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Evaluation of intron containing potential reference gene-specific primers to validate grapevine nucleic acid samples prepared for conventional PCR and RT-PCR

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Summary

Previously we proved the usefulness of an intron containing reference gene, phosphoenolpyruvate carboxylase (PEP) to validate cDNA synthesis for detection of grapevine viruses by conventional RT-PCR from crude nucleic acid preparations. Thus amplicons derived from residual genomic DNA (gDNA) and cDNA can be clearly distinguished by their sizes. Here we designed novel sets of primers which encompass one or two intron containing sequences of grapevine housekeeping genes such as actin, tubulin and elongation factor 1-α. Using these primers the expected sequences were amplified from gDNAs of the tested 24 grapevine cultivars. Thereafter they were challenged using cDNAs prepared from total nucleic acid samples isolated from cambial scrapings of dormant canes, leaf laminas, petioles and *in vitro* leaves of 12 grapevine cultivars. All of these novel, and the previously published PEP gene-specific primers generated the amplification of the expected shorter DNA fragments without introns. Thus they are suitable to check the quality of nucleic acid preparations and to validate subsequent cDNA synthesis prior to pathogen detection assays.

K e y w o r d s : cDNA synthesis; polymerase chain reaction; *Vitis vinifera.*

Introduction

Grapevines are affected by several pathogens including viroids, viruses, phytoplasmas, bacteria and fungi that spread with propagating material (Bisztray *et al*. 2012, Wilcox *et al*. 2015). Thus their detection and identification has an emerging importance in production of healthy stock material. To this end mostly DNA-amplification based protocols (e.g. conventional and real-time PCR) are used that target the genome (RNA or DNA) of the given pathogens.

Viroids and most viruses infecting grapevines contain RNA as genetic material (Martelli 2014, Maliogka *et al*. 2015, Gago-Zachert 2016, Steger and Perreault 2016, Gucek *et al.* 2017), but recently an increasing number of DNA viruses has also been described in *Vitis vinifera* (Zhang *et al*. 2011, Al Rwahnih *et al*. 2013, Poojari *et al*. 2013, Basso *et al*. 2015, Al Rwahnih *et al*. 2017). DNA viruses, phytoplasmas, bacteria and fungi can be detected and/or identified directly from crude plant nucleic acid samples by PCR while viroids and RNA viruses are detected from extracted RNAs by RT-PCR. To prevent false negatives in PCR reactions it is necessary to use an internal control to check the quality of nucleic acid preparations and/or the efficiency of cDNA synthesis. For such control experiments usually housekeeping genes are used, e. g., *18S rRNA* or *actin* genes (Bruisson *et al*. 2017, Gambino and Gribaudo 2006, Osman and Rowhani 2008).

Recently we designed a novel set of primers surrounding two or three introns in the *phosphoenolpyruvate carboxylase* gene (*PPC3*, VIT_212s0028g02180) to check the quality of cDNA samples. Thus amplicons derived from residual genomic DNA (gDNA) and cDNA produced by reverse transcription of RNAs could be clearly distinguished on the basis of their sizes since cDNAs lack the intron sequences. These primers amplified the expected bands from total nucleic acid samples prepared from leaves of 24 grapevine cultivars grown *in vitro* or in the greenhouse (Oláh *et al*. 2017). Here we describe three additional pairs of intron encompassing primer sequences that can be used as internal reference controls for the validation of gDNA as well as cDNA prior to detection of grapevine pathogens by conventional PCR. The expression of these genes was evaluated in 12 various grapevine cultivars as well as in different grapevine tissues/organs.

Material and Methods

For initial experiments to test the potential suitability of the newly designed reference gene specific primers gDNA samples of 24 rootstock, table and wine grape cultivars were included (see legend to Fig. 2). For subsequent gene expression studies four rootstocks, namely *V. berlandieri* x *V. riparia* '5C', *V. berlandieri* x *V. riparia* 'SO4', (*V. berlandieri* x *V. riparia*) x *V. vinifera* 'Georgikon 28' and *V. riparia* x

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V. cinerea 'Börner', and eight *Vitis vinifera* cultivars, namely 'Kövidinka', 'Sárfehér', 'Kadarka', 'Zefír', 'Furmint', 'Esther', 'Muscat Ottonel' and 'Welschriesling' were used.

Total nucleic acids (DNA and RNA) were isolated from (i) cambial scrapings of one year old wooden canes collected during the winter (January, 2017), (ii) leaves and (iii) petioles collected from field during the vegetation period (June, 2017), and from (iv) *in vitro* grown plants using a simplified CTAB-based protocol (Xu *et al*. 2004). Briefly, ca. 50 mg of plant material was directly lysed without washing in 1.0 ml of lysis buffer and after repeated extractions with chloroform:isoamyl alcohol nucleic acids were precipitated with 0.8 volumes of isopropanol at room temperature for 30 min. Finally, the precipitated nucleic acid samples were washed in 70 % ethanol, redissolved in 200 µL of sterile water, and checked in agarose gel by ethidium bromide (EtBr) staining. Revert Aid First Strand cDNA kit (Thermo Scientific, #K1622) was used for reverse transcription of 0.3-0.4 µg total RNA in 20 µl volume with random hexamer, and simultaneously with oligodT primers according to the supplier's instructions.

For primer design the Primer3 program (Untergasser *et al*. 2012) was used. Schematic structures of the potential *V. vinifera* reference genes included in this study with approximate position of primers are shown in Fig. 1. Polymerase chain reactions were carried out with KAPA *Taq* polymerase (KAPA Biosystems, KK1015) as proposed by the enclosed protocol. Reactions were performed in two independent experiments with an initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 54 or 58 °C (Table) for 30 sec, and extension at 72 °C for 1 min 30 sec. The reactions were closed with a final elongation step at 72 °C for 3 min. PCR products were separated by gel electrophoresis in 1.5 % (w/v) agarose followed by EtBr staining.

Results and Discussion

Primers surrounding one or two intron(s) were designed for three reference gene candidates showing stable expression in different tissues, cultivars or under abiotic or biotic stress conditions in previous studies. These include *actin* (Reid *et al*. 2006, Shinde *et al*. 2016), *tubulin* (Shinde *et al*. 2016, Upadhyay *et al*. 2015) and *elongation factor 1-α* (Monteiro *et al*. 2013, Reid *et al*. 2006, Shinde *et al*. 2016) genes. The published primers of potential reference genes designed for qPCR were usually tested only on one or two grapevine cultivars (Reid *et al*. 2006, Monteiro *et al*. 2013, Borges *et al*. 2014, Upadhyay *et al*. 2015, Katayama-Ikegami *et al*. 2016, Shinde *et al*. 2016) and were mostly positioned onto a single exon. We designed new sets of primers, which enclose one or two introns (Oláh *et al*. 2017 and this work) and generate amplicons optimal for conventional PCR (ca. 250-1500 bp) at commonly used cycling parameters. Thus products derived from gDNAs and cDNAs can be clearly distinguished on the basis of their sizes. The newly designed primers specific for *actin*, *tubulin* and *elongation*

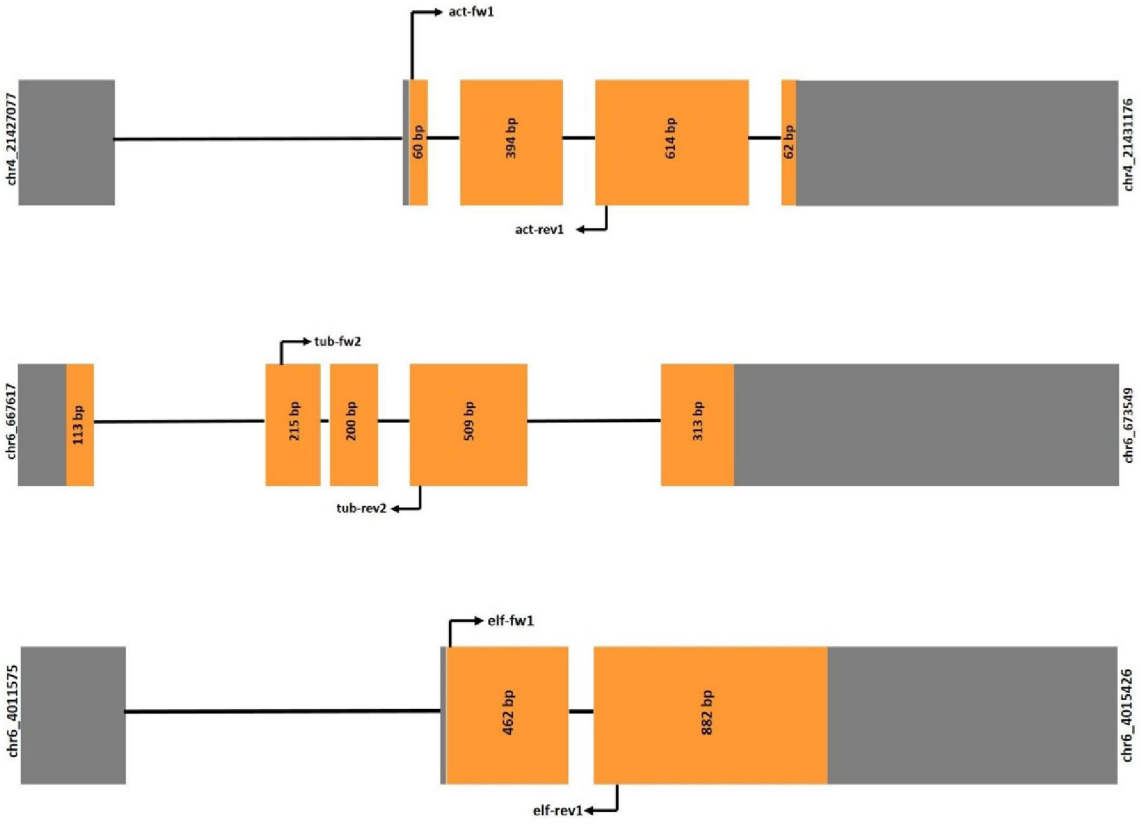

Fig. 1: Schematic representation of the *Vitis vinifera actin* (*ACT7*, VIT_204s0044g00580.1), *tubulin* (*TUA5*, VIT_206s0004g00480.3) and *elongation factor1-α* (*EF1-α*, VIT_206s0004g03240.1) genes. Introns are indicated by full black lanes. Exons are depicted by orange, 5' and 3' untranslated regions by grey colors. Approximate positions of the designed primers are indicated by arrows.

Table

Primers tested as potential internal controls

Primer name	Target gene	Sequence (5'→3')	Primer length (nt)	Annealing temperature (°C)	Amplified fragment length in bp (cDNA/genomic DNA)	Reference
PepSfw PepSrev	*V. vinifera phosphoenolpyruvate carboxylase* gene (*PPC3*, VIT_212s0028g02180)	GTCCTTACAGCACATCCTACTC CCCACCCATCCAAGAAGAAA	22 20	58	354/1597	Oláh *et al*. 2017
act-fw1 act-rev1	*V. vinifera actin* gene (*ACT7*, VIT_204s0044g00580.1)	GGCCGATACTGAAGATATCCAG ACCAGAATCCAGCACAATACC	22 21	54	472/664	This work
tub-fw2 tub-rev2	*V. vinifera tubulin* gene (*TUA5*, VIT_206s0004g00480.3)	CACGATGCTTTCAACACCTTC CTTCATTGTCCAAGAGCACAG	21 21	54	487/898	This work
elf-fw1 elf-rev1	*V. vinifera elongation factor1* gene (*EF1-α*, VIT_206s0004g03240	GGGTAAGGAGAAGGTTCACATC TGCCTTGGAGTACTTTGGTG	22 20	54	493/579	This work

factor 1-α genes, were first tested on the genomic DNA samples as templates prepared from *in vitro* grown plants of 24 grapevine cultivars. Each primer combination directed the synthesis of the expected amplicons. Namely, the *actin* gene-specific primers amplified a 664 bp, the *tubulin* gene-specific primers amplified an 898 and the *elongation factor 1-α* gene-specific primers amplified a 579 bp sequence (Fig. 2). These data show that the target sequences used for primer design are highly conserved among grapevine cultivars with different origin including rootstocks.

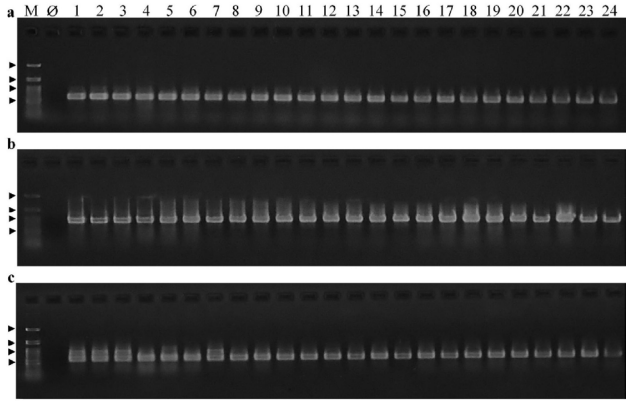

Fig. 2: PCR analysis of crude nucleic acid samples (containing both DNA and RNA) extracted from greenhouse and *in vitro* grown plants of 24 grapevine cultivars. Amplifications were carried out with act-fw1/act-rev1 (**a**), tub-fw2/tub-rev2 (**b**) and elf-fw1/elf-rev1 (**c**) primer pairs. M: H3 RTU DNA ladder (NIPPON Genetics Europe GmbH, Cat. no.: MWD 100). Arrowheads from top to bottom indicate 3000, 1500, 1000 and 500 bp long DNA fragments, respectively. Ø: Nucleic acid free control, lanes 1 to 24 are PCR products of grapevine cultivars *Vitis berlandieri* x *V. riparia* '5C', *V. berlandieri* x *V. riparia* 'SO4', *V. berlandieri* x *V. riparia* '5BB', (*V. berlandieri* x *V. riparia*) x *V. vinifera* 'Georgikon 28', *V. riparia* x *V. cinerea* 'Börner', *V. berlandieri* x *V. rupestris* 'Ruggieri 140' and *V. berlandieri* x *V. rupestris* '110 Richter'. *Vitis vinifera* cultivars tested were 'Kövidinka', 'Sárfehér', 'Kunleány', 'Miklóstelep 7', 'Kadarka', 'Kék bakator', 'Juhfark', 'Neoplanta', 'Pintes', 'Zefír', 'Furmint', 'Esther', 'Muscat Ottonel', 'Welschriesling', 'Vulcanus', 'Zervin' and 'Piros bakator'.

Next we have chosen 12 cultivars from the previously tested material for gene expression studies (see Material and Methods). Total nucleic acids prepared from cambial scrapings of dormant canes, from leaf laminas and petioles, as well as from *in vitro* grown plantlets were used for subsequent cDNA synthesis generated with random hexamer, and simultaneously with oligo dT primers. These cDNAs were challenged with conventional PCR using the four reference gene candidate-specific primers (Table). The expected shorter amplicons without introns which were deleted from cDNAs by RNA splicing were produced from all samples prepared from cambial scrapings, leaf laminas and petioles, as well as from *in vitro* grown grapevine leaves (see also Oláh *et al*. 2017). The *PEP, tubulin* and *elongation factor 1-α* gene-specific primers amplified both the 1597 bp, 898 bp and 579 bp fragment from residual gDNAs, as well as the 354 bp, 487 bp and 493 bp fragments from cDNAs, respectively (Oláh *et al*. 2017 and this work). Unexpectedly, the *actin* gene-specific primers produced only the smaller (472 bp) cDNA specific amplicon, but the larger 664 bp fragment was not clearly recovered from the residual gDNAs (Fig. 3). PCR reactions carried out with oligo dT-directed cDNAs produced the same results as shown for *tubulin* gene-specific assays in Fig. 4.

Grapevines may be infected by several viroids, RNA and DNA viruses, phytoplasmas, bacteria and fungi which are disseminated by propagating material. For the PCR-based detection of viroids and RNA viruses purified plant RNAs are reverse transcribed and cDNAs should be first validated by PCR using a reference gene-specific primer pair. To distinguish amplification products derived from residual gDNA and cDNA previously we designed *PEP* gene-specific primers pairs that encompass two or three introns (Oláh *et al*. 2017). Since the remnant DNA does not inhibit the reverse transcription reaction its removal that takes additional time and costs during preparation of nucleic acid templates for viroid and RNA virus detection is unnecessary. Therefore total plant nucleic acid preparations containing both DNAs and RNAs can be used as starting material for the detection of pathogens listed above as proposed by several

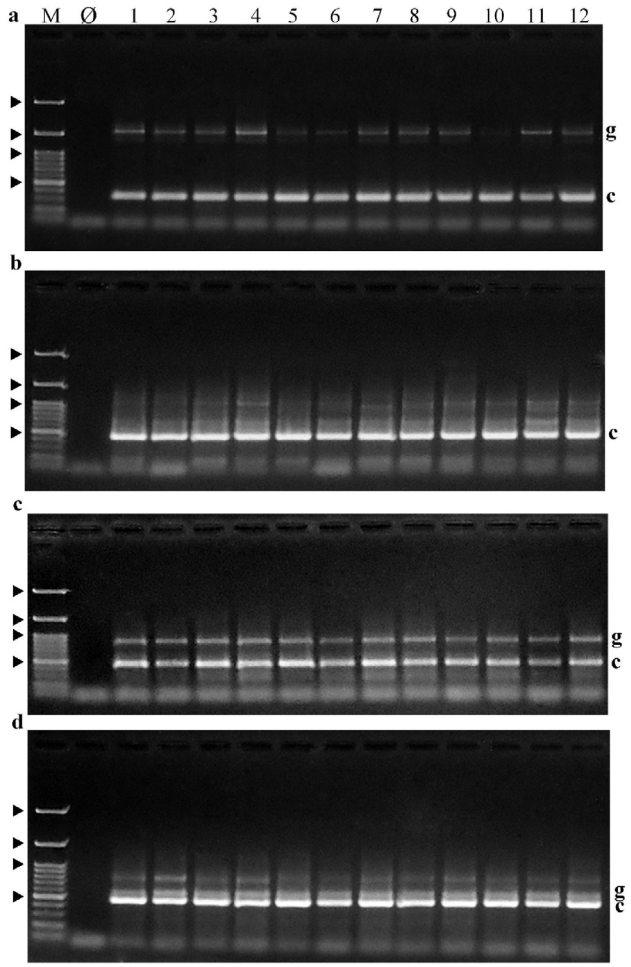

Fig. 3: PCR analysis of cDNAs started from total RNA samples extracted from leaves of field grown plants. Amplifications were carried out with PepSfw/PepSrev (**a**), act-fw1/act-rev1 (**b**), tub-fw2/tub-rev2 (**c**) and elf-fw1/elf-rev1 (**d**) primer pairs. M: H3 RTU DNA ladder (NIPPON Genetics Europe GmbH, Cat. no.: MWD 100). Arrowheads from top to bottom indicate 3000, 1500, 1000 and 500 bp long DNA fragments, respectively. Ø: Nucleic acid free control, lanes 1 to 12 are PCR products from cDNAs of *Vitis berlandieri* x *V. riparia* '5C'*, V. berlandieri* x *V. riparia* 'SO4', (*V. berlandieri* x *V. riparia*) x *V. vinifera* 'Georgikon 28', *V. riparia* x *V. cinerea* 'Börner' rootstocks followed by *V. vinifera* cultivars 'Kövidinka', 'Sárfehér', 'Kadarka', 'Zefír', 'Furmint', 'Esther', 'Muscat Ottonel' and 'Welschriesling'. **g**: amplicons derived from residual gDNAs, **c**: amplicons derived from cDNAs.

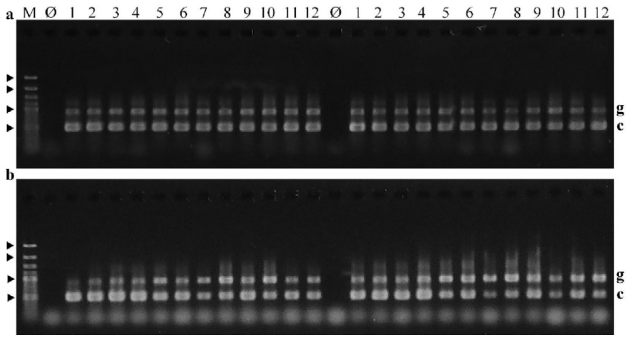

Fig. 4: PCR analysis of cDNAs started from total RNA samples extracted from cambial scrapings (**a**) and from leaves of *in vitro* grown plants (**b**). cDNAs were synthetized with random hexamer (left side) and oligo dT (right side) primers. Amplifications were carried out with tub-fw2/tub-rev2 primers. M: H3 RTU DNA ladder (NIPPON Genetics Europe GmbH, Cat. no.: MWD 100). Arrowheads from top to bottom indicate 3000, 1500, 1000 and 500 bp long DNA fragments, respectively. Ø: Nucleic acid free control, lanes 1 to 12 are PCR products from cDNAs of *Vitis berlandieri* x *V. riparia* '5C'*, V. berlandieri* x *V. riparia* 'SO4', (*V. berlandieri* x *V. riparia*) x *V. vinifera* 'Georgikon 28', *V. riparia* x *V. cinerea* 'Börner' rootstocks followed by *V. vinifera* cultivars 'Kövidinka', 'Sárfehér', 'Kadarka', 'Zefír', 'Furmint', 'Esther', 'Muscat Ottonel' and 'Welschriesling'. **g**: amplicons derived from remnant gDNAs, **c**: amplicons derived from cDNAs.

authors (Osman and Rowhani 2008, Margaria *et al*. 2009, Sun *et al*. 2014, Arruabarrena *et al*. 2016, Fiore *et al*. 2016). Here we have described three new sets of potential reference gene specific primers encompassing one or two introns thus amplicons derived from residual gDNAs and cDNAs can be clearly distinguished by their sizes since introns are deleted from cDNAs by RNA splicing. These, and the previously described *PEP* gene-specific (Oláh *et al*. 2017) primers direct the synthesis of approx. 600-1500 bp amplicons from gDNA and 350-500 bp amplicons from cDNA which are optimal with commonly used cycling parameters. Conventional direct PCR assays showed that the tested four genes were clearly amplified from the gDNAs of 24 grapevine cultivars and they are reliably expressed in the cambial scrapings of dormant canes, in leaves and petioles collected from field, as well as in *in vitro* grown plants. Thus these primers can be used as internal controls for any pathogen detection assays including virus surveys both at dormancy and in the vegetation period, as well as for preliminary selection of *in vitro* propagated plants produced from apical meristems or through somatic embryogenesis for virus elimination. Further studies are needed to determine if the described housekeeping gene-specific primers can be combined with different pathogen specific primers in duplex/multiplex PCR.

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