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 prepared from 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.

Key words: 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, Bassso 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 Griibaudo 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_21250028g02180) 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...
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 (Olah 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 (TU15, VIT_206s0004g00480.3) and elongation factor 1-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.
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 bp 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.

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
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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References


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GAMBINO, G.; GRIBAUDO, I.; 2006: Simultaneous detection of nine grapevine viruses by multiplex reverse transcription-polymerase chain reaction with coamplification of a plant RNA as internal control. Phytopathology 96, 1223-1229.


XU, Q.; WEN, X.; DENG, X.; 2004: A simple protocol for isolating genomic DNA from chestnut rose (Rosa roxburghii Tratt) for RFLP and PCR analyses. Plant Mol. Biol. Rep. 22, 301a-301g.
