Short Communication

A MULTIPLEX RT-PCR FOR SIMULTANEOUS DETECTION OF THE AGENTS OF YELLOW SPECKLE AND VEIN BANDING DISEASES IN GRAPEVINE

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SUMMARY

Six viroids have been reported to infect grapevine and among which only *Grapevine yellow speckle viroid*-1 (GYSVd-1) and *Grapevine yellow speckle viroid*-2 (GYSVd-2) are able to induce yellow speckle (YS) disease, characterized by yellowish flecks scattered on the leaf blade or massed along the veins. The mixed infection of GYSVd-1 or GYSVd-2 with *Grapevine fanleaf virus* (GFLV) may elicit vein banding (VB), a syndrome characterized by chrome-yellow flecks along the main veins of mature leaves and expanding through the interveinal tissues of infected leaves. In the present study, we have developed a multiplex RT-PCR (mRT-PCR) for simultaneous detection of GYSVd-1, GYSVd-2 and GFLV. *Hop stunt viroid* (HSVd), another viroid that infects most grapevines worldwide is used as a positive control instead of a host-derived internal control. The test developed in this study proved to be reliable for simultaneous detection of three pathogens, *i.e.* GYSVd-1, GYSVd-2 and GFLV in grapevine; hence it is useful for detection of YS and VB in large-scale surveys and certification programs.

Keywords: Detection, PCR, Viruses, Viroids, GYSVd-1, GYSVd-2, GFLV.

Grapevine (*Vitis vinifera*) is an important crop plant cultivated worldwide. It is susceptible to infection by at least 63 virus species (Martelli, 2014) and six viroids (Zhang *et al*., 2014), some of which cause diseases. Six viroids of different genera belonging to the family *Pospiviroidae* have been isolated from grapevine, i.e. *Hop stunt viroid* (HSVd), *Citrus exocortis viroid* (CEVd), *Grapevine yellow speckle viroid*-1 (GYSVd-1), *Grapevine yellow speckle viroid*-2 (GYSVd-2), *Australian grapevine viroid* (AGVd) (Little and Rezaian, 2003) and *Grapevine latent viroid*, which has been recently described (Zhang *et al*., 2014).

In grapevine, only GYSVd-1 and GYSVd-2 are able to induce yellow speckle (YS), a disease first reported by Taylor and Woodham (1972) in Australia. The role of these viroids in YS symptom expression was shown by Koltunow *et al*. (1989). YS is characterized by tiny yellow spots distributed on the leaf surface or concentrated along the veins usually appearing in the height of summer (Stellmach and Goheen, 1988). Mixed infections by GYSVd-1 and/or GYSVd-2 and GFLV have been shown to elicit vein banding (VB) (Szychowski *et al*., 1995), a disease characterized by chrome yellow mottling or banding which localized along the main veins of mature leaves, then expanding into the interveinal areas of affected vines (Goheen and Hewitt, 1962). The presence of these pathogens is not sufficient for inducing YS or VB symptoms in vines. Probably various other factors, including climate conditions (Salman *et al*., 2014), sequence variability of viroids (Polivka *et al*., 1996; Salman *et al*., 2014) and virus and/or viroids titer (Szychowski *et al*., 1995), are involved in their symptoms appearance. Actually these infectious agents are frequently latent in grapevine hence they can be missed from the sanitary selection programs.

In Iran, grapevine is cultivated mainly in three distinct regions, including North-West, North-East and South, where GFLV and four of the six known grapevine viroids have been reported (Sokhandan-Bashir and Hajizadeh, 2007; Pourrahim *et al*., 2007; Hajizadeh *et al*., 2010). In the northwest of Iran, VB was found in 16% of vines infected by GYSVd-1, GYSVd-2 and GFLV, whereas YS symptoms, which occurred in 10% of the tested plants, were always shown in vines infected only by GYSVd-1 and/or GYSVd-2 (Hajizadeh *et al*., 2015a).

Up to now, just a few reports exist on the incidence of mixed infections with GFLV and viroids (Krake and Woodham, 1983; Prota *et al*., 1985; Szychowski *et al*., 1995), probably due to the lack of fast and reliable laboratory procedures for contemporary detection of these infectious agents. A multiplex RT-PCR (mRT-PCR) has been validated for simultaneous detection of five grapevine viroids (Hajizadeh *et al*., 2012). Also, a few mRT-PCR have been optimized for detecting several viruses in grapevine

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Table 1. Primer pairs used for detection of GYSVd-1, GYSVd-2, HSVd and GFLV by multiplex RT-PCR.

Target	Primer name[a]	Sequence (5'-3')	Positions[b]	Expected cDNA (bp)	Reference
GFLV	GFL-mF	TCCCGGGGTGTATGTGGAAG	2851-2870	451	This work
	GFL-mR	GCTCCCACTGCTCTT(A/G)CCAA	3301-3282		This work
HSVd	HSVd-mF	TGAGCCCCTCTGGGGAATTC	294-11	302	This work
	HSVd-mR	AGAGAGGATCCGCGGCAGA	293-275		This work
GYSVd-2	GYSVd-2-P1	ACTAGTACTTTCTTCTATCTCCGAAGC	184-204	229	Jiang *et al*., 2009
	GYSVd-R[c]	CGGAGGCCTTCCGAGGTG	49-32		This work
GYSVd-1	GYSVd-1-mF	CAAAGCCCTTTTTCTTTCAACTGAG	287-311	126	Hajizadeh *et al*., 2015a
	GYSVd-R[c]	CGGAGGCCTTCCGAGGTG	48-31		This work

[a]Forward primers are indicated in bold.
[b]Positions of primers with respect to the reference variant.
[c]This primer is common for detecting both GYSVd-1 and GYSVd-2.

(Gambino and Gribaudo, 2006; Digiaro *et al*., 2007; Gambino, 2015). In the present study, a new mRT-PCR assay for simultaneous detection of GYSVd-1, GYSVd-2 and GFLV has been developed, which will be useful for further investigation on YS and VB in grapevine, also for certification programs.

Selection and design of specific primers for each target species is a key step in the development of mRT-PCR. When optimizing a multiplex PCR, several other factors must be taken into consideration, including differences in the size of the expected amplicons, balance of melting temperature of several primer pairs, optimization of primer concentration, and amplification cycles. In the present work, for detection of GYSVd-1 and GYSVd-2, the reverse primer was designed based on a conserved region between these viroids. The conserved region was identified by alignment of Iranian viroid sequences along with those already deposited in the GenBank database. The forward primers for detection of GYSVd-1 and GYSVd-2 were those reported by Hajizadeh *et al*. (2012) and Jiang *et al*. (2009), respectively. The specific primer for GFLV detection was designed based on the conserved region of the coat protein gene (Table 1). The specificity of the candidate primers for detection of GYSVd-1 and GYSVd-2 were further tested *in silico,* using Amplify (version 3.1.4) software (Engels, 1993) in the presence of cDNAs belonging to the other grapevine viroids. The designed primers were expected to amplify three specific fragments for the three infectious agents, varying in their size (100 to 450 bp), and differing in length from each other by at least 80 bp. All primer pairs were separately tested by single RT-PCR (sRT-PCR) to evaluate their ability to amplify the expected fragments and determine the best amplification conditions. cDNA preparation from a vine (Bm9) naturally infected by GYSVd-1, GYSVd-2 and GFLV was used in these preliminary tests to assess primer specificity.

To develop this test, grapevine leaves with typical symptoms of infections by i) GFLV (vein yellowing, leaf deformation, mosaic, zigzag stems), ii) GYSVd-1 and GYSVd-2 (yellow speckle), and iii) GYSVd-1, GYSVd-2 and GFLV (vein banding) or with no apparent symptoms were collected from Iranian vineyards (East and West Azerbaijan and Kurdistan provinces) during 2010-2015. A viroid-free grapevine of cv. Nebbiolo regenerated from somatic embryos (Gambino *et al*., 2011) was used as a negative control. The silica-capture extraction method (Foissac *et al*., 2000) was used for preparing total nucleic acid (TNA) extracts with minor modifications (Hajizadeh *et al*., 2012) and subsequently subjected to cDNA synthesis. Reverse transcription (RT) reactions were carried out in 10 µl final volume. For each reaction 1 µl (0.2 µg/ml) of random hexamer primer (100 mM) and 4 µl aliquot of total RNA suspension were added to 5 µl cDNA synthesis kit (HyperScript™ Reverse Transcriptase, GeneAll, Seoul, Korea). The mixture was incubated at 55°C for 60 min to activate the reverse transcriptase enzyme for cDNA synthesis in a BioRad thermocycler (BioRad, USA).

To determine optimum conditions for mRT-PCR, the following factors were tested: (i) a range of annealing temperatures (52-60°C), of which 56°C showed the best result; (ii) four different cycle numbers (28, 30, 32 and 35), with reliable results observed when 32 cycles was used; (iii) several concentrations of each primer from 0.1 µM to 0.5 µM: all the pathogens were successfully detected using 0.2 µM of the GFLV primer pairs, 0.2 µM of the GYSVd-1 and GYSVd-2 forward primers and 0.4 µM of the GYSVd-1 and GYSVd-2 reverse primer.

mRT-PCR was performed with 1 µl of cDNA in a final reaction volume of 12.5 µl consisting of 1× PCR Master Mix (Amplicon, GeneAll, Seoul, Korea) (Tris-HCl pH 8.5, $(NH4)_2SO_4$, 3 mM $MgCl_2$, 0.2% Tween 20, 0.4 mM dNTPs, 0.2 Units/µl Ampliqon *Taq* DNA polymerase) in a BioRad thermocycler (BioRad, USA) and a final primer concentration of 0.2 µM for GFLV primers, GYSVd-1 and GYSVd-2 forward primers and of 0.4 µM for GYSVd-1 and GYSVd-2 reverse primers. Cycling conditions were as follows: initial denaturation at 94°C for 3 min followed by 32 cycles at 94°C for 30 s, 56°C for 30 s, 72°C for 1 min and a final extension step at 72°C for 7 min. Reaction products were visualized in ethidium bromide-stained 1.8% agarose gels. cDNA prepared from a virus and viroid-free sample (cv. Nebbiolo) (Gambino *et al*., 2011) and a preparation

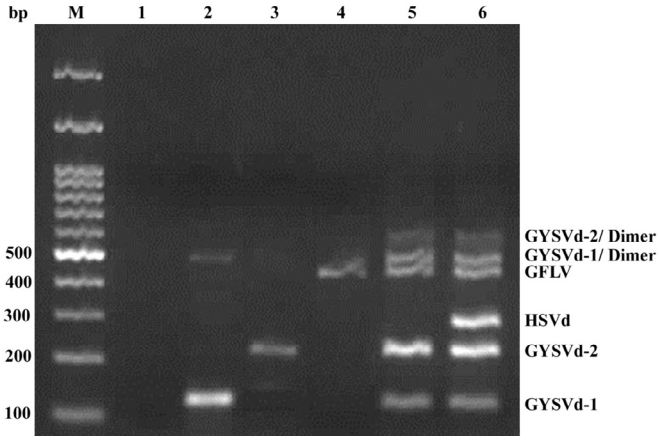

Fig. 1. Agarose gel electrophoresis analysis of DNA fragments amplified from infected grapevines by single RT-PCR (sRT-PCR) and multiplex RT-PCR (mRT-PCR). Total nucleic acid (TNA) preparation from an Iranian grapevine (BM9) co-infected by GYSVd-1, GYSVd-2, HSVd and GFLV tested by sRT-PCR with primer pairs specific for GYSVd-1 (lane 2), GYSVd-2 (lane 3), GFLV (lane 4), and by mRT-PCR, mixing together the same primers (lane 5) and the same sample used in lane 6 were tested after adding an additional primer pair designed to amplify full length of HSVd as internal positive control; lane 1, healthy control and lane M, 1 Kb ladder with fragment sizes reported on the left.

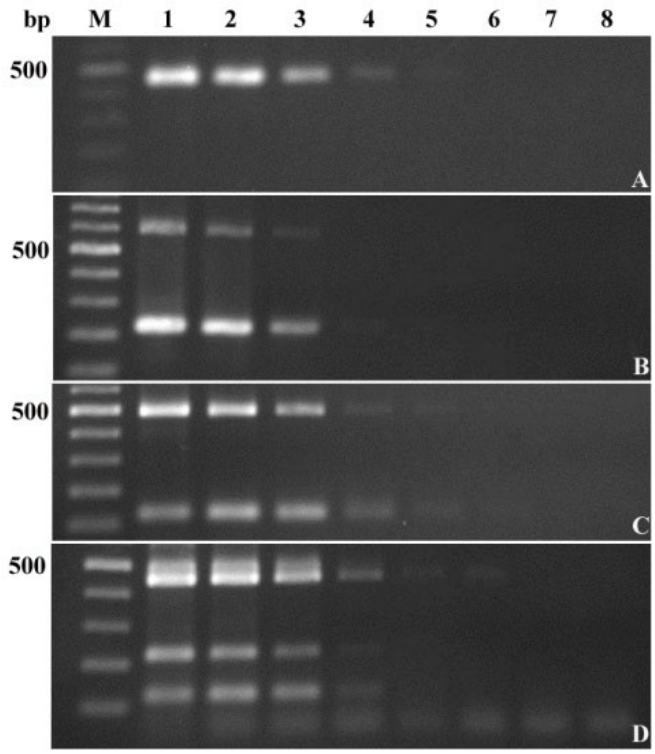

Fig. 2. Agarose gel electrophoresis analysis of DNA fragments amplified by single RT-PCR (sRT-PCR) and multiplex RT-PCR (mRT-PCR). TNA preparation from a positive control naturally infected by GFLV, GYSVd-2 and GYSVd-1 was serially diluted in TNA extracted from healthy plants. Panels A, B and C: sRT-PCR performed using GFLV, GYSVd-2 and GYSVd-1-specific primer pairs separately; D, mRT-PCR. Details of the primers used in these tests are provided in Table 1. Lane M, DNA 100 bp ladder; Lane 1, undiluted; Lane 2, 10^{-1} dilution; lane 3, 10^{-2} dilution; lane 4, 10^{-3} dilution; lane 5, 10^{-4} dilution; lane 6, 10^{-5} dilution; lane 7, 10^{-6} dilution; lane 8, healthy control.

from a vine naturally infected by GYSVd-1, GYSVd-2 and GFLV were used as negative and positive control, respectively.

In order to test the mRT-PCR, cDNAs prepared from TNA extracts of infected plants with GFLV and/or GYSVd-1 and/or GYSVd-2 were subjected to mRT-PCR using the PCR conditions as mentioned above and were successfully detected in all virus and viroid combinations (Fig. 1, lane 5). In most cases, in addition to the expected fragments, extra bands of a larger size (475 bp for GYSVd-1 and 580 bp for GYSVd-2) were detected (Fig. 1, Fig. 3). The sizes of these bands are consistent with the expected amplicons plus full length of the two viroids. Therefore, these bands were not non-specific amplicons, but corresponded to the specific viroid-derived cDNAs.

To verify the PCR results, the amplified fragments were ligated into the pTG19 Vector according to the manufacturer's instructions (SinaClon, Iran) and the recombinant plasmids were sequenced using the M13 universal forward primer by Macrogen Inc. (Seoul, South Korea). The amplicons showed a high sequence identity with variants of the causal agents of VB (data not shown).

To determine and compare the detection limits of standard RT-PCR (sRT-PCR) and mRT-PCR, the TNA preparation from the Bm9 positive control was serially diluted with a TNA preparation from a non-infected vine before reverse transcription. cDNAs synthesized by the random hexamer primers were then amplified by sRT-PCR, using the primer pairs specific for GYSVd-1, GYSVd-2 and GFLV separately, and by combining all the primers in the amplification mixture of mRT-PCR. Detection limits were 10^{-3} (GYSVd-2) to 10^{-4} (GYSVd-1 and GFLV) in sRT-PCR (Fig. 2, panels B, C and A, respectively). Surprisingly, amplification by mRT-PCR had more sensitivity for GFLV, while GYSVd-2 had same sensitivity, and GYSVd-1 was less sensitive. GYSVd-1, GYSVd-2 and GFLV had a detection limit of 10^{-3}, 10^{-3} and 10^{-5}, respectively in mRT-PCR (Fig. 2, panel D).

We used HSVd as a reliable internal positive control to show false negative results due to RT-PCR inhibitors, which are prevalent in grapevine. Previous studies showed that HSVd is widespread in Iranian grapevines and infect almost all the cultivated grapes, hence it was used as positive internal control in mRT-PCR to detect five different viroids at the same time (Hajizadeh *et al.*, 2012, 2015a, 2015b). We did the same in the novel mRT-PCR assay aimed to detect GYSVd-1, GYSVd-2 and GFLV. Advantages of using a viroid instead of host-derived RNA (Gambino *et al.*, 2009) as an internal positive control in our mRT-PCR protocol are: i) HSVd shares many structural characteristics with the other grapevine viroids and

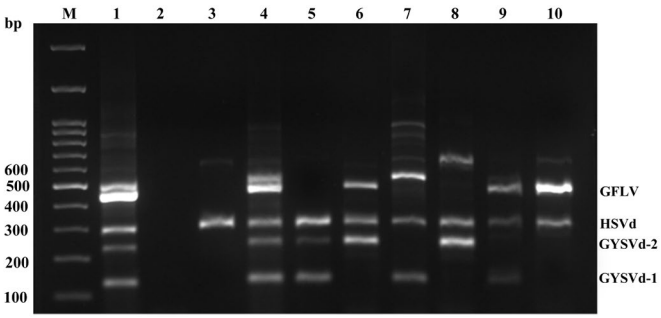

Fig. 3. Detection of natural viroid infections in grapevine by mRT-PCR. mRT-PCR was performed using TNA preparations from naturally infected grapevines representative of the single and mixed infections found in the Iranian samples. The viroids and GFLV specific primers used in the mRT-PCR are detailed in Table 1. Lane M, 100 bp ladder; lane 1, positive control; lane 2, healthy control; lanes 3 to 10, naturally infected grapevines.

ii) being an exogenous RNA, DNase treatment is not needed before reverse transcription, saving time and costs for the diagnostic system. Throughout this study, HSVd worked as a valid positive control for the mRT-PCR-based tests (Fig. 3).

The novel mRT-PCR method was able to detect both single as well as mixed viroid infections in 24 tested samples, allowing the unequivocal diagnosis of all natural combinations of GYSVd-1, GYSVd-2 and GFLV which were previously reported in Iranian vines (Hajizadeh *et al*., 2010, 2015a) (Table 2; Fig. 3) based on sRT-PCR performed using specific primer pairs described by other authors (Polivka *et al*., 1996; Wan Chow Wah and Symons, 1997; MacKenzie *et al*., 1997). In addition, all the tested samples positive to GYSVd-1, GYSVd-2 and GFLV by conventional sRT-PCR (Hajizadeh *et al*., 2010), were also positive to the same viroids and GFLV when assayed by mRT-PCR (Table 2) except for some few samples. One vine tested positive to GFLV in sRT-PCR, but gave a negative result in mRT-PCR; meanwhile, two and five additional samples, which tested negative in sRT-PCR (Table 2, gray background), were positive to GYSVd-1 and GFLV by mRT-PCR, respectively. The difference might be due to the better efficiency of the new primers, which were designed considering the sequence variability among the Iranian isolates of GYSVd-1 and GFLV compared to those reported in other geographic areas (Hajizadeh *et al*., 2015a; Polivka *et al*., 1996; Szychowski *et al*., 1998; Naraghi-Arani *et al*., 2001; Vigne *et al*., 2004).

Efficiency of our new mRT-PCR was further proved by testing 36 additional samples. Although GFLV with GYSVd-1 and/or GYSVd-2 were mostly found in symptomless plants, alone or in mixed infections, it is noteworthy that VB and YS were always shown by vines in which both GYSVd-1 and GYSVd-2 were present without (YS) or together with GFLV (VB) (Table 2; Table 3). Symptom expression may involve various factors such as nucleotide mutations at certain positions in the GYSVd-1 genome, the titer of viroids (Szychowski *et al*., 1995; Salman *et al*., 2014; Szychowski *et al*., 1988) and high temperatures, which might be different among individual plants of the same variety and even in the same plant (Habili and Randles, 2010).

In conclusion, the mRT-PCR protocol described here is a highly specific and sensitive technique for rapid and cost effective detection of GYSVd-1, GYSVd-2 and GFLV in one reaction and has a great potential to be used routinely for large-scale surveys and certification programs.

Table 2. Comparing analysis of grapevine samples from North-West Iran by mRT-PCR (MP) (this study) and single RT-PCR (SP) (Hajizadeh *et al*., 2010)[a].

Sample	Yellow speckle	Vein banding	GYSVd-1		GYSVd-2		GFLV	
			SP	MP	SP	MP	SP	MP
Cl3	–	–	+	+	+	+	+	+
Cl17	–	–	+	+	+	+	–	+
Cl20	–	–	+	+	+	+	+	+
Cl24	–	–	+	+	+	+	–	+
Cl29	–	–	–	+	–	–	–	–
Btg6	–	+	+	+	–	–	+	+
Btg15	–	–	+	+	–	–	–	–
Btg17	–	–	–	+	+	+	+	+
Bu	–	–	+	+	–	–	–	–
As1	–	–	+	–	–	–	–	–
As5	–	–	–	–	–	–	–	+
As6	–	–	–	–	–	–	–	–
As8	–	–	+	+	–	–	–	–
Bt812	–	–	–	–	–	–	+	+
Ao17	+	–	+	+	–	–	–	–
Bs3	–	–	+	+	+	+	+	+
Cm3	–	–	+	+	+	+	–	+
Bm7	–	+	+	+	+	+	–	+
Bm9	–	+	+	+	+	+	+	+
Bm11	–	+	+	+	+	+	+	+
Bm12	–	+==	+	+	+	+	+	+
Bm13	–	+	+	+	+	+	+	+
Cf3	–	–	+	+	+	+	–	–
Bt23	–	+ =+	–	–	+	+	+	+

[a]Positive and negative samples are indicated by +, –, respectively; gray background highlights samples that tested positive to mRT-PCR, but negative to sRT-PCR.

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Table 3. Incidence of two viroids and a virus from naturally infected field samples of grapevine assayed by developed multiplex RT-PCR (Positive and negative samples are indicated by +, –, respectively).

Sample	Host	VBS	YSS	GYSVd-1	GYSVd-2	GFLV
Ss1	*Vitis vinifera* cv. Farkhi	+	–	+	+	+
Ss3	*V. vinifera* cv. Gazne	–	–	–	–	–
Ss4	*V. vinifera* cv. Sahani	–	+	–	+	–
Ss5	*V. vinifera* cv. Gazne	+	–	–	+	+
Skh1	*V. vinifera* cv. Korda kojha	+	–	+	+	+
Skh2	*V. vinifera* cv. Korda kojha	+	–	+	+	+
Sh4	*V. vinifera* cv. Asgari	+	–	+	+	+
Sb1	*V. vinifera* cv. Khoshnav	–	–	+	–	–
Sb2	*V. vinifera* cv. Asgari	–	–	+	+	–
Sb3	*V. vinifera* cv. Khoshnav	–	–	+	+	–
Sb4	*V. vinifera* cv. Farkhi	–	–	–	+	+
Sb5	*V. vinifera* cv. Asgari	–	–	–	+	+
Sb6	*V. vinifera* cv. Farkhi	–	–	+	–	–
Sb7	*V. vinifera* cv. Farkhi	–	–	–	–	–
M1	*V. vinifera* cv. Korda kojha	–	–	–	–	–
M2	*V. vinifera* cv. Korda kojha	–	–	–	–	–
M3	*V. vinifera* cv. Rasha	–	–	+	+	–
M4	*V. vinifera* cv. Asgari	–	–	+	+	–
M5	*V. vinifera* cv. Rasha	–	–	+	+	–
M6	*V. vinifera* cv. Rasha	–	–	–	–	–
M7	*V. vinifera* cv. Farkhi	–	–	+	+	–
M8	*V. vinifera* cv. Farkhi	–	–	+	+	–
M9	*V. vinifera* cv. Asgari	–	–	+	+	–
M10	*V. vinifera* cv. Sahani	–	–	+	–	–
Q1	*V. vinifera* cv. Asgari	–	–	+	–	–
Q2	*V. vinifera* cv. Gazne	–	–	–	–	–
Q3	*V. vinifera* cv. Rasha	–	–	+	+	–
Bm1	*V. vinifera* cv. Farkhi	–	–	+	–	–
Bm4	*V. vinifera* cv. Gazne	–	–	+	+	–
Bm5	*V. vinifera* cv. Rasha	–	–	+	+	–
Ma_g	*V. vinifera* cv. Asgari	–	–	+	–	–
Ma_3	*V. vinifera* cv. Rasha	–	–	–	+	–
K1	*V. vinifera* cv. Rasha	–	–	–	–	–
K2	*V. vinifera* cv. Rasha	–	–	–	–	–
K3	*V. vinifera* cv. Rasha	–	–	+	+	–
K4	*V. vinifera* cv. Rasha	–	–	–	–	–

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