Water Deficit Increases Stilbene Metabolism in Cabernet Sauvignon Berries

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The impact of water deficit on stilbene biosynthesis in wine grape (Vitis vinifera) berries was investigated. Water deficit increased the accumulation of trans-piceid (the glycosylated form of resveratrol) by 5-fold in Cabernet Sauvignon berries but not in Chardonnay. Similarly, water deficit significantly increased the transcript abundance of genes involved in the biosynthesis of stilbene precursors in Cabernet Sauvignon. Increased expression of stilbene synthase, but not that of resveratrol-O-glycosyltransferase, resulted in increased trans-piceid concentrations. In contrast, the transcript abundance of the same genes declined in Chardonnay in response to water deficit. Twelve single nucleotide polymorphisms (SNPs) were identified in the promoters of stilbene synthase genes of Cabernet Sauvignon, Chardonnay, and Pinot Noir. These polymorphisms resulted in eight changes within the predicted cis regulatory elements in Cabernet Sauvignon and Chardonnay. These results suggest that cultivar-specific molecular mechanisms might exist that control resveratrol biosynthesis in grapes.

KEYWORDS: Grape berry; stilbene synthase; stilbenes; Vitis vinifera; water deficit

INTRODUCTION

Resveratrol, a polyphenolic compound, exhibits anti-inflammatory, antioxidative, and antiproliferative properties (1–4) in humans. Resveratrol in wines is thought to contribute to the well-known health benefits of red wine, including protective effects against cardiovascular diseases (5) and extension of life span in animals (6–8). Its average concentration in red wines is 1.9 mg L\(^{-1}\) trans-resveratrol, ranging from nondetectable levels to 14.3 mg L\(^{-1}\) and is recognized to account, in part, for the famous “French Paradox” (9–12). Low concentrations of stilbenes, especially resveratrol and piceid, were found to protect against Alzheimer’s disease (13–16) and skin cancer (17). In the plant kingdom, resveratrol (3,4',5-trihydroxystilbene) biosynthesis occurs in various plants including grapes, berries, and peanuts (18) and is found in leaves, skin, and seed coats of the fruit (10, 19). Multiple stilbene-derived compounds, including isomers, polymers, and glycosylated forms, have also been characterized in grapes (11, 18, 19).

The resveratrol biosynthetic pathway consists of four enzymes: phenylalanine ammonia lyase (PAL), cinnamic acid 4-hydroxylase (C4H), 4-coumarate:CoA ligase (4CL), and resveratrol synthase, also known as stilbene synthase (STS) (18). Only plants with STS, the last enzyme in the resveratrol biosynthetic pathway, are capable of synthesizing resveratrol (18). Downstream of these reactions, a resveratrol glucosyltransferase transfers a glucose moiety onto the resveratrol backbone to produce piceid-derived compounds. This last enzyme was identified recently in Vitis labrusca grape berries (20). Likewise, another step in this pathway was recently characterized in grapes involving a resveratrol O-methyl transferase (ROMT) cDNA associated with the biosynthesis of pterostilbene, which has attracted much attention recently because of its promising pharmacological properties (21, 22).

Grape resveratrol biosynthesis appears to be dependent upon multiple factors including environmental and fungal stresses on the vine (23). Stilbene synthesis in Vitis spp. leaves can be influenced by fertilizer application, with less resveratrol accumulating with increasing nitrogen supply (24). UV light irradiation greatly induces STS steady state transcript abundance in unripe berries (25). In addition, ectopic expression of STS genes improves pathogen resistance in several plant species (26–30).

In the recently sequenced Vitis genome, 43 members of the STS gene family were identified (31). However, only 20 of them appear to be expressed in grapes, based upon transcript evidence. Previously, we reported that water deficit increases the specific steady state transcript abundance of a STS gene and phenylpropanoid metabolism in general in Cabernet Sauvignon berries (32). Here, we provide evidence in support of the hypothesis that stilbene concentrations are increased in drought stressed grapes.
Table 1. The Set of Transcripts Differentially Expressed by Water Deficit and Associated with the Resveratrol Biosynthetic Pathway

| aﬀy probe set ID | putative function     | treatment effect | p-value | adjusted p-value | cultivar A treatment effect | p-value | adjusted p-value | cultivar B treatment effect | p-value |
|------------------|-----------------------|------------------|---------|-----------------|-----------------------------|---------|-----------------|-------------------------------|---------|
| 160732_at        | chalcone synthase     | X                | 9.54 × 10⁻³| 2.87 × 10⁻²  | X                           | 9.54 × 10⁻³| 2.87 × 10⁻² | --                            | --      |
| 1610415_at       | cinnamate 3′hydroxylase| X                | 1.73 × 10⁻²| 4.64 × 10⁻³  | X                           | 2.75 × 10⁻⁶| 1.34 × 10⁻⁴| X 1.08 × 10⁻³                    | 1.80 × 10⁻³|
| 1610821_at       | cinnamate 4 hydroxylase| X                | --       | --              | --                          | --       | --              | X 2.56 × 10⁻⁵                  | 2.98 × 10⁻³|
| 1619119_s_at     | cinnamate 4 hydroxylase| X                | --       | --              | --                          | --       | --              | X 2.56 × 10⁻⁵                  | 2.98 × 10⁻³|
| 1609307_at       | 4-coumaroyl-CoA ligase| X                | --       | --              | --                          | --       | --              | --                            | --      |
| 1608094_at       | cinnamoyl-CoA reductase| X                | 1.42 × 10⁻³| 6.13 × 10⁻³  | X                           | 1.42 × 10⁻³| 6.13 × 10⁻³| --                            | --      |
| 1619513_at       | cinnamoyl-CoA reductase| X                | 6.09 × 10⁻³| 2.00 × 10⁻²  | --                          | --       | --              | --                            | --      |
| 1621163_at       | cinnamoyl-CoA reductase| X                | 1.47 × 10⁻⁴| 9.30 × 10⁻⁴  | --                          | --       | --              | --                            | --      |
| 1613113_at       | phenylalanine ammonia lyase| X                | --       | --              | --                          | --       | --              | --                            | --      |
| 1611285_at       | 4 Coumarate CoA Ligase| X                | 3.48 × 10⁻⁴| 1.90 × 10⁻³  | --                          | --       | --              | --                            | --      |
| 1617708_at       | resveratrol glucosyl transferase| X                | 7.96 × 10⁻⁴| 3.78 × 10⁻⁴  | --                          | --       | --              | --                            | --      |
| 1608009_s_at     | stilbene synthase     | X                | 1.92 × 10⁻⁴| 1.15 × 10⁻³  | X 1.55 × 10⁻¹⁰| 6.27 × 10⁻⁸| --              | --                            | --      |

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a, X, significantly diﬀerentially expressed. —, Not signiﬁcantly diﬀerentially expressed for the treatment eﬀect, cultivar X treatment eﬀects, cultivar X treatment X development eﬀects. **Multiple testing correction applied to p-value.**

**MATERIALS AND METHODS**

Field Experiments and Physiological Data. Grape berries harvested at seven diﬀerent developmental stages from Cabernet Sauvignon and Chardonnay (Vitis vinifera) vines, respectively, were collected during the summer of 2004 from the Shenandoah Vineyard in Plymouth, CA, and the Valley Road experimental vineyard belonging to the University of Nevada, Reno, NV, USA. Additional details about the training system, plant density, ripeness parameter (total soluble solids, titratable acidity) and stem water potentials were published previously (33). Berry cluster samples were collected on a weekly basis, and seven time points encompassing the growing season were selected to perform a global transcript profiling (33). To avoid any row effects, plants used for this experiment were located in the middle of the vineyard. All vines were equipped with drip irrigation. Irrigation was withheld until the desired range of stem water potentials was reached. Stem water potentials were measured with a pressure chamber as described previously (34). Two grape clusters were harvested weekly on the south (sunny) and the north (shady) side of each plant. The clusters were pooled together in order to avoid any light and temperature eﬀects. No visible symptoms of disease were observed on the grapevines or grape clusters. Clusters from three plants under diﬀerential water regime supply in Cabernet Sauvignon and Chardonnay were compared for their global transcript proﬁles. The eﬀect of water deﬁcit on the resveratrol biosynthetic pathway in Cabernet Sauvignon and Chardonnay grape varieties was investigated during berry development. Global transcript proﬁling throughout berry development was performed on Cabernet Sauvignon (CS) and Chardonnay (CH) cultivars of wine grape with two irrigation treatments - water deﬁcit stressed grapevines were maintained between 0.025% TFA in water and acetonitrile. The analytical HPLC separations were performed with an Agilent HPLC 1100 system on a Speedvic 200 column (Framingdale NY, USA) and recovered with 100 µL of methanol and 1 mL of water. The extract was chromatographically separated on a cation exchange resin column (6 mm × 40 mm) and eluted with 75% (v/v) aqueous methanol to obtain polyphenols. Analysis of polyphenols was performed with an Agilent HPLC 1100 system on a Prontosil Eurobound C18 (5 µm) reverse-phase column (4 mm i.d. × 250 mm) (Bischoff, Stuttgart, Germany). Solvents used for the separation were A, water with 0.1% trifluoroacetic acid; and B, acetonitrile with 0.1% trifluoroacetic acid. The elution program at a rate of 1 mL min⁻¹ was as follows: 0 min, 10% B in A; 50 min, 35% B in A; 51 min, 100% B in A; 60 min 100% B in A; and 61 min 10% B in A. The chromatogram was monitored at 286 and 306 nm. Stilbene contents were estimated according to calibration curves prepared with standards of trans-resveratrol (Sigma-Aldrich, Saint Quentin-Fallavier, France) and trans-piceid (Sequioa Research Products, Pangbourne, UK). For mass spectrometry identiﬁcation, the same solvents were used, but with less trifluoroacetic acid, which provokes signal suppression. The solvent concentrations were 0.025% TFA in water and acetonitrile. The analytical HPLC separations were also conducted in the same column and the HPLC eluent was introduced into the electrospray source in a postcolumn splitting ratio of 9:1. The mass spectrometry was acquired on a Thermo Finnigan LCQ Advantage ion trap spectrometer, equipped with an electrospray source. The software used for data acquisition and treatment was Xcalibur (http://www.xcalibur.com/). Data were obtained both in positive and negative ion modes with an ion spray voltage of 3.5 kV and an auxiliary gas temperature of 300 °C.
negative ionization modes; most of the compounds were assigned by their positive [ionization] mass spectra (higher sensitivity).

**DNA Extraction and Amplification of the STS Promoter Region.** DNA was extracted from the rachis of Chardonnay and Cabernet Sauvignon grape berry clusters according to Lodhi et al. (38). The transcript sequence used for the amplification of the STS promoter was related to the probe set (1608009_s_at). The annotated identifier from Genoscope (39) for this transcript was GSVIVT00008253001. Primers were designed upstream using Primer3 software, based on the sequence of the STS gene deposited on the Genoscope Web site, which contained the 8X assembly of the partially inbred derived Pinot noir genome (http://www.genoscope.cns.fr). PCR products were cloned into a pGEM-T vector (PROMEGA, Madison, WI). Clones of interest were sequenced in both strands by an ABI prism 3730 DNA analyzer using the Sanger method (40). Alignment of the sequence of the promoters from the two cultivars was performed using MEGA4 software (http://www.megasoftware.net/) (41). Aligned sequences were edited using MacVector 10 (Cary, NC; http://www.macvector.com/).

**RESULTS AND DISCUSSION**

Transcript abundance of genes related to the resveratrol biosynthetic pathway has been investigated in two cultivars with

![Figure 1. Expression of potential candidate unigenes associated with the resveratrol pathway. (a) Phenylalanine ammonia lyase (1613113_at; GSVIVT00018175001); (b) cinnamate-4-hydroxylase (1610821_at; GSVIVT00023932001); (c) 4-coumarate:CoA ligase (1609307_at; GSVIVT00031383001); (d) stilbene synthase (1608009_s_at; GSVIVT00008253001); (e) resveratrol-O-glycosyltransferase (1617078_at; GSVIVT00036670001); (f) resveratrol-O-methyltransferase (1617632_at; GSVIVT00003032001). The symbol ** indicates a nonsignificant difference observed for the treatment, cultivar-treatment interaction effects, and the cultivar-treatment-development interaction effects. Symbols represent means ± standard errors (SE); n = 3.](image-url)
two irrigation levels: sufficient water and water deficit. These transcripts were among the most differentially expressed genes that exhibited significant cultivar, treatment, and time interaction effects (Table 1). One probe set associated with stilbene synthase genes present in the Affymetrix microarrays, which represent a total of 8 genes out of the 42 genes identified in the Pinot Noir genome (42).

The first committed step associated with the resveratrol biosynthetic pathway is controlled by phenylalanine ammonia-lyase. One gene (1613113_at; GSVIVT00018175001) was found to have a statistically different mRNA expression pattern (Figures 1a and 2). This unigene encodes a protein that shares strong homology with phenylalanine ammonia-lyase-lyase in Arabidopsis thaliana and in Saccharomyces cerevisiae and in Escherichia coli of 4-coumarate:coenzyme A ligase (AtPAL2), which has functional specialization in abiotsically, environmentally triggered flavonoid synthesis (43). In our study, there was a coordinated and gradual decline in transcript abundance of this gene over berry development in both cultivars under well-watered conditions. In Cabernet Sauvignon, both genes had coordinately increased transcript abundance in water-deficit-treated compared to well-watered berries from the véraison stage through harvest (Figures 1a and 2). In contrast, transcript abundance in Chardonnay was reduced in water-deficit-treated berries. This indicates cultivar specificity with respect to the relative transcriptional regulation of these two genes.

The second step in the resveratrol biosynthetic pathway is catalyzed by cinnamate 4-hydroxylase (C4H). In Arabidopsis, the C4H gene responds to various biotic stresses and abiotic stresses, such as light and wounding, indicating that it might have diverse functions in phenylpropanoid metabolism (44, 45). In Cabernet Sauvignon, one C4H transcript (1610821_at; GSVIVT00023932001) showed increased abundance under water deficit from véraison through harvest, whereas the expression of this same gene was not affected in Chardonnay (Figures 1b and 2).

The third step of resveratrol biosynthesis is related to the expression of the 4-coumarate coenzyme A:ligase (4CL) gene (1611265_at; GSVIVT00009148001). There was a significant increase in transcript abundance due to water deficit in Cabernet Sauvignon following véraison through harvest, but a decrease in transcript abundance in water-deficit-treated Chardonnay berries during the same period (Figures 1c and 2). The closest ortholog of this gene is the tobacco 4-coumarate coenzyme A ligase 2 gene, which was found to utilize 4-coumarate as a substrate, supporting its direct involvement in the biosynthesis of coumaroyl-CoA, precursor of trans-resveratrol (46). Constitutive expression in Saccharomyces cerevisiae and in Escherichia coli of 4-coumarate:coenzyme A ligase 4CL from tobacco enhances resveratrol through harvest, whereas the expression of this enzyme was affected in Chardonnay (Figures 1b and 2).

The next step in the resveratrol biosynthetic pathway is catalyzed by stilbene synthase (STS) or resveratrol synthase (RS). The most remarkable and statistically significant difference was seen with a probe set (1608009_s_at; GSVIVT00008253001) that underwent a 10-fold increase upon water deficit one week following véraison in Cabernet Sauvignon, reaching maximal expression five weeks after véraison (Figures 1d and 2). In contrast,
Figure 3. Gene expression profiles of other stilbene synthases on the microarray. (a) 1606750_at, GSVIVG00009225001, stilbene synthase 1), (b) 160967_at, GSVIVT00005194001, stilbene synthase), (c) 1610070_at, GSVIVT00009232001, stilbene synthase), (d) 1611190_s_at, GSVIVT00010117001, resveratrol synthase 1), (e) 1616575_at, GSVIVT00009225001, stilbene synthase 2), (f) 1610850_at, GSVIVT00009216001, stilbene synthase 1), (g) 1612804_at, GSVIVT00004047001, stilbene synthase 3). The lines and symbols are the same as in Figure 1. Symbols represent means ± SE; n = 3.
the transcript abundance of this gene in Chardonnay was always higher in well-watered vines than in vines exposed to water deficit. Interestingly, the expression of seven other, but less strongly expressed, STS isogenes showed very similar transcript expression patterns to GSVIVT00008253001 (Figure 3). However, these results should be interpreted with caution because the expression of these isogenes was not statistically different between well-watered and water-deficit-stressed plants in both cultivars.

Trans-resveratrol is converted to trans-piceid by resveratrol-O-glucosyltransferase. One gene was recently characterized in *Vitis labrusca*; however, other substrates such as *p*-hydroxybenzoic and hydroxycinnamic acids might be acted up by this enzyme (48). The steady state abundance of the transcript of resveratrol-O-glucosyltransferase (1617078_at; GSVIVT00036670001) declined steadily over the course of development and was unaffected by water deficit in either cultivar. Two other genes (GSVIVT00036668001; GSVIVT00036670001) potentially associated with this enzyme are present in the Pinot Noir genome according to the 8X assembly. However, none of them had a related probeset (Figures 1e and 2). The transcript abundance of resveratrol-O-methyltransferase (1617632_at; GSVIVG00003032001) was unaffected by water deficit even though the pattern of expression was different between the two cultivars (Figure 1f). Because of the crosstalk between monolignol and the phenylpropanoid biosynthetic pathways, the expression of genes encoding other enzymes including chalcone synthase (CHS), which leads to flavonoid production, and cinnamate 3'-hydroxylase (C3H) and cinnamoyl CoA reductase (CCR), which lead to monolignol production, was surveyed. The transcript abundance of four out of six genes analyzed showed small increases upon water deficit in Cabernet Sauvignon after véraison (Figure 4), and three of these transcripts showed similar increases in water-deficit-stressed Chardonnay. Upon the basis of the above transcript abundance changes, stilbene concentrations were hypothesized to increase following water deficit treatment. Stilbene accumulation was determined by HPLC-coupled mass spectrometry (MS) with the same berry samples used for the transcriptomic analyses. Cabernet Sauvignon and Chardonnay were analyzed from whole berries harvested at six and eight weeks, after véraison, respectively. The abundance of two stilbene-derived compounds, trans-piceid and trans-resveratrol, was not significantly different between the two cultivars when well-watered (Figure 5). The most abundant stilbene in both cultivars was trans-piceid, the glycosylated form of trans-resveratrol. Trans-piceid concentration significantly increased 5-fold in response to water deficit in berries of Cabernet Sauvignon, but not in Chardonnay. Given that resveratrol-O-glucosyltransferase gene expression was unaffected by water stress, STS gene expression appears to be a regulatory step for the accumulation of

Figure 4. Expression profiles of unigenes related to flavonoid and monolignol biosynthesis. (a) 1621163_at, GSVIVT00024039001, cinnamoyl CoA reductase, (b) 1617740_at, GSVIVT00001045001, cinnamate 3’ hydroxylase, (c) 1607732_at, GSVIVT00037969001, chalcone synthase. The lines and symbols are the same as in Figure 1. Symbols represent means ± SE; n = 3.

Figure 5. The concentrations of trans-piceid and trans-resveratrol in whole berries for both cultivars at EL-stage 38 (harvest stage) for the two irrigation treatments. CH = Chardonnay, CS = Cabernet Sauvignon, WW = well watered, WD = water deficit. Values are means ± SE; n = 6. The * indicates a significant difference from well-watered plants (P < 0.001; two-way ANOVA), (inset) mass spectrometric data with fragmentation patterns to confirm the presence of trans-piceid and trans-resveratrol in the samples.
trans-piceid. This result is consistent with findings in *Sorghum bicolor* in which ectopic expression of STS enhanced the accumulation of cis-piceid (49).

In summary, the hypothesis that resveratrol biosynthesis would increase due to water deficit in Cabernet Sauvignon berries was supported by both transcript and metabolite data. Transcript data revealed that the majority of resveratrol biosynthetic genes exhibited increased steady state transcript abundance following water deficit in Cabernet Sauvignon, but not in Chardonnay. These results were confirmed by the metabolite profiling of resveratrol and piceid, indicating that under water deficit, the synthesis of stilbenes might be differentially regulated at the transcriptional level between the two cultivars.

Interestingly, the impact of water deficit on stilbene synthesis was previously investigated in *V. vinifera* cv. Barbera (50). In this study, the application of water deficit did not significantly influence the stilbene concentrations in Barbera berries, whereas cumulative applications of methyl jasmonate did induce trans-resveratrol synthesis at the ripening stage (50). Piceid concentrations were not measured, so it is uncertain whether this result was due to cultivar differences.

To provide a preliminary assessment of the genomic origin of the cultivar-specific interactions with water deficit in the transcriptional regulation of STS genes, a 1.2 kb proximal upstream region of the most differentially expressed stilbene synthase gene of this experiment (GSVIVT00008253001) was sequenced for Chardonnay and Cabernet Sauvignon. The sequences were compared to the corresponding promoter region from the partially inbred derived Pinot Noir genome (51). Interestingly, the nucleic acid alignment of this promoter region indicates a closer homology between Pinot Noir and Cabernet Sauvignon, even though Chardonnay is a progeny of Pinot Noir (51).

Computational analysis of this particular promoter region in both cultivars, using the PLACE algorithm (http://www.dna.affrc.go.jp/PLACE/) revealed 12 single nucleotide polymorphisms (SNP) resulting in the modification of eight canonical cis-elements within the promoter region (Table 2). Particularly interesting were two single nucleotide changes in regions that are related to abiotic stress regulation (Table 2). The first polymorphism, positioned at −407 from the transcription start site, lies within a regulatory element named the RAV1AAT-box. This polymorphism present in 50% of the cloned Cabernet Sauvignon sequences surveyed by PCR amplification indicates that only one allele is affected by this mutation. This particular cis-element is bound by the RAV transcription factor family, from which ectopic expression results in enhanced plant tolerance to biotic and abiotic stresses (52). Moreover, the transcript abundance of one member of this transcription factor family was found to be induced by abscisic acid (ABA) in soybean (53). Further investigations will be needed to correlate the potential role of ABA in the resveratrol biosynthetic pathway, and a more detailed functional analysis of the resveratrol promoter region will be critical to clearly identify potential ABA regulatory elements of this promoter.

The second nucleotide change located at −273, from the transcription start site, leads to the formation of an I-Box core in 50% of the clones sequenced from Cabernet Sauvignon, but not in Chardonnay, indicating a heterozygous state for this cis-element in Cabernet Sauvignon. This I-Box core is commonly found upstream of light-regulated genes (54, 55). In grapevine, water deficit alters canopy architecture by reducing shoot growth and basal shoot foliage, leading to higher levels of sun exposure within the cluster zone (56). Therefore, transcriptional regulation of some target genes might be due to light or heat effects, as already proposed for the flavonoid pathway in vines exposed to water deficit (57, 58). Recently, comparative analysis between gene expression and protein accumulation of resveratrol synthase indicated that both are strongly influenced by UV–C irradiation (59). Whether this environmental cue is directly involved in a differential cis-regulatory activity of the STS gene between cultivars should be tested experimentally. Polymorphisms in promoter regions have been identified previously in *Vitis*. For instance, some of them are associated with traits selected during domestication that yield greater color (60, 61).

To the best of our knowledge, this is the first report showing an increase in trans-piceid concentration in grape berry under water deficit conditions. Water deficit does not induce the transcript abundance of the gene most directly related to the piceid accumulation (resveratrol-O-glycosyltransferase) but rather affects the expression of the STS gene. Even though this glycosylated derivative is not the most active form of the stilbene compounds in wine, hydrolysis of trans-piceid releases trans-resveratrol in the small intestine and liver of humans (62). The elucidation of differences in the promoter of STS gene might allow both cultural and genetic manipulation of this important class of nutraceuticals, leading to increased concentrations of stilbene-derived compounds in grape berry. In addition to the potential human health benefits, increased stilbene concentrations in berries may contribute to improved vine resistance to pathogens and reduce the use of pesticides in vineyards.

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**Table 2. Putative cis-Acting Elements Harboring Polymorphisms within the Proximal Region of the STS2 Promoter in Cabernet Sauvignon and Chardonnay**

| nature of mutation | Chardonnay sequences | Cabernet Sauvignon sequences | metabolism or response | metabolism or response |
|--------------------|----------------------|------------------------------|------------------------|------------------------|
| −823 substitution in CH | GAATGG | GAATGA | — | — |
| −553 substitution in CH | CAATGGTGT | CTAAGACAA (RAV1-Box) | drought stress response elements | — |
| −407 substitution in CH | GACA | AGTCCTTTA | light regulated response elements | — |
| −273 substitution in CH | AGTCTTTA | CACAAATA | — | — |
| −190 deletion in CH | CACATA | AACGCAT | — | — |
| −181 substitution in CH | AACACAT | TAGGCTTTA | — | — |
| + 20 substitution in CH | TAGGCCTTA | — | — | — |

*The symbol * means that the DNA sequencing of 50% of the PCR products result in the presence of a cytosine residue.*
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