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Production of Virus-free Vitis Vinifera ‘koshu’ Plantlets From Shoot Tips or Axillary Buds

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Research note

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Abstract

Objective: *Vitis vinifera* 'Koshu' is the most important grape cultivar for producing white wine in Japan. Most Koshu vines are heavily infected with viruses of multiple types, such as grapevine leafroll-associated viruses, which severely interfere with normal development and physiology such as photosynthesis of grapes. The meristem culture method has been the most commonly and successfully employed to eliminate viruses from grapevines. Koshu is, however, known for its difficulty of regenerating plantlets from meristems.

Results: We have established a robust method of regenerating plantlets with high frequency from shoot apical meristem tissues of 'Koshu' grapevines. The key points of this method are the use of (1) no or low concentrations of 6-benzyl-aminopurine (BAP) (0.22 μM) for shoot regeneration, and (2) a synthetic auxin, such as 10^{-5} M 4-chloroindole-3-acetic acid (4-Cl-IAA), for rooting. This method should be very useful and important for producing 'Koshu' virus-free vines.

Key Message

A regeneration protocol has been established for virus-free *Vitisvinifera* Koshu. Virus-free plants are expected to bear grapes of high sugar content, which should make Koshu an attractive white wine cultivar.

Introduction

Vitisvinifera 'Koshu,' assumedly brought into Japan from China around the early 8th century, is the most popular variety for making white wine in Japan [1, 2]. 'Koshu' is a hybrid variety of *V.vinifera* (71.5%) and *V.davidii* or its closely related wild species (28.5%) [3], and in 2010, was officially registered by the International Organization of Vine and Wine. Although its quality has recently been improved by changing the vine canopy management and by employing advanced winemaking technology, Koshu's sugar content is still insufficient to satisfy high-end consumers. Since virus-free vines usually yield grapes of higher sugar content than virus-infected vines, a robust method of obtaining virus-free vines is strongly desired to further improve the quality of Koshu wine. Among more than 50 different viruses that infect grapes, five grapevine leafroll-associated viruses (GLRaVs) affect significantly the sugar content of grapes [4]. The sugar contents of virus-free grapes have long been considered a few % higher than those of virus-infected counterparts [5].

Several therapeutic methodologies have been developed to eliminate viruses in propagation materials, and among them the meristem culture method has been one of the most commonly and successfully employed [6, 7]. To date, however, there have been no published reports about robust and stable production of virus-free 'Koshu'. Although we have produced 'Koshu' plantlets cleaned of viruses by shoot-tip cultures, including those for shoot apical meristem (SAM) (Nakagawa et al., unpublished results), the frequency of obtaining virus-free 'Koshu' was very low. Thus, we need a better protocol for

obtaining them at much higher frequency. Here, we describe and present our results obtained from a robust method of regenerating virus-free ‘Koshu’ plantlets with high frequency from shoot tips and axillary buds.

Methods

We used a low concentration of 6-benzyl-aminopurine (BAP), or none, for shoot regeneration, and then applied synthetic auxin to these regenerated shoots for rooting. With this method, we have succeeded in obtaining ‘Koshu’ plantlets from SAM tissues at reasonably high frequency. We have so far obtained more than 20 plantlets from meristem tissues of the parental virus-free‘Koshu’ vines. They were grown in the green house of Chubu University in about 20 pots containing a 1:1 (v/v) mixture of culturing soil and vermiculite. Shoot tips and axillary buds including meristem tissues were cut at ca. one cm from the top, and then collected in 50 ml tubes containing fresh distilled water. The shoot tips and axillary buds were surface-sterilized for 10 min with 20-fold diluted sodium hypochlorite solution (min. 0.25% effective chlorine concentration) and 0.09% (v/v) neutral detergent, and washed ca. 5 times with sterile distilled water. A meristem dome of ca. 0.2 mm was excised from the cut shoot tips or axillary buds under a stereomicroscope, and then cultured on the following medium: 1/2 Murashige and Skoog (MS) Plant Mixture basal [8] (Nihon Pharmaceutical CO. LTD, Tokyo) adjusted to pH5.7 containing 3% sucrose, 0.8% agar, 3 mg/l thiamine hydrochloride, 5 mg/l nicotinic acid, 0.5 mg/l pyridoxine hydrochloride, and various concentrations of phytohormones. Meristem tissues were cultured under a regimen of white light at 50 μmol m−2 s−1 for 16 h and darkness for 8 h daily at 26°C).

Results

The use of low concentrations of BAP for shoot regeneration

The presence of BAP was required for shoot tip and axillary bud cultures of *V. vinifera* ‘Napoleon,’ ‘Cabernet Sauvignon,’ ‘Riesling Italian,’ [9-11]; and five Iranian cultivars [12], and for shoot regeneration from foliar tissues of *Vitis* rootstocks. In the case of explants from rootstock leaves, no calli or shoots were obtained without BAP, but the development of calli and shoots was observed under high BAP concentrations between 2.21 and 8.87 μM [10]. A high BAP of 5 mg/l (22 μM) was also required for shoot regeneration and proliferation of shoot tips and axillary buds of ‘Cabernet Sauvignon’ and ‘Riesling Italian’ [11]. In contrast, a low BAP of 0.5 mg/l (2.2 μM) was used for shoot tip and axillary bud culturing of ‘Napoleon’ [9] and shoot apical meristem (SAM) cultures of five Iranian cultivars [12]. Based on these previous findings, we also used several different concentrations of BAP in this study. The effects of various BAP concentrations on shoot regeneration are shown in Table 1. SAM cultured on BAP-containing medium was induced to produce organogenic calli, which formed shoots 20 days after their transfer to phytohormone-free medium.. After testing with various concentrations, 0. 22μM BAP was selected for later experiments as the optimal shoot induction treatment because of its high viability and rate of shoot regeneration (Table 1). To add, it is notable that the BAP concentration suitable for shoot regeneration of ‘Koshu’ seems to be low as compared with that of other grapes [6], since the regenerated shoots were

observed from phytohormone-free medium (Table 1). However, this result does not necessarily mean that BAP concentrations in these SAM tissues were low, and requires further investigation for verification.

Root regeneration of the grapevine 'Koshu' *by using synthetic auxins*

Roots appeared within 15 days after the developed small plantlet was transferred from the leaf explant onto phytohormone-free medium [10]. Phytohormone-free medium was also used for rooting shoots that developed by culturing shoot tips or axillary buds for 6 weeks [9]. On the contrary, the number of roots per shoot for 'Cabernet Sauvignon,' 'Riesling Italian,' and commercial cultivars of *Vitis vinifera* L. was increased by pretreating them with 1 to 2 mg/l of Indole-3-butyric acid (IBA) [11, 12]. Since the frequency of root regeneration from regenerated shoots of 'Koshu' was low with IBA or NAA, we examined this frequency by using the new synthetic auxins 4-chloroindole-3-acetic acid (4-Cl-IAA), 5,6-dichloroindole-3-acetic acid (5,6-Cl_2-IAA), and 4-trifluormethylindole-3-acetic acid (4-CF_3-IAA), the shoot regeneration activities of which were, respectively, 15, 4, and 1.5 times as high as that of IBA in black gram [13, 14]. The regenerated shoots obtained were transferred to MS medium containing no auxin, 10^{-4} M to 10^{-6} M of 1-naphthaleneacetic acid (NAA), 4-Cl-IAA, 5,6-Cl_2-IAA, and 4-CF_3-IAA from 20 to 35 days post-cultivation. Calli without and calli with roots were obtained at 7 and 14 days after their transfer onto no auxin and 10^{-5} M or 10^{-6} M synthetic auxin (Table 2, Fig. 1 C, D). Notably, the frequency of root formation was highest at the 10^{-5} M 4-Cl-IAA concentration (Table 2, Fig. 1 C, D). This is the first report of successful root regeneration of grapevines by using synthetic auxins. The rooting plantlets were transferred to hormone-free medium, and their development was observed (Fig. 1 E – H). We have succeeded in obtaining 14 plantlets from the SAM tissues of 'Koshu.' The plantlets were morphologically normal (Fig. 1 G, H) and would then be transferred into soil with covering by plastic wrap to maintain humidity. Timing of humidity reduction and/or elevation of light intensity might be important for further plant development.

Discussion

We described and presented the data obtained from the robust method of obtaining plantlets with high frequency from shoot tips and axillary buds of virus-free *V.vinifera* 'Koshu.' By using a low concentration of the cytokinin BAP, we were able to increase the frequency of shoot regeneration from shoot apical meristems of 'Koshu' grapes. 'Koshu' is known to have a strong growth potential, suggesting that its concentration of endogenous cytokinins may be higher than those of various European cultivars. Cytokinins are central regulators of plant growth and development [15, 16], and function as both local and long-distance regulatory signals for the coordination of plant development [17]. It has also been reported that the function of cytokinins is regulated not only by changes in quantity but also by side-chain modification [18, 19]. Thus, it would be interesting to compare the endogenous amount and composition of cytokinins in Koshu with those in European cultivars..

Our results also showed that the frequency of root formation, a promising feature that eventually leads to successful plantlets, was highest at 10^{-5} M 4-Cl-IAA among all auxins tested. 4-Cl-IAA was originally isolated from immature seeds of *Pisum sativum* [20], and much evidence for its high biological activity

has been reported [13, 21, 22]. Both 4-Cl-IAA and 4-CF_3-IAA promote root formation in black gram (*Vigna mungo*) [13]. It is recently reported that 4-Cl-IAA stimulates pericarp growth and gibberellin biosynthesis in *Pisum sativum* [23]. Although how 4-Cl-IAA affects grapevines needs further investigation, it might be possible that 4-Cl-IAA also promotes vines' root formation as in black gram. Further work examining more samples will be required to confirm appropriate concentrations of these synthetic auxins. It would be also worthwhile to use these synthetic auxins for other plants that rarely regenerate roots and thus full-fledged plants from shoot tips.

Limitations

The possible limitation for this report is availability of the novel synthetic auxins, well trained hands, and meristems. Another shortcoming might be a small sample size for the number of the regenerated shoots allocated for the various concentrations of different auxins that were tested for root formation.

Declarations

Declarations

Not applicable

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Authors'contributions

AD, SA, KT and SK performed the experiments. SA, JM, CM and SM wrote the manuscript. All authors read and approved the final manuscript.

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Ethics approval and consent to participate

Not applicable

Availability of data and material

The data obtained and analyzed during the current study are available from corresponding authors on reasonable request.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Tables

Due to technical limitations, table 1-2 is only available as a download in the Supplemental Files section.

Figures

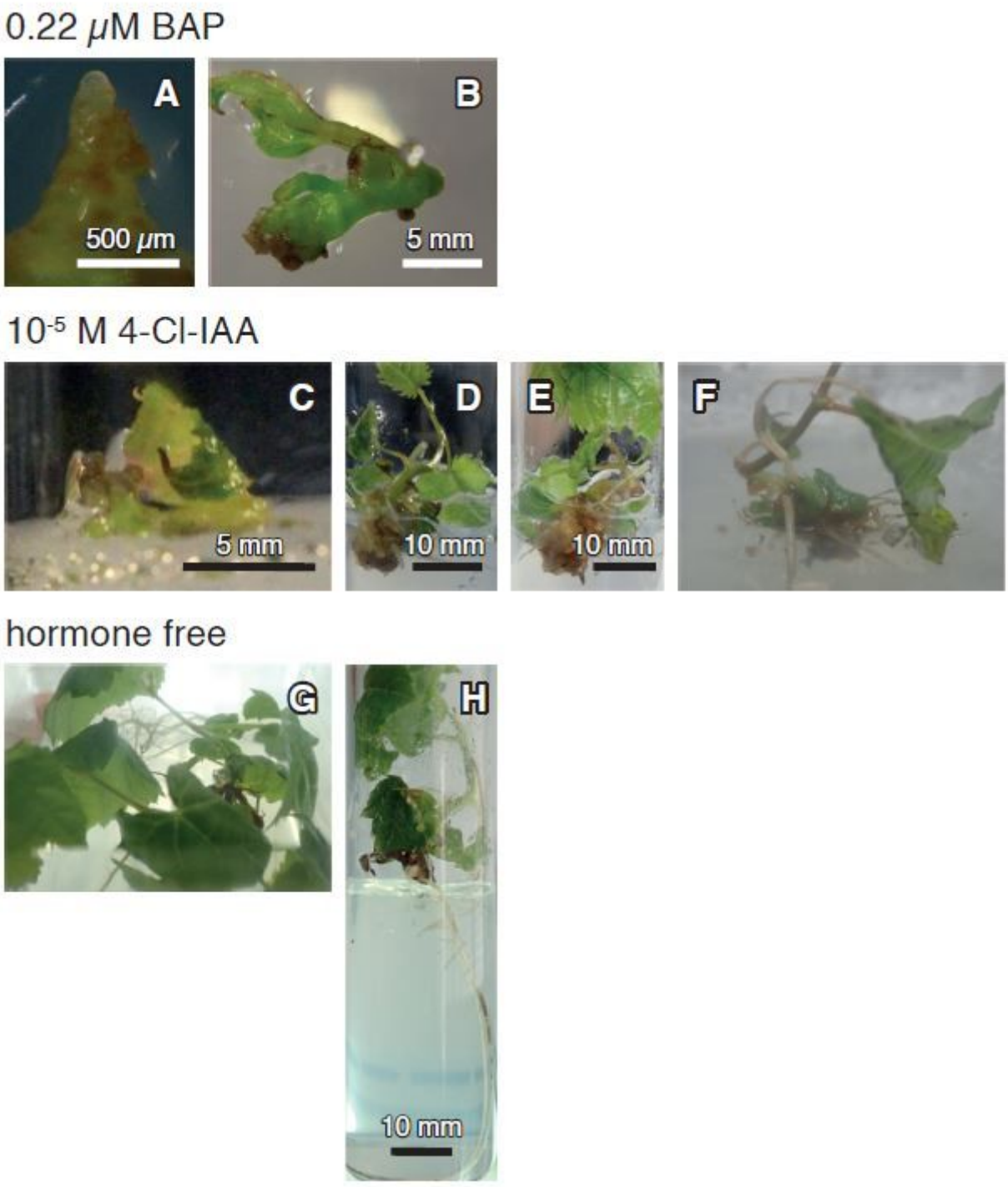
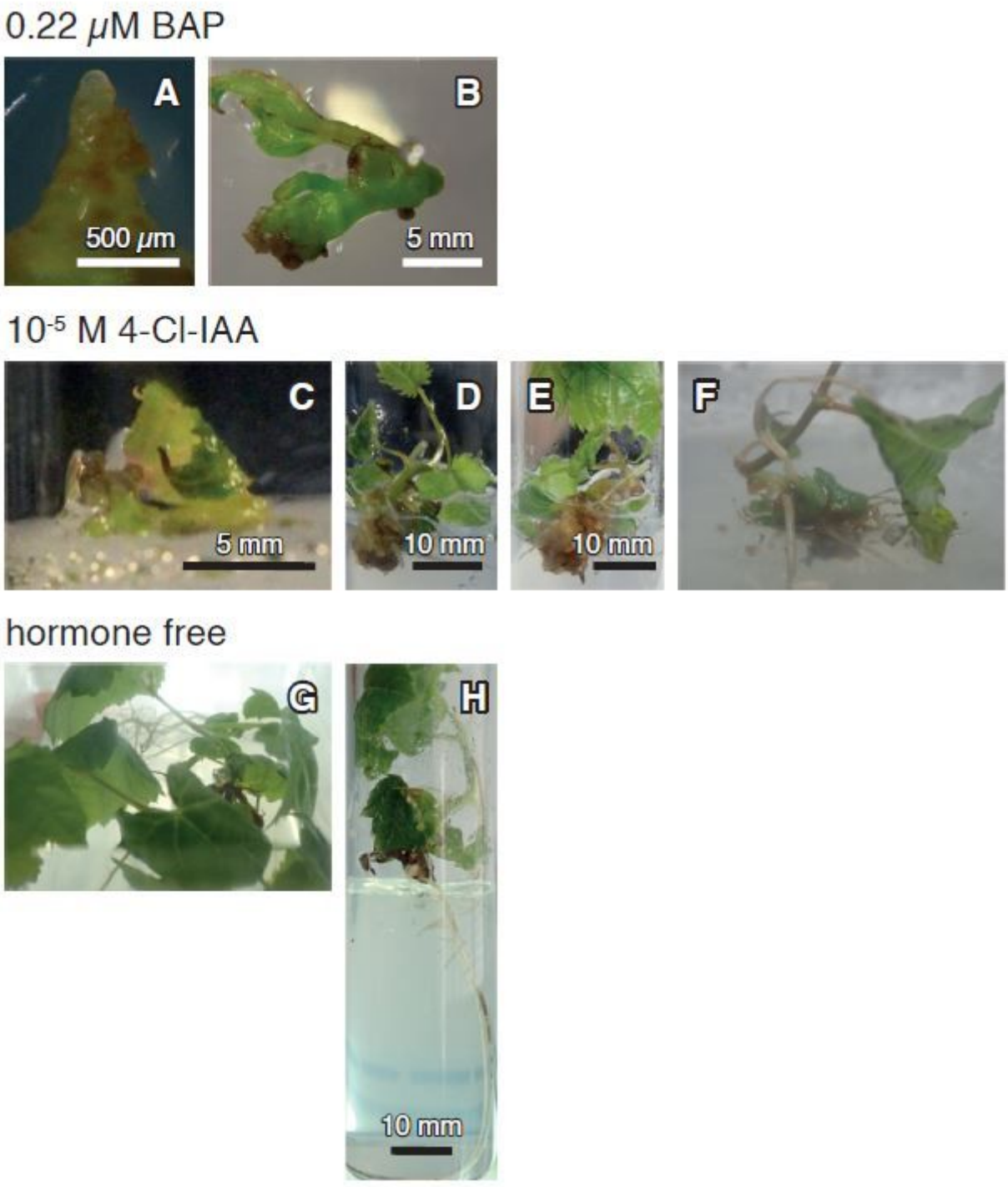


Figure 1

Virus-free regenerated plantlet from V. vinifera ‘Koshu’ SAM tissues. ‘Koshu’ meristem at day 0 (A) and after 35 days (B) on BAP 0.22 µM. ‘Koshu’ shootlet after 7 days (C) and 21 days (D) on 10-5 M 4-Cl-IAA. ‘Koshu’ shootlet with roots after 7 days (E) and 14 days (F) hormone free. ‘Koshu’ plantlet after 35 days (G, H) hormone free.



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‘Koshu’ shootlet with roots after 7 days (E) and 14 days (F) hormone free. ‘Koshu’ plantlet after 35 days (G, H) hormone free.

Supplementary Files

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