Eur J Plant Pathol (2009) 124:521–526
DOI 10.1007/s10658-008-9424-9

Development of a TaqMan allelic discrimination assay for the distinction of two major subtypes of the grapevine yellows phytoplasma Bois noir

Jennifer Berger · Josef Dalla Via · Sanja Baric

Received: 13 June 2008 / Accepted: 11 December 2008 / Published online: 16 January 2009

Abstract Bois noir is a grapevine yellows disease that is gaining importance in many regions of Europe. The Bois noir phytoplasma ("*Candidatus* Phytoplasma solani") was shown to be transmitted by the planthopper *Hyalesthes obsoletus*, which normally feeds on herbaceous weeds, and occasionally also on grapevines. Three subtypes of the Bois noir phytoplasma have been described and were shown to be associated with distinctive host plants. In this study, we developed a novel and rapid real-time PCR allelic discrimination assay for the distinction of the two major Bois noir phytoplasma subtypes, VK type I and II. Two TaqMan probes carrying different fluorescent dyes were designed to specifically bind to a polymorphism characteristic for the two Bois noir phytoplasma subtypes, thereby allowing discriminative amplification in a single-tube and single-step assay. A total of 259 bois noir-positive grapevine samples collected over 5 years were analysed using the conventional PCR-RFLP method and our newly developed TaqMan allelic discrimination assay. 257 out of 259 samples could be typed with the TaqMan method, compared to 200 out of 259 samples when using the conventional method. The overall concordance of the two methods was 100%. Our newly developed TaqMan assay represents a useful tool for fast and reliable determination of Bois noir phytoplasma subtypes in infected grapevine, insect vector, and host plant samples. The test is suitable for high-throughput analysis and will thereby facilitate further characterisation of Bois noir epidemiology.

Keywords "*Candidatus* Phytoplasma solani" · Real-time PCR · Vergilbungskrankheit · *Vitis vinifera*

Abbreviations

BN	Bois noir
C_T	threshold cycle
delta Rn	relative fluorescence intensity
FD	Flavescence dorée
MGB	minor groove binder
RFLP	restriction fragment length polymorphism
SNP	single nucleotide polymorphism
tuf	elongation factor TU
VK	Vergilbungskrankheit

Flavescence dorée (FD) and Bois noir (BN; or Vergilbungskrankheit, VK) are the two economically most important phytoplasmic diseases of the grapevine *Vitis vinifera*, affecting vineyards in large parts of central and southern Europe. The FD phytoplasma ("*Candidatus* Phytoplasma vitis"; 16SrV or Elm yellows group) occurs mainly in the western Mediterranean area and is thought to be the more pernicious grapevine yellows disease due to the epidemic spreading (Caudwell 1990; Boudon-Padieu

J. Berger · J. Dalla Via · S. Baric (✉)
Research Centre for Agriculture and Forestry Laimburg,
I-39040 Auer/Ora (BZ), Italy
e-mail: sanja.baric@provinz.bz.it

2002). In recent years however, BN has become an increasing problem, especially in areas not affected by FD. The BN phytoplasma ("*Ca.* Phytoplasma solani") belongs to the 16SrXII-A or Stolbur group (Lee et al. 1998), and was shown to be transmitted by the planthopper *Hyalesthes obsoletus* (Maixner 1994; Sforza et al. 1998). These phloem-sucking insects preferentially feed on herbaceous host plants, such as the bindweed *Convolvulus arvensis* or the stinging nettle *Urtica dioica*. Occasionally, they also feed on grapevines, thereby transmitting the BN phytoplasma. A previous study by Langer and Maixner (2004) reported the existence of three BN subtypes in Germany, which are associated with different host plants of *H. obsoletus*. VK type I seems to be mainly associated with *U. dioica*, whereas VK type II was found predominantly in *C. arvensis*, but also in other plants such as *Solanum nigrum* and *Prunus spinosa*. VK type III was found in *Calystegia sepium* and appeared to be geographically restricted to the Mosel valley in Germany. All three subtypes could be detected in the insect vector *H. obsoletus* as well as in BN-diseased grapevines (Langer and Maixner 2004). Recently, we reported the occurrence of VK types I and II in grapevine samples from South Tyrol (northern Italy). In contrast to Germany where VK type II was the predominant BN phytoplasma subtype, we found that VK type I was the only BN phytoplasma subtype detected in South Tyrol until the first appearance of VK type II in 2003 (Baric and Dalla Via 2007). Other studies confirmed the occurrence of VK type I and II in infected Italian grapevines as well as weeds and insect vectors (Pasquini et al. 2007; Riolo et al. 2007).

The method currently employed for the distinction of VK subtypes is based on PCR amplification of the *tuf* gene followed by RFLP analysis (Langer and Maixner 2004). A major disadvantage of the PCR-RFLP method is however that it consists of a rather time-consuming multi-step analysis with limited sensitivity, and is not suitable for high-throughput applications. We therefore decided to develop an allelic discrimination assay based on real-time PCR, which has been increasingly used for the detection of phytoplasmas (Angelini et al. 2007; Baric and Dalla Via 2004; Baric et al. 2006; Galetto et al. 2005). The new test represents a single-step assay for fast and reliable distinction of the two main VK phytoplasma subtypes, *U. dioica*-associated VK type I and *C. arvensis*-associated VK type II. Similar assays have been used successfully for genotyping of single nucleotide polymorphisms (Mamotte 2006).

In order to develop specific TaqMan probes for allelic discrimination, the exact location of the *Hpa*II SNP site within the elongation factor TU (*tuf*) gene was determined by cloning and sequencing PCR fragments obtained by amplification of four DNA isolates with fTufAy and rTufAy primers (Schneider et al. 1997). Two of the DNA isolates had been previously typed as VK type I and two as VK type II (Baric and Dalla Via 2007). PCR products were purified and cloned using the pGEM-T Easy Vector-System II (Promega, Madison, WI, USA). Plasmids containing inserts were isolated with the Eppendorf Perfectprep Plasmid Isolation Kit (Eppendorf, Hamburg, Germany) and cycle-sequenced in both directions using the CEQ DTCS Quick Start Kit (Beckman Coulter, Fullerton, CA, USA) with Primer 20mer (Novagen, EMD Biosciences, Darmstadt, Germany) and Sequencing Primer M13 -47 (Beckman Coulter). The cycling conditions were set to 30 cycles of 20 s at 96°C, 20 s at 50°C and 4 min at 60°C. Sequencing products were separated and visualised on a CEQ 8000 Genetic Analysis System (Beckman Coulter). Sequences were aligned using the computer programme SEQUENCHER Version 4.7 for Windows (Gene Codes Corporation, Ann Arbor, MI, USA), and deposited in GenBank under the accession numbers FJ394551-FJ394552. DNA sequences from isolates of the same VK type were identical, whereas two point mutations distinguishing the VK types were found at positions 543 and 604 (Fig. 1). The *Hpa*II SNP site at position 543, which was identical to the one used for VK type distinction by RFLP analysis, was chosen for the development of the TaqMan allelic discrimination assay. Using PrimerExpress software (Version 1.1, Applied Biosystems, Foster City, CA, USA), two primers flanking the *Hpa*II SNP site were designed to amplify an 80-bp product (forward, qBN-AD-F, 5′-CCT TTC TTA ATG CCA GTC GAA-3′; reverse, qBN-AD-R, 5′-TGT CCT CTT TCA ACT CTA CCA GTA A-3′). In order to allow single-tube analysis, two fluorogenic minor groove binder (MGB) probes spanning the *Hpa*II SNP site and binding to the antisense strand were designed with different fluorescent dyes. One probe was targeted to VK type I (qBN-VKI, 5′-6FAM-TCC TCT ACC GGT GAT AG-3′), whereas the other was specific for VK type II

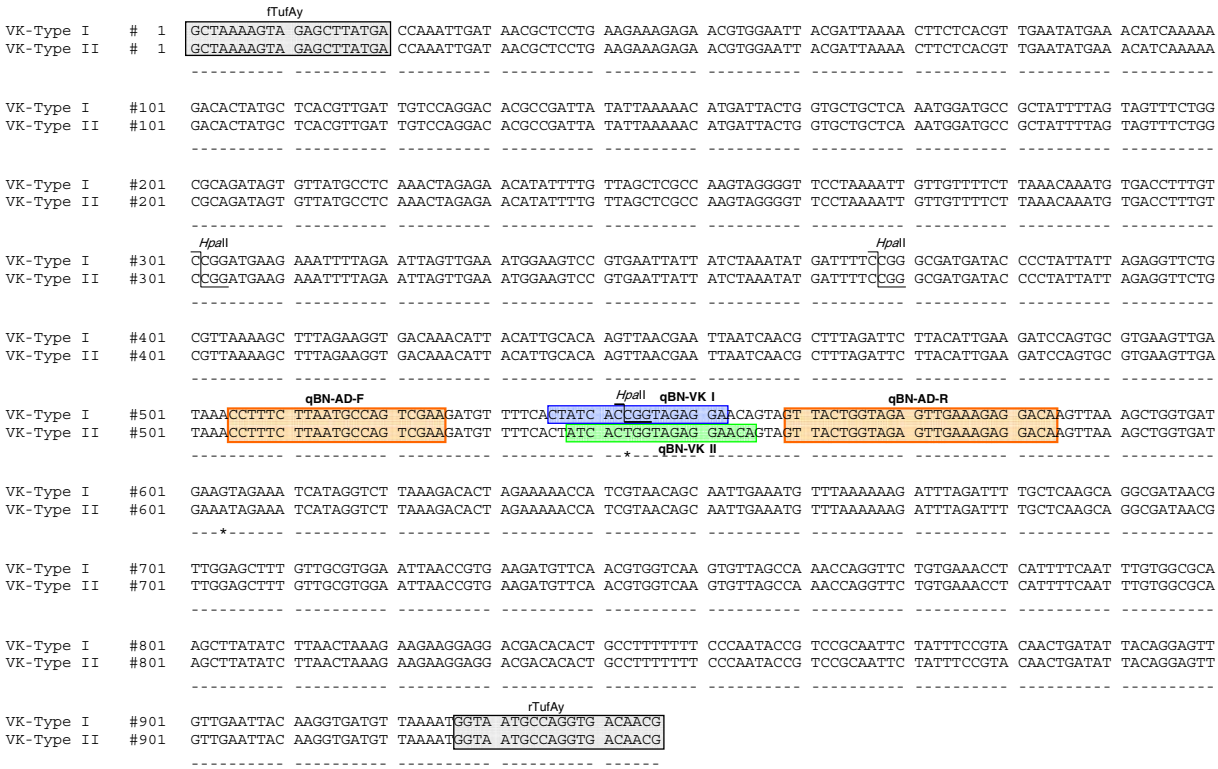

Fig. 1 Alignment of the *tuf* gene sequences of the two major Bois noir subtypes with the positions of primers (*grey boxes*) and *Hpa*II restriction sites used for PCR-RFLP analysis as well as primers and probes for the TaqMan allelic discrimination assay (*coloured boxes*). The point mutations between the sequences of the two subtypes are indicated by *asterisks*

(qBN-VKII, 5′-VIC-TGT TCC TCT ACC AGT GAT-3′) (Fig. 1). Real-time PCR analysis was performed in duplicates in 25 µl-reactions, containing 12.5 µl TaqMan Universal PCR Master Mix (Applied Biosystems), 500 nM of each primer, 250 nM of qBN-VKII probe, 100 nM of qBN-VKI probe, and 2 µl of sample DNA. Thermocycling was carried out on the ABI PRISM 7000 Sequence Detection System (Applied Biosystems), and consisted of 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C, and 1 min at 57°C or 61°C, respectively.

During optimisation of real-time PCR conditions, it emerged that in the case of an infection with VK type II phytoplasma, the qBN-VKI probe also produced a positive signal when the real-time PCR was run at a lower annealing temperature (57°C). This was presumably due to non-specific binding of the qBN-VKI probe to the VK type II sequence which harbours only a single mismatch. Non-specific binding of the qBN-VKII probe to VK type I phytoplasma sequences was never observed. However, specific and non-specific

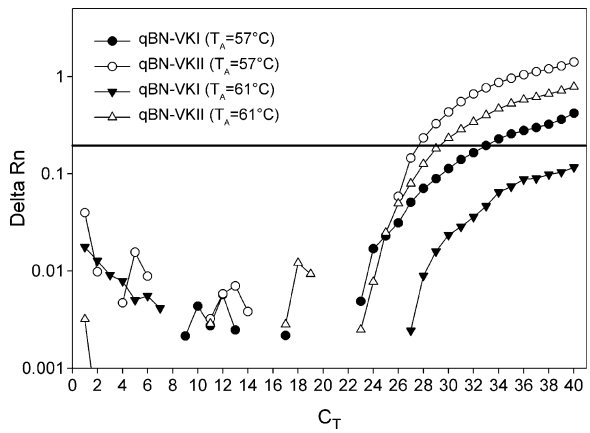

Fig. 2 Real-time PCR amplification plots for a representative VK type II-positive grapevine sample run at annealing temperatures (T_A) of 57°C and 61°C. Fluorescence intensity (Delta Rn) is plotted against the threshold cycle number (C_T). The threshold (indicated by the *horizontal line*) was set at 0.2. At T_A of 57°C fluorescence signals generated by both probes, qBN-VKII and qBN-VKI, pass the threshold. At T_A of 61°C only the specific qBN-VKII probe produces positive fluorescence intensity

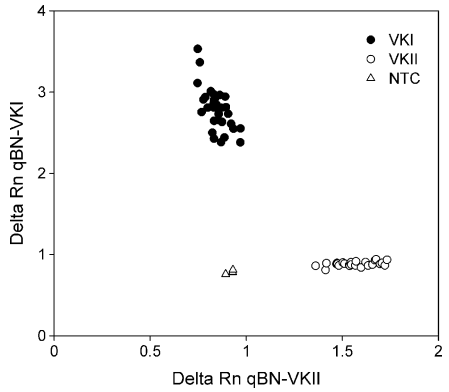

Fig. 3 Allelic discrimination plot of 29 representative BN-positive grapevine samples. 16 VK type I (*black circles*), 13 VK type II samples (*white circles*), and controls containing no template (NTC; *triangles*) were amplified in duplicate by the TaqMan allelic discrimination method. Results were plotted by using the absolute fluorescence of each probe

amplification curves could be easily distinguished according to their shape: the specific qBN-VKII curve displayed a sigmoid progression typical for logarithmic amplification, whereas the curve obtained by non-specific binding of the qBN-VKI probe was almost linear in shape, and the relative fluorescence intensity (delta Rn) reached after 40 cycles was much lower (Fig. 2). When simulating a mixed infection by experimentally combining VK type I and II DNA, the amplification plot revealed two clear sigmoid curves (data not shown). This indicates that our method can distinguish between a mixed infection and non-specific amplification at 57°C.

At a higher annealing temperature (61°C), specific detection of VK type II phytoplasma was achieved without fluorescence levels from cross-amplification by the qBN-VKI probe passing the threshold set at 0.2 (Fig. 2). However, this increase in specificity results in a loss of sensitivity: in the example shown in Fig. 2, the C_T value at 61°C was 29.5 compared to 27.5 at 57°C, which corresponds to a 4-fold lower sensitivity. As BN phytoplasma titers in grapevine samples appear to be relatively low, we decided to perform the real-time PCR at an annealing temperature of 57°C, and to distinguish between specific and non-specific signals according to the shape of the amplification curves. However, when phytoplasma titers are high and sensitivity is not an issue, we recommend running the TaqMan real-time PCR at 61°C to achieve higher specificity. After PCR amplification using the more stringent conditions, a post-read allelic discrimination assay was performed on the ABI PRISM 7000 Sequence Detection System. An example for an allelic discrimination plot used to assign the two BN phytoplasma subtypes in 29 representative grapevine samples is displayed in Fig. 3. The two subtypes were easily discriminated by their distribution along either the qBN-VKII probe or qBN-VKI probe axis.

To assess the newly established TaqMan real-time PCR assay, we decided to apply this method on DNA samples isolated from symptomatic grapevines located in South Tyrol (northern Italy), which had been previously analysed using conventional PCR-RFLP (Baric and Dalla Via 2007). A further 76 samples collected in the year 2006 and processed in the same

Table 1 Results of PCR-RFLP analyses and TaqMan allelic discrimination assays for the assignment of the BN subtype in BN-positive grapevine samples collected from 2002 until 2006

Year	n tested	PCR-RFLP[1]			TaqMan		
		VK I	VK II	ND[b]	VK I	VK II	ND
2002	29	29	-	NA[c]	29	-	-
2003	31	31	-	NA	31	-	-
2004	41	36	5	NA	35	5	1
2005	82	63	19	NA	62	19	1
2006[a]	76	13	4	59	61	15	-
Total	259	172	28	59	218	39	2

[1] Baric and Dalla Via (2007)

[a] All BN-positive samples from 2006 were tested with the TaqMan method, whereas in years 2002–2005 only those samples were analysed that produced a positive result with the PCR-RFLP method.

[b] *ND* Not detectable

[c] *NA* Not applicable

way, which were not included in the earlier study, were assayed in parallel using both methods. Two reference samples VK type I and VK type II (kindly provided by M. Maixner) were included as controls. Of the samples isolated in years 2002–2005, only those that produced an informative result with the PCR-RFLP method were analysed by TaqMan PCR. In contrast, samples from 2006 testing positive for BN phytoplasma were assayed in parallel using both methods. A detailed comparison of the two methods is shown in Table 1. Results obtained by the TaqMan assay corresponded with those from the PCR-RFLP method in all samples tested, indicating that the overall concordance of the two methods was 100%. However, two samples that could be typed by PCR-RFLP soon after nucleic acid isolation produced no informative results when analysed with the TaqMan method two years later. PCR-RFLP analysis was repeated and also proved unable to re-amplify these samples, thereby confirming that DNA degradation had occurred in the meantime (data not shown).

A major requirement for the accuracy and sensitivity of molecular diagnostic techniques is high quality DNA. It is known that infected grapevines usually contain a high concentration of PCR inhibitors (Boudon-Padieu et al. 2003), a fact that often leads to detection problems. Difficulties with analysing the samples from 2006 by PCR-RFLP were presumably due to low quantities of phytoplasmic DNA or the presence of PCR inhibitors. The TaqMan-based allelic discrimination assay was less affected by this, producing informative results for 76 samples from the year 2006, compared to only 17 samples when using PCR-RFLP. Another reason for the increased sensitivity of the real-time PCR may be the fact that PCR fragments generated by TaqMan PCR were much shorter than those obtained by conventional PCR for RFLP analysis (80 bp versus 946 bp), thereby increasing PCR efficiency. It has been generally observed that real-time PCR has a higher sensitivity than conventional PCR (Baric et al. 2006). In this respect, it should also be taken into account that the detection of BN phytoplasmas in DNA isolates normally requires a nested PCR where two rounds of PCR are performed in series (Maixner et al. 1995). This greatly increases the detection sensitivity which is necessary because of the apparent low phytoplasma titers in affected plants. The *tuf* gene PCR for VK type distinction by PCR-RFLP consists of a single PCR run only, thereby potentially causing problems when analysing low titre phytoplasma infections, which can be overcome by using the TaqMan-based allelic discrimination assay.

In summary, we developed a fast and sensitive TaqMan-based allelic discrimination assay for the distinction of the two BN phytoplasma subtypes VK type I and II. The conventional PCR-RFLP method is a time-consuming multi-step procedure, and is often limited by the low quantities of phytoplasma DNA present in grapevine samples. A sensitive and high-throughput method such as our newly developed TaqMan allelic discrimination assay would be of great value for studying the epidemiology of BN in greater detail.

Acknowledgements We thank M. Maixner for providing DNA extracts of the different VK types, K. Mair and A. Morandell for supplying grapevine samples and C. Kerschbamer for technical assistance. This study was funded by the Autonomous Province of Bozen/Bolzano, Italy.

References

Angelini, E., Bianchi, G. L., Filippin, L., Morassutti, C., & Borgo, M. (2007). A new TaqMan method for the identification of phytoplasmas associated with grapevine yellows by real-time PCR assay. *Journal of Microbiological Methods*, *68*, 613–622.

Baric, S., & Dalla Via, J. (2004). A new approach to apple proliferation detection: a highly sensitive real-time PCR assay. *Journal of Microbiological Methods*, *57*, 135–145.

Baric, S., & Dalla Via, J. (2007). Temporal shifts of Bois Noir phytoplasma types infecting grapevine in South Tyrol (Northern Italy). *Vitis*, *46*, 101–102.

Baric, S., Kerschbamer, C., & Dalla Via, J. (2006). TaqMan real-time PCR versus four conventional PCR assays for detection of apple proliferation phytoplasma. *Plant Molecular Biology Reporter*, *24*, 169–184.

Boudon-Padieu, E. (2002). Flavescence dorée of the grapevine: knowledge and new developments in epidemiology, etiology and diagnosis. *ATTI Giornate Fitopatologiche*, *1*, 15–34.

Boudon-Padieu, E., Béjat, A., Clair, D., Larrue, J., Borgo, M., Bertotto, L., et al. (2003). Grapevine yellows: Comparison of different procedures for DNA extraction and amplification with PCR for routine diagnosis of phytoplasmas in grapevine. *Vitis*, *42*, 141–149.

Caudwell, A. (1990). Epidemiology and characterization of flavescence dorée (FD) and other grapevine yellows. *Agronomie*, *10*, 655–663.

Galetto, L., Bosco, D., & Marzachi, C. (2005). Universal and group-specific real-time PCR diagnosis of flavescence dorée (16Sr-V), bois noir (16Sr-XII) and apple proliferation (16Sr-X) phytoplasmas from field-collected plant

hosts and insect vectors. *Annals of Applied Biology*, *147*, 191–201.

Langer, M., & Maixner, M. (2004). Molecular characterisation of grapevine yellows associated phytoplasmas of the stolbur-group based on RFLP-analysis of non-ribosomal DNA. *Vitis*, *43*, 191–199.

Lee, I. M., Gundersen-Rindal, D. E., Davis, R. E., & Bartoszyk, I. M. (1998). Revised classification scheme of phytoplasmas based on RFLP analyses of 16S rRNA and ribosomal protein gene sequences. *International Journal of Systematic and Evolutionary Microbiology*, *48*, 1153–1169.

Maixner, M. (1994). Transmission of German grapevine yellows (Vergilbungskrankheit) by the planthopper *Hyalesthes obsoletus* (Auchenorrhyncha: Cixiidae). *Vitis*, *33*, 103–104.

Maixner, M., Ahrens, U., & Seemüller, E. (1995). Detection of the German grapevine yellows (Vergilbungskrankheit) MLO in grapevine, alternative hosts and a vector by a specific PCR procedure. *European Journal of Plant Pathology*, *101*, 241–250.

Mamotte, C. (2006). Genotyping of single nucleotide substitutions. *Clinical Biochemist Reviews*, *27*, 63–75.

Pasquini, G., Ferretti, L., Gentili, A., Bagnoli, B., Cavalieri, V., & Barba, M. (2007). Molecular characterization of stolbur isolates collected in grapevines, weeds and insects in central and southern Italy. *Bulletin of Insectology*, *60*, 355–356.

Riolo, P., Landi, L., Nardi, S., & Isidoro, N. (2007). Relationships among *Hyalesthes obsoletus*, its herbaceous host plants and "bois noir" phytoplasma strains in vineyard ecosystems in the Marche region (central-eastern Italy). *Bulletin of Insectology*, *60*, 353–354.

Schneider, B., Gibb, K. S., & Seemüller, E. (1997). Sequence and RFLP analysis of the elongation factor Tu gene used in differentiation and classification of phytoplasmas. *Microbiology*, *143*, 3381–3389.

Sforza, R., Clair, D., Daire, X., Larrue, J., & Boudon-Padieu, E. (1998). The role of *Hyalesthes obsoletus* (Hemiptera: Cixiidae) in the occurrence of bois noir of grapevines in France. *Journal of Phytopathology*, *146*, 549–556.