of *V. albo-atrum* PG1 or PG2 pathotype-specific AFLP markers into more diagnostically applicable SCAR markers. From 16 developed SCARs, two markers (S-9-1INT and S-9-1EXT), specific for the detection of the highly virulent PG2 hop pathotype, retain specificity under testing on a wide range of isolates from important hop-growing regions in Europe where hop wilt has appeared to date, as well as isolates from different hosts and *Verticillium* spp. The remaining SCAR pathotype-specific markers may be useful for the identification of *V. albo-atrum* isolates from Slovene hop-growing regions. The first hop pathotype-specific SCAR markers presented in this study are of potential importance for future diagnostic studies and for complementation in genetic analysis of *Verticillium* isolates.

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Fig. 3. Detection of *Verticillium albo-atrum* PG2 hop pathotype in xylem tissue of artificially infected hop cultivars. Lane M: 100-bp ladder; lanes 1 through 4: total genomic DNA of cvs. Celeia, Fuggle, Wye Challenger, and Wye Target, respectively, infected by PG2 isolate Tr99; lanes 5 through 8: the same cultivars infected by PG1 isolate KresCer; lane F₂: positive control (genomic DNA of Tr99 isolate); Lane F₁: negative control (genomic DNA of KresCer isolate); H: hop genomic DNA; P: plasmid positive control; W: water control. **A**, Amplification products from single polymerase chain reaction (PCR) of total genomic DNA by using primers for sequence-characterized amplified region marker S-9-1EXT. **B**, Nested PCR amplification products obtained with external primers S-9-1E-For/S-9-1E-Rev, followed by S-9-1I-For/S-9-1I-Rev internal primer pair obtained from amplified fragment length polymorphism 9-1 sequence.

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