Original Paper

Establishment of the optimal condition for shoot regeneration in *Vitis vinifera* 'Koshu'

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Abstract

Vitis vinifera 'Koshu' is an indigenous Japanese grape often praised as the best white wine grape suited for Washoku, traditional Japanese cuisine. Koshu is known to have a very low sugar content, however, perhaps partially due to its viral infection. Thus, to obtain Koshu with a higher sugar content, we aimed to establish a microshoot tip culture method for Koshu, as it is most commonly used for elimination of viruses from various wine grape varieties. We found that the highest frequency for shoot regeneration was reached when Koshu microshoot tips were cultured in the range of 0.1 to 0.2 mg/L (0.44 to 0.88 μM) 6-benzylaminopurine, which was lower than those for other *Vitis vinifera* cultivars. We also obtained a Koshu plant, fully regenerated from a microshoot tip, that was tested to be free from the economically most devastating virus GLRaV-3.

1. Introduction

Vitis vinifera 'Koshu', possibly the earliest among grapevines brought to Japan in the early 8th century from China, has been the most important cultivar for winemaking in Japan since the late 19th century, and has lately been re-evaluated as a wine grape best suited for pairing with Washoku. A recent genetic analysis revealed that 71.5% of Koshu was derived from *V. vinifera*, whilst the remaining 28.5% was most likely derived from wild East Asian *Vitis* species, such as *V. davidii* (Gotoh-Yamamoto et al. 2015). The Koshu grape has pink skin and contains higher amounts of phenolics (phenylpropanoids and flavanols) than do popular European grapes such as 'Chardonnay', 'Sauvignon Blanc', and 'Merlot' (Kobayashi et al. 2011). These metabolic components provide Koshu with its unique color and flavor. All of these characteristics, together with its historical background, render Koshu uniquely important for winemaking in Japan. Thus, great wines produced from Koshu are highly coveted. Koshu's very low sugar content, however, is a bottleneck for making great wines. Kobayashi et al. (2009) reported that the Brix value for ripened Koshu grapes could be raised at most up to 17, whereas those for typical *V. vinifera* cultivars such as Sauvignon Blanc and Chardonnay reach 21 and 25.8,

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respectively (Peyrot des Gachons 2005; Walker and Blackmore 2012).

We have an abundance of reports that virus infection of grapevines may not only threaten sustainable production of wine grapes (*Vitis vinifera* L.) but reduce their sugar level as well (Harmon and Weinberger 1956; Namba et al. 1979; Saito et al. 2007; Lee and Martin 2009; Alabi et al. 2016). Among virus associated symptoms, grapevine leafroll reflects the most important and economically damaging vine disease (Atallah et al. 2011; Naidu et al. 2015). Of all leafroll-associated viruses, GLRaV-3 is known to cause the most serious leafroll symptoms, substantially affecting grapevine vigor and grape quality (Adiptra et al. 2018). Grapevines infected with GLRaV-3 show reduced levels of anthocyanin, organic acid, and sugar (Lee and Martin 2009; Lee et al. 2009; Vega et al. 2011), as well as delayed grape ripening (Montero et al. 2016). In contrast, vines from which GLRaV-3 was eliminated showed increased vigor and higher yield compared with virus-infected vines (Komar et al. 2007; Mannini et al. 2012).

The microshoot tip culture method has been most frequently and successfully used to obtain virus-free vines for many *Vitis vinifera* cultivars and other *Vitis* spp (Golino et al. 2017). By this method, dissected sterile microshoot tips are incubated with cytokinin- and/or auxin-including medium for multiple shoot formation, and then incubated with auxin for rooting. For example, the optimum cytokinin concentration for shoot regeneration of Cabernet Sauvignon was 2 mg/L (8.8 μM) 6-benzylaminopurine (BAP) (Barlass and Skene 1978), and that for axillary bud formation of Pinot Noir was 1-2 mg/L (4.4-8.8 μM) BAP (Heloir et al. 1991). The optimized BAP concentrations for shoot regeneration of other *V. vinifera* cultivars were also in the range over 1 mg/L (>4.4 μM) (Lee and Wetzstein 1990; Gray and Benton 1991; Gribaudo et al. 2006). Although several papers described for Koshu have been published (Sasahara et al. 1981; Ueno et al. 1983; Yamakawa et al. 1985), the optimal cytokinin concentration range for Koshu is not yet well known.

In this study, we attempted to find the optimal cytokinin concentration for shoot regeneration from Koshu microshoot tips. And, also here, we obtained a fully-regenerated Koshu plant named Fuji No.1, which was free from one of the most economically damaging viruses for grapevines, GLRaV-3.

2. Materials and Methods

2-1. Microshoot tip culture

Microshoot tips were obtained from *Vitis vinifera* Koshu grown in an enclosed greenhouse. Microshoot tip culture was conducted according to Sim et al. (2006). Microshoot tips were harvested from fresh canes of shoot-regenerated clones that we called simply "parents", which we purchased from Tenko-en (Yamagata, Japan). The harvested canes were washed with a solution containing 0.5% hypochlorite and 0.02% detergent (Charmy V Compact, Lion Corporation, Tokyo). The microshoot tips were removed from leaves and stems aseptically by using tweezers and scissors under a stereomicroscope (Stemi DV4, Carl Zeiss). The 0.4 - 0.5 mm microshoot tips from several leaf primordia were incubated on 1/2 Murashige and Skoog medium (pH5.8) (MS medium, Murashige and Skoog 1962) containing vitamins, 3% sucrose, 0.6% agar, and different concentrations of BAP (Fujifilm Wako, Co. LTD, Osaka, Japan) at 22°C, under 16-hour light, 8-hour dark conditions. Media were refreshed every 2 weeks. Explants cultured from microshoot tips, as above, were incubated on 1/2 MS medium (pH5.8) containing vitamins, 3% sucrose, 0.6% agar, and 0 or 0.5 mg/L (0 or 2.6 μM) 1-Naphthylacetic acid (NAA) (Fujifilm Wako).

2-2. RT-PCR

Canes of Koshu Makioka3 (hereinafter designated as

Makioka3) harvested from a vineyard in Makioka town (Yamanashi, Japan) and those of Fuji No. 1 and its parent were used for RNA extraction. Frozen canes crushed with a frozen hammer were powdered at 26 Hz x 30 sec., twice on a Tissue lyser (Qiagen, Hilden, Germany). RNA was extracted with a modified procedure of McKenzie et al. 1997. cDNA was synthesized with random d(N)6 primers and ReverTra Ace (TOYOBO CO., LTD., Osaka, Japan) according to the manufacturer's instructions. GLRaV-3 was detected by RT-PCR with KOD FX Neo (TOYOBO CO., LTD., Osaka, Japan) and a set of the three primers GLRaV-3 CP (coat protein gene)-F (5'- TACGTTAAGGACGGG ACACAGG-3') and GLRaV-3 CP-R (5'- TGCGGCAT TAATCTTCATTG-3') (Gambino and Gribaudo 2006), GLRaV3 HSP70h (heat shock protein 70 homologue gene)-F (5'- AAGTGCTCTAGTTAAGGTCAGGAGT GA-3') and GLRaV3 HSP70h-R (5'- GTATTGGACTA CCTTTCGGGAAAAT-3') (Osman and Rowhani 2006), and GLRaV3 RdRP (RNA-dependent RNA polymerase gene)-F (5'- AAATGGGAATTTCAACGC CG-3') and GLRaV3 RdRP-R (5'-GCCCTTTTGTCCA ACCAATC-3') (Walsh and Pietersen 2013).

2-3. Sequencing and alignment of RT-PCR products from the Coat Protein gene

The RT-PCR products from the CP gene were cloned into pGEM-T easy plasmids (Promega, Madison, WS, USA) and amplified according to the provider's manual. The amplicons were sequenced by using M13 forward (5'-GTAAAACGACGGCCAGT-3') or SP6 reverse (5'-ATTTAGGTGACACTATAG-3') primers. The DNA sequences of amplicons primed for the GLRaV-3 CP gene from Makioka3 were aligned and compared with the NY-1 GLRaV-3 isolate (GenBank database accession number AF037268) (Ling et al. 2004).

3. Results and Discussion

We cultured parental microshoot tips with 1/2 MS medium containing 7 different BAP concentrations. Shoots were grown from most of the microshoot tips regardless of the BAP concentrations used, except for no BAP (0 mg/L BAP) in MS medium in which shoots did not grow but turned brown (Type I, Figure 1a). When microshoot tips were cultured on BAP-containing MS media in concentrations of 0.05 to 1 mg/L, 27 to 86% of the microshoot tips grew to become green shoots, but then these shoots stopped growing (Type II, Figures 1a and 1b). Among the microshoot tips cultured with 0.05 to 0.5 mg/L BAP, 6% to 24% of shoots appeared (Type III, Figures 1a and 1b). Our present result shows that the optimal cytokinin concentration for shoot regeneration of Koshu was in the range of 0.1 to 0.2 mg/L (0.44 to 0.88 μM) (Figure 1b), which was lower than those for most other *Vitis vinifera* cultivars. In several plant species, different *in*

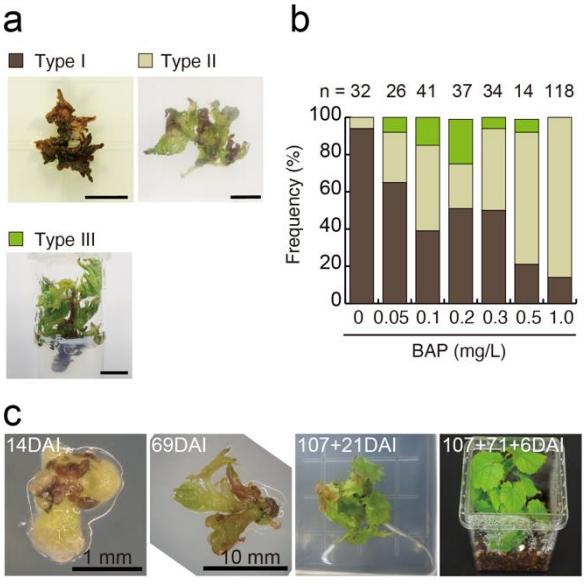

Figure 1
Lower concentrations of cytokinin induce new shoots from parental Koshu microshoot tips.
(a) Representative photos of microshoot tips cultured after incubation with BAP and NAA. Type I, shoot appears but growth stops; Type II, shoot appears and remains green; Type III, shoot and new meristem appear. Bar indicates 10 mm.
(b) Frequencies of explants observed in microshoot tip cultures incubated with different concentrations of BAP.
(c) Growth of the regenerated explant of Koshu (Fuji No.1).

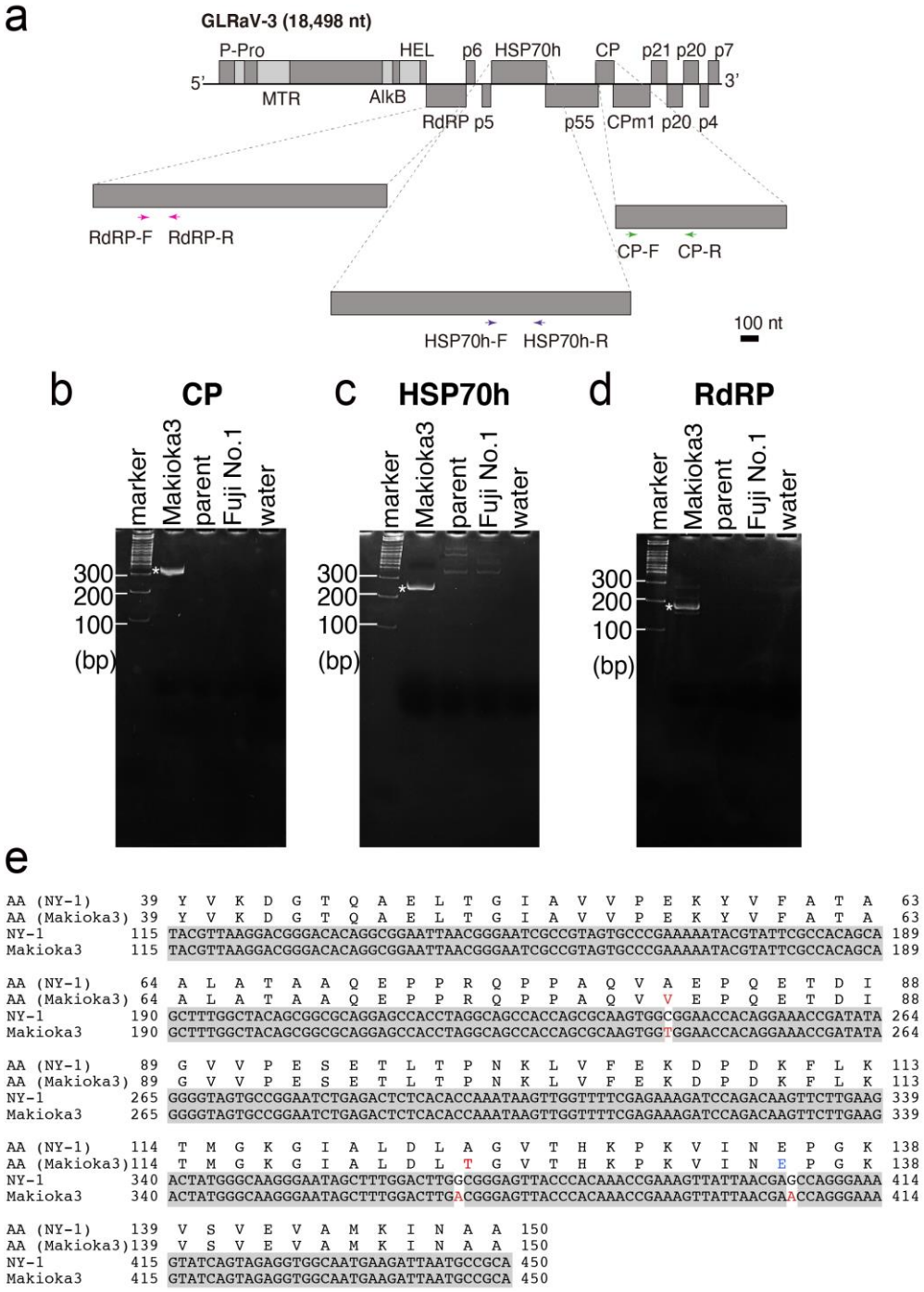

Figure 2

Detection of GLRaV-3 from the canes of Makioka3.

(a) Genetic map of GLRaV-3 genome (Martelli et al. 2012). Arrows indicate positions of primers in genes for coat protein (CP), heat shock protein 70 homologue (HSP70h), and RNA-dependent RNA polymerase (RdRP).

(b-d) Polyacrylamide gel electrophoresis of the DNA products by RT-PCR using GLRaV-3 CP F+R primers as shown in (b), GLRaV-3 HSP70h F+R primers in (c), and GLRaV-3 RdRP F+R primers in (d). Asterisks indicate the expected sizes (336 bp for CP, 230 bp for HSP70h, and 177 bp for RdRP).

(e) Multiple alignment of the CP fragment amplified with GLRaV-3 CP F+R. DNA sequences and deduced amino acid (AA) sequences for the CP gene of the NY-1 isolate and Makioka3 are shown. Red letters indicate changed AA or DNA residues in Makioka3, whereas a blue letter indicates an unchanged AA residue. Shadowed are sequences of the CP gene from Makioka3 that match those of the NY-1 isolate. Numbers flanking the AA or DNA sequences indicate positions from the translational start site of the CP gene.

vitro responses to hormones have been suggested to reflect genotypic differences in the endogenous content of hormones (Alvarez et al. 1989; Grönroos et al. 1989).

The present result that the optimal cytokinin concentration for shoot regeneration from Koshu microshoot tips was lower than those of other *Vitis* cultivars suggests that the endogenous cytokinin concentration in Koshu might be higher than those of other cultivars. In the present study, roots were also regenerated from several shoots grown when the shoots were cultured on MS medium with 0.05 - 0.5 mg/L (0.22 - 2.2 μM) of BAP. Among those shoots with regenerated roots, however, only one survived to become an explant, as we describe below. After we incubated the explant on 0.5 mg/L BAP-containing MS medium for 107 days and transferred it onto the MS medium containing no NAA, that is, 0 mg/L (0 μM) NAA, followed by a 21-day incubation, new roots appeared from the plantlet. At 107 + 71 days after incubation (DAI), the plantlet was acclimatized onto soil under ambient air conditions (Figure 1c). We obtained one complete plant with regenerated shoots and roots, and named it Fuji No.1. Leaves from Fuji No.1 had no leafroll symptoms.

Although we do not know reasons why we obtained such a low frequency for rooting explants, it is possible that high endogenous cytokinin concentrations in Koshu might have inhibited rooting; there is a report that high concentrations of cytokinin inhibited the initiation of root formation (Laplaze et al. 2007).

We obtained Fuji No.1 from one of parents. To test that Fuji No.1 was not infected with GLRaV-3, we conducted RT-PCR. Amplicons were detected with all of the primer sets for the GLRaV-3 Coat Protein (CP) gene, its Heat Shock Protein 70 homologue (HSP70h) gene, and its RNA-dependent RNA Polymerase (RdRP) gene when Makioka3 was used as a template, whereas no amplicons were detected when either the Fuji No.1 or its parent was used as the template (Figure 2b, 2c, 2d). The result indicates that Fuji No.1 was not infected with GLRaV-3.

We also read sequences of 9 clones, all of which were amplicons primed for the GLRaV-3 CP gene from Makioka3, ligated into pGEM-T easy vectors. Shown in Figure 2e is the DNA sequence of the GLRaV-3 CP gene from Makioka3 that was aligned and compared with that of the NY-1 GLRaV-3 isolate (Ling et al. 2004). The CP gene sequence of GLRaV-3 from Makioka3 showed 99.4% identity with that for GLRaV-3 CP of the NY-1 isolate.

As also shown in Figure 2e, the amino acid sequence deduced from the CP gene sequence indicates that A81 is changed to V (nonpolar to nonpolar), A129 is changed to T (nonpolar to polar), and E140 is silently changed.

In the present study, we did produce the new Koshu clone Fuji No 1 by microshoot tip culturing method. To obtain regenerated explants more constantly and efficiently, however, we need to further investigate hormonal conditions necessary for Koshu root regeneration. Establishment of an efficient and stable microshoot tip culture method that we aimed to achieve here in this study should be one most important step to produce virus-free good quality Koshu grapes, which is a prerequisite to the making of great Koshu wine.

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要　旨

Vitis vinifera ‘甲州’におけるシュート再生の最適条件の確立

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Vitis vinifera ‘甲州’は日本固有のブドウ品種であり, 伝統的な日本料理「和食」に最も合う白ワイン用ブドウとして期待されている. しかしながら甲州は, 糖度が非常に低いことが知られており, ウイルス感染が原因のひとつであろうと考えられている. そこで, 我々は, 糖度の高い甲州ブドウを得るために, 他のワイン用ブドウ品種のウイルス除去方法である茎頂培養法を用いて, 甲州種におけるその効率的な条件の確立を目指した. その結果, 甲州の茎頂部を 0.1～0.2 mg/L (0.44～0.88 μM) の 6-ベンジルアミノプリンを含む培地で培養した場合に, シュートの再生効率が最も高いことがわかった. この濃度は, 他の *Vitis vinifera* 種における最適濃度よりも明らかに低いことがわかった. さらに, 我々は, この茎頂培養法を用いて, 甲州の再生個体を一株得ることができた. この個体は, ブドウの生産に最もダメージを与えると言われているブドウ葉巻病随伴ウイルス 3 (GLRaV-3) のゲノムが検出されないことも確認した.