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Original Paper

Stability of variegation in plants propagated by tissue culture of three variegated cultivars of *Farfugium japonicum* (Asteraceae), a Japanese traditional ornamental plant

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Abstract Stability of the variegation was examined in the plants obtained by tissue culture of three variegated cultivars of *Farfugium japonicum*, 'Ukigumo-nishiki', 'Temboshi' and 'Kinkan' by culturing shoot tips on plant growth regulator-free 1/2 Murashige and Skoog (MS) medium and rhizome and leaf blade segments on MS medium supplemented with 1 mg l^{-1} 6-benzylaminopurine and 1 mg l^{-1} α-naphthaleneacetic acid, respectively. In 'Ukigumo-nishiki', shoots regenerated from rhizome and leaf blade segments had only green leaves in the former and either green or albino in the latter, respectively, whereas those developed from the culture of shoot tips exhibited the same variegation as the original plant. In this cultivar, however, shoots propagated secondary by transferring the variegated shoots obtained by shoot tip culture onto 1/2MS medium containing 0.2 mg l^{-1} 6-furfurylaminopurine segregated into either green or albino shoots without maintaining the original variegation phenotype. These results indicate the chimeric nature of variegation in 'Ukigumo-nishiki'. In contrast, shoots derived from all explants of 'Temboshi' and 'Kinkan' exhibited the same variegation as original plants during the multiplication process, indicating no chimeric nature of both cultivars. In these two non-chimeric variegated cultivars, approximately 3.2 times proliferation rate in average was obtained after ca. 40 days of culture on gellan gum-solidified medium containing 0.2 mg l^{-1} 6-furfurylaminopurine. These shoots were successfully rooted on 1/2MS medium containing 0.01 mg l^{-1} α-naphthaleneacetic acid and established in the soil.

Key words: Chimera, *Farfugium japonicum*, micropropagation, tissue culture, variegation.

Farfugium japonicum (L.) Kitam., Japanese name: "Tsuwabuki", is a species belonging to Family Asteraceae. It is an evergreen perennial plant having glossy green leaves with long stalks, short and thick rhizomes, and bearing yellow flowers (Ohwi 1975) in autumn to winter (Kitamura et al. 1994). It grows mainly on rocky coastal cliffs in southern Japan, and is also distributed to South Korea, Taiwan and the middle part of Mainland China (Kitamura 1981; Koyama 1968). In the Edo era (1603–1867), wild *F. japonicum* plants were planted mainly in the gardens (Kitamura et al. 1994), and some variants were selected (Okuno et al. 2007) and probably cultivated in pots. Since six cultivars with variations in leaf morphology or variegation in leaves were described in 'Somoku-kinyo-shu' (Mizuno 1829), which is the oldest literature concerning variegated plants in Japan, the number of cultivars has increased and recently attained to more than one hundred (Okuno et al. 2007). Although variegated cultivars of this species have high ornamental value and are ranked as one of the most important ornamental plants in the Japanese garden, it is difficult to supply stably to the horticultural markets due to low propagation rate of the plants by division, which is a conventional method for this species. Therefore establishment of a micropropagation system is urgently needed to solve this problem.

Leaf variegations are usually caused by chimera consisting of normal green tissues and mutated non-green tissues, or by non-chimeric genetically determined ones. When chimeric plants such as a variegated strain of *Yucca elephantipes* (Pierik and Steegmans 1983) and flower color variants of *Chrysanthemum morifolium* (Earle and Langhans 1974a, b, Bush et al. 1976) and *Dianthus caryophyllus* (Hackett and Anderson 1967) were used for tissue culture, dechimerization was encountered in the propagules. George and Sherrington (1984) also listed some variegated plants which showed the same phenomenon in *Cordyline terminalis*, *Crypthanthus*, *Dracaena marginata*, *Dracaena deremensis* and *Hosta*. Such dechimerization in tissue

Abbreviations: BAP, 6-benzylaminopurine; kinetin, 6-furfurylaminopurine; MS, (Murashige and Skoog 1962); NAA, α-naphthaleneacetic acid
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culture is considered to occur by the adventitious shoot formation from one cell or a few cells of either one cell layer consisting of the chimera. However, no information has been available so far on the stability of variegation in *in vitro* culture of non-chimeric variegated plants.

The leaves in variegated cultivars of *F. japonicum* represent a lot of variegation patterns, i.e. margined, centered, splashed, veined, dusted, mottled, spotted, etc. In the present study, we describe variegation nature in plants obtained from explants of different organs and those from subsequent micropropagation in three representative variegated cultivars of *F. japonicum*, which have the following characteristics. 'Ukigumo-nishiki' (Figure 1A), English name: 'Argentea', has leaves displaying irregular yellowish-white variegation on the margin. 'Temboshi' (Figure 1B) has leaves displaying irregular yellow spots variegation over all, which is similar to 'Kimon-tsuwabuki', the most famous cultivar in this species, described by Hooker (1862) in Curtis's Botanical Magazine (Tab. 5302) as *Ligularia kaempferi* Siebold et Zucc. var. *aureomaculata* Hook.. Since 20 to 50% of the F_1 hybrids obtained by reciprocal crosses between 'Temboshi' and non-variegated plant showed the same variegation pattern, the variegation of 'Temboshi' seemed to have caused by genetic factors (Okuno et al. 2007). 'Kinkan' (Figure 1C) has leaves displaying thin yellow variegation on the margin which is very rare in variegated plants. Both 'Temboshi' and 'Kinkan' have a common characteristic; the boundary between variegated area and green area is unclear. In contrast, a clear border is detectable between the two areas in variegation of 'Ukigumo-nishiki'.

Materials and methods

Plant materials

Potted plants of three traditional variegated cultivars (Figures 2A, 3A, 4A) and a wild plant collected in Osaka Prefecture, Japan (Figure 5A) were used in this study. Leaf color of the wild plant as control is green throughout. Under the Japanese climatic conditions, a plant of *F. japonicum* emerges new leaves twice a year from early spring to early summer and from late summer to late autumn. Leaf variegation of 'Temboshi' is distinct from autumn to spring and indistinct in summer. In 'Kinkan', leaf variegation usually appears in spring and becomes obscure from summer to winter. In contrast, leaf variegation of 'Ukigumo-nishiki' is stably expressed all the year around.

Shoot tip culture

Shoot tips (ca. 3 mm long) were excised from each strain, surface sterilized for 25 min with 0.5% NaOCl solution added with a few drops of Tween 20, and rinsed twice with sterilized distilled water. Five shoot tips were prepared for each strain, and each explant was inoculated in a glass tube (21 mm in inside diameter ×150 mm in length) containing 10 ml of 1/2MS (Murashige and Skoog 1962) medium which consisted of half strength MS mineral salts and full strength organic constituents, 20 g l^{-1} sucrose and 2.5 g l^{-1} gellan gum (Wako Pure Chemical Industries, LTD., Osaka, Japan) without adding any plant growth regulators. Each shoot developed from shoot tip culture was transferred into a glass bottle (300 ml) containing 70 ml of rooting medium, i.e. 1/2MS medium containing 30 g l^{-1} sucrose, 0.01 mg l^{-1} α-naphthaleneacetic acid (NAA) and 3 g l^{-1} gellan gum. The pH of all the media were adjusted to 5.8 before autoclaving, and all the cultures were kept at 25°C under light condition of 24 h photoperiod at 43 μmol m^{-2} s^{-1}. After 45 days of culture on rooting medium, plantlets with growing roots were potted in 9 cm diameter pots filled with an equal mixture of Akadama soil and Kanuma soil, and maintained in the greenhouse for acclimatization.

Shoot regeneration from rhizomes and leaf blades

Rhizomes were collected from potted plants, washed with running tap water and subjected to strip off the surface of ca. 1 mm thick with a scalpel to minimize the chance of microbial contamination, whereas leaf blades of each strain were sterilized and washed by the same method as described for shoot tip culture. Rhizome segments (ca. 10 mm×10 mm wide ×3 mm thick) were prepared by splitting laterally and leaf blade segments were obtained from green colored parts in variegated leaves by cutting squarely (ca. 10 mm×15 mm wide) for each kind of explants. Each of the 15 rhizome and 50 leaf blade explants was placed in a glass tube (21 mm in inside diameter ×150 mm in length) with 10 ml of shoot regeneration

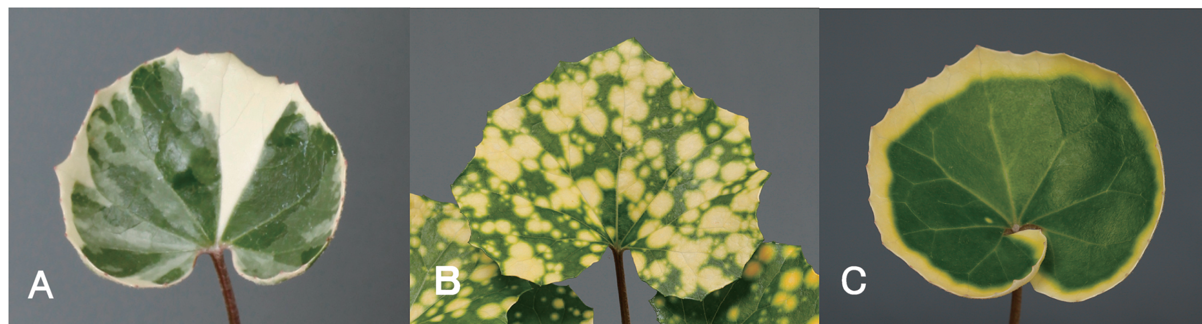

Figure 1. Leaf variegation patterns in three cultivars of *Farfugium japonicum*. (A) *F. japonicum* 'Ukigumo-nishiki': the clear boundary between variegate and green regions. (B) *F. japonicum* 'Temboshi': the unclear boundary between variegate and green regions. (C) *F. japonicum* 'Kinkan': the unclear boundary between variegate and green regions.

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medium, i.e. MS basal medium containing 30 g l^{-1} sucrose, 1 mg l^{-1} 6-benzylaminopurine (BAP), 1 mg l^{-1} NAA and 3 g l^{-1} gellan gum. The number of adventitious shoots was counted every 10 days after placing. Adventitious shoots produced on rhizome and leaf blade segments were transferred into glass bottles (300 ml) with 70 ml of the same rooting medium as that used for shoot tip culture. The acclimatization method in this culture was the same as that in shoot tip culture.

Shoot multiplication and stability of variegation

To confirm the stability of variegation in the variegated shoots obtained by the method described above, two variegated shoots developed from shoot tips of 'Ukigumo-nishiki', 70 variegated shoots regenerated from leaf blade segments each of 'Temboshi' and 'Kinkan', and 70 green shoots regenerated from leaf blade segments of a wild plant were further proliferated on propagation medium in glass bottles (300 ml). In 'Ukigumo-nishiki', one shoot was cultured in each bottle containing 70 ml of 0.25% gellan gum-solidified propagation medium which consisted of half strength MS mineral salts, full strength organic constituents, 20 g l^{-1} sucrose and 0.2 mg l^{-1} 6-furfurylaminopurine (kinetin). In other two cultivars and a wild strain, however, 7 shoots were cultured in each bottle containing the same propagation medium. Proliferated shoots on propagation medium were cut into single shoots and transferred onto the same rooting medium as that used in shoot tip culture.

Results

Some of the shoot tips of each strain grew normally without contamination, and produced one or a few roots occasionally on plant growth regulator-free 1/2MS medium. After transfer to the rooting medium containing 0.01 mg l^{-1} NAA, all shoots produced many roots. After acclimatization, plants derived from shoot tips in all the strains examined exhibited the same variegation as original plants (Table 1).

In the culture of both rhizome and leaf blade segments, adventitious shoots were directly regenerated from each explant without callusing on medium containing 1 mg l^{-1} of both NAA and BAP in all strains, whereas indirect regeneration was not observed. Number of the directly regenerated shoots that could be transferred to rooting medium was from one to three per successful explant. Although percentages of explants with shoot formation from rhizome segments 90 days after the starting of culture were relatively low in all the strains, i.e. 13.3, 18.2, 11.0 and 16.7% in 'Ukigumo-nishiki', 'Temboshi', 'Kinkan' and a wild plant, respectively, leaf blade segments showed much higher responses, 60.5, 65.9, 61.7 and 77.3%, in 'Ukigumo-nishiki', 'Temboshi', 'Kinkan' and a wild plant, respectively (Table 2). After transfer to the rooting medium, all shoots except for albino shoots produced

Table 1. Leaf variegation in shoot tip culture-derived plants in 3 cultivars and a wild plant of *Farfugium japonicum*

Cultivar name	No. of explants cultured	No. of explants forming shoot*	No. of acclimatized plants		
			variegated leaf	green leaf	albino
'Ukigumo-nishiki'	5	3	3	0	0
'Temboshi'	5	2	2	0	0
'Kinkan'	5	2	2	0	0
wild plant	5	3	0	3	0

*All the explants which survived without contamination developed into single shoots.
All the explants were cultured on 1/2MS medium without any plant growth regulators. Data were scored after 90 days of culture in glass tubes.

Table 2. Effect of the type of explants on leaf variegation of the micropropagated shoots of *Farfugium japonicum*

Cultivar name	Region used as explants	No. of explants	No. of survived explants	No. of explants			No. of total explants forming shoot	Frequency of explants with shoot formation (%)	No. of total shoots	No. of acclimatized plants
				variegated shoot	green shoot	albino shoot				
'Ukigumo-nishiki'	rhizome	15	15	0	2	0	2	13.3	4	2
	leaf blade	50	43	0	18	8	26	60.5	39(13*)	25
'Temboshi'	rhizome	15	11	2	0	0	2	18.2	4	3
	leaf blade	50	41	27	0	0	27	65.9	42	42
'Kinkan'	rhizome	15	9	1	0	0	1	11.0	2	2
	leaf blade	50	47	29	0	0	29	61.7	51	48
wild plant	rhizome	15	12	0	2	0	2	16.7	5	4
	leaf blade	50	44	0	34	0	34	77.3	79	77

*Number of albino shoots.
All explants were cultured on MS medium containing 30 g l^{-1} sucrose, 1 mg l^{-1} BAP and 1 mg l^{-1} NAA. Data were scored 90 days after starting of culture.

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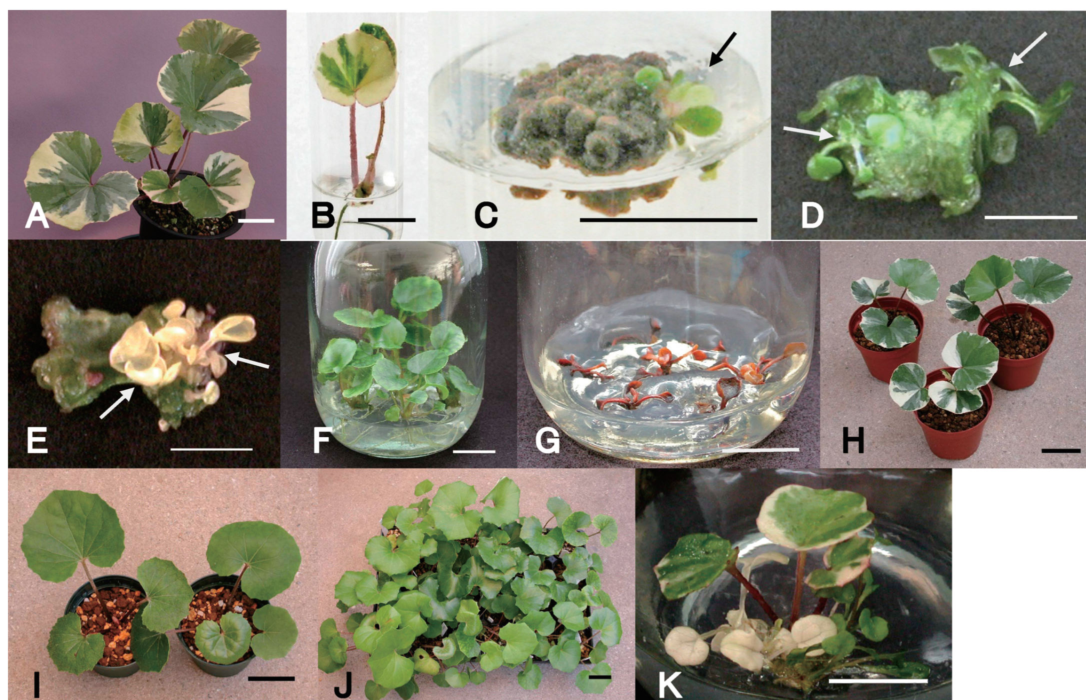

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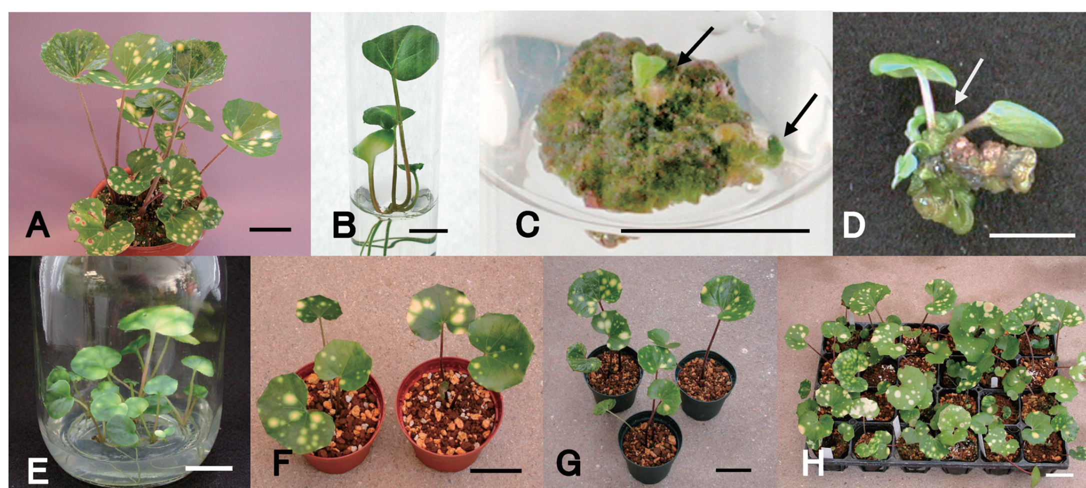

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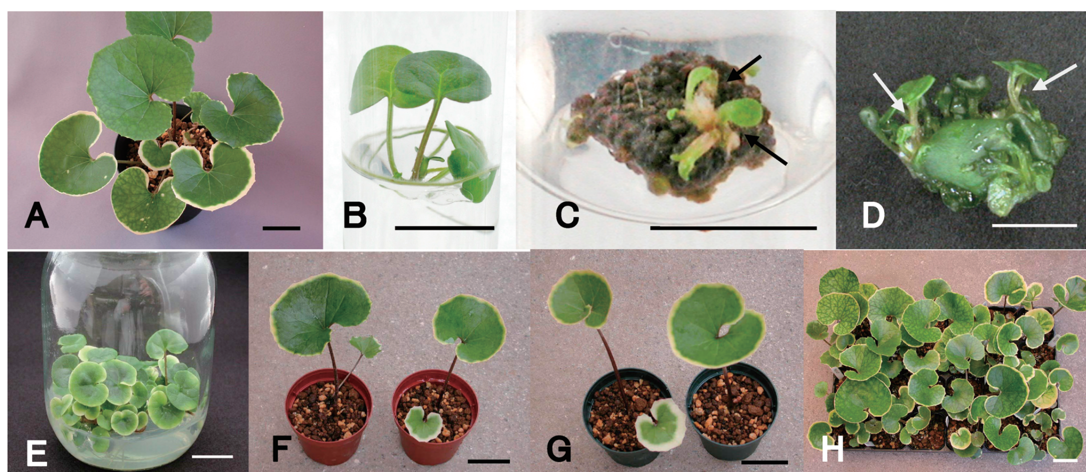

Figure 4.

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many roots as in the case of shoot tip-derived shoots.

In 'Ukigumo-nishiki', *in vitro* shoots raised from shoot tips appeared distinct variegation (Figure 2B), whereas shoots regenerated from rhizome segments were green (Figure 2C) and those from leaf blade segments were either green or albino (Figure 2D, E). On rooting medium, variegation was stably maintained in plantlets developed from shoot tips. In contrast, green shoots developed from rhizome and leaf blade segments remained green even after producing roots (Figure 2F), whereas albino shoots obtained from leaf blade segments eventually died without producing roots (Figure 2G). After acclimatization, plants derived from shoot tips exhibited the same variegation as original plants (Figure 2H), but plants developed from rhizomes and leaf blades exhibited uniformly green coloration without showing any sign of variegation (Figure 2I, J).

In 'Temboshi' and 'Kinkan', shoots raised from all the three kinds of explants showed extremely indistinct variegation (Figures 3B, C, D, 4B, C, D). However plantlets produced from all these explants showed distinct variegation after transfer onto rooting medium in glass bottles (Figures 3E, 4E). After acclimatization, plants derived from all explants of these two cultivars exhibited the same variegation as original plants (Figures 3F, G, H, 4F, G, H).

In a control wild plant, shoots, plantlets and acclimatized plants stably exhibited the same green color as the original plant (Figure 5B, C, D, E, F, G, H).

Shoot multiplication

On propagation medium, several shoots were produced from axils of transplanted shoots irrespective of the origin of the initial explants (Figure 6). In 'Ukigumo-nishiki', shoot tip-derived variegated shoots only produced either green or albino shoots and no variegated shoots were obtained (Figure 2K). In contrast, all proliferated shoots in 'Temboshi' and 'Kinkan' showed the same variegation as original plants and those of a wild plant were green throughout. Shoot multiplication rates after each subculture for 40 days were approximately 2.5, 2.9, 3.5 and 3.4 times in 'Ukigumo-nishiki', 'Temboshi', 'Kinkan' and a wild plant, respectively (Table 3).

Discussion

In tissue culture of variegated geranium, *Pelargonium zonale* 'Mme Salleron', plants obtained directly through adventitious shoot formation from petiole segments never showed variegation and were either green or albino, whereas all the plants raised from shoot-tip culture showed the typical marginal variegation (Cassells et al. 1980). These results suggest that this geranium cultivar was periclinal chimera. Due to the same tissue culture results obtained in the present study, the variegation in 'Ukigumo-nishiki' is also considered to be a periclinal chimera with marginal variegation as suggested previously (Yokoi 1990). In leaf segments culture, it can be considered that albino shoots were regenerated from the region composed of cells lacking for chlorophyll synthesis, and green shoots were from the region without chlorophyll deficiency. In the culture of rhizomes, variegated colorless region located at the outer surface might be completely removed by stripping treatment to avoid the microbial contaminations, which resulted in regeneration of only green shoots.

Although several shoots could be obtained from the shoot tip-derived variegated shoots of 'Ukigumo-nishiki' on the propagation medium, they never showed variegation, representing either green or albino phenotype. Moreover, these shoots gave the same results as that observed in leaf blade segment culture; green shoots only yielded green plants, whereas albino shoots finally died on rooting medium. Although it is rather difficult to explain why the shoots originated from the axils could

Figure 2. Tissue culture of *Farfugium japonicum* 'Ukigumo-nishiki'. (A) A representative plant of the cultivar. (B) A variegated shoot developed from a shoot tip. (C) Green shoots regenerated from a rhizome segment. (D) Green shoots regenerated from a leaf blade segment. (E) Albino shoots regenerated from a leaf blade segment. (F) Green plantlets derived from leaf blades on rooting medium. (G) Dead albino shoots derived from leaf blades on rooting medium. (H) Acclimatized variegated plants derived from shoot tips. (I) Acclimatized green plants derived from rhizomes. (J) Acclimatized green plants derived from leaf blades. (K) Shoot multiplication: a green shoot and an albino shoot proliferated from a variegated shoot on the propagation medium. Arrows indicate regenerated shoots. Scale bars indicate 5 cm (A, H, I, J), 1 cm (B, C, D, E) and 2 cm (F, G, K), respectively.

Figure 3. Tissue culture of *Farfugium japonicum* 'Temboshi'. (A) A representative plant of the cultivar. (B) An indistinctly variegated shoot developed from a shoot tip. (C) Indistinctly variegated shoots regenerated from a rhizome segment. (D) An indistinctly variegated shoot regenerated from a leaf blade segment. (E) Variegated plantlets derived from leaf blades on rooting medium. (F) Acclimatized variegated plants derived from shoot tips. (G) Acclimatized variegated plants derived from rhizomes. (H) Acclimatized variegated plants derived from leaf blades. Arrows indicate regenerated shoots. Scale bars indicate 5 cm (A, F, G, H), 1 cm (B, C, D) and 2 cm (E), respectively.

Figure 4. Tissue culture of *Farfugium japonicum* 'Kinkan'. (A) A representative plant of the cultivar. (B) An indistinctly variegated shoot developed from a shoot tip. (C) Indistinctly variegated shoots regenerated from a rhizome segment. (D) Indistinctly variegated shoots regenerated from a leaf blade segment. (E) Variegated plantlets derived from leaf blades on rooting medium. (F) Acclimatized variegated plants derived from shoot tips. (G) Acclimatized variegated plants derived from rhizomes. (H) Acclimatized variegated plants derived from leaf blades. Arrows indicate regenerated shoots. Scale bars indicate 5 cm (A, F, G, H), 1 cm (B, C, D) and 2 cm (E), respectively.

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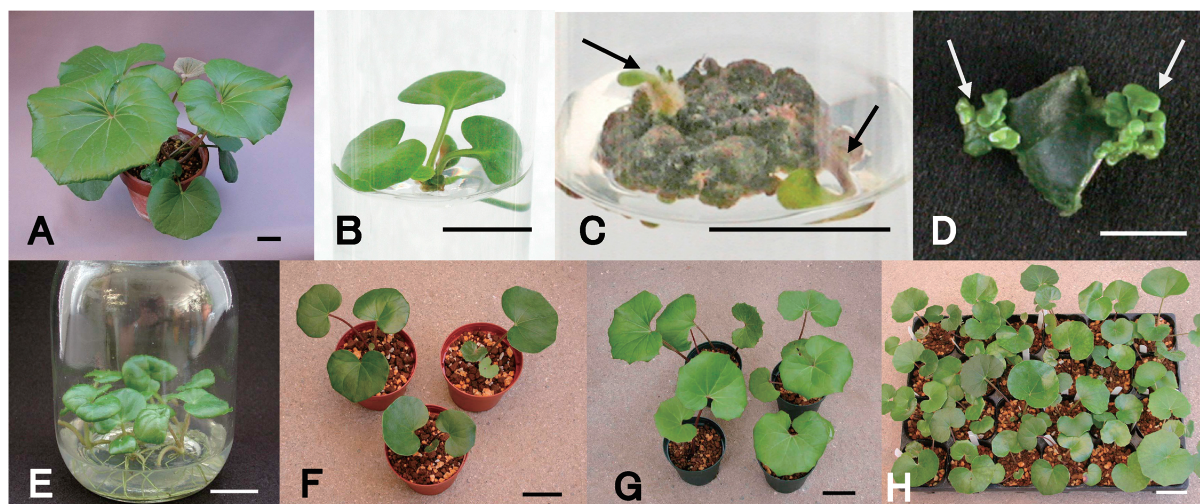

Figure 5. Tissue culture of a wild plant of *Farfugium japonicum*. (A) A representative plant of the cultivar. (B) A green shoot developed from a shoot tip. (C) Green shoots regenerated from a rhizome segment. (D) Green shoots regenerated from a leaf blade segment. (E) Green plantlets derived from leaf blades on rooting medium. (F) Acclimatized green plants derived from shoot tips. (G) Acclimatized green plants derived from rhizomes. (H) Acclimatized green plants derived from leaf blades. Arrows indicate regenerated shoots. Scale bars indicate 5 cm (A, F, G, H), 1 cm (B, C, D) and 2 cm (E), respectively.

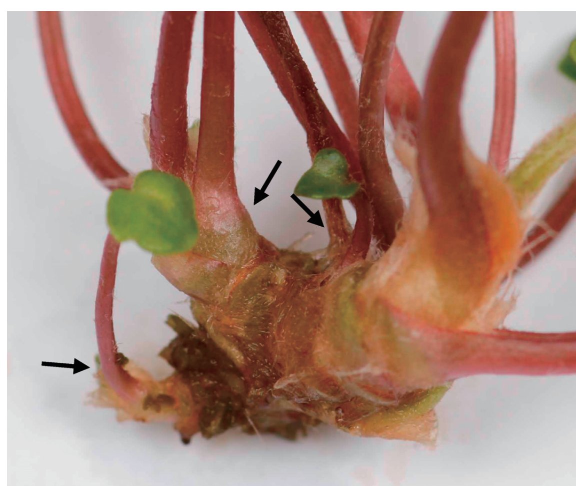

Figure 6. Shoots produced from axils of a transplanted shoot irrespective of the origin of the initial explant. Arrows indicate propagated shoots.

Table 3. Multiplication of shoots after subculture of the tissue culture-derived initial shoots of *Farfugium japonicum*

Cultivar name	No. of cultured shoots	No. of shoots totally obtained	Multi-plication rate
'Ukigumo-nishiki'	2	5	2.5
'Temboshi'	70	206	2.9
'Kinkan'	70	243	3.5
wild plant	70	239	3.4

Shoots were cultured on 1/2MS medium containing 20 $g\,l^{-1}$ sucrose and 0.2 $mg\,l^{-1}$ kinetin. Data were scored after 40 days of culture.

not maintain the chimeric nature, it might be that precocious development of axillary buds in tissue culture condition, which never occurs under natural condition, caused breakage of chimeric organization in apical meristem of axillary buds, which finally resulted in the regeneration of non-chimeric shoots. Since a chimeric variegated cultivar of *Yucca elephantipes* was successfully propagated without loosing chimeric character by inducing axillary branching, which was achieved by applying 1 $mg\,l^{-1}$ BAP (Pierik and Steegmans 1983). In the present study, it is possible that low concentration of kinetin (0.2 $mg\,l^{-1}$) in the propagation medium induced the breakdown of normal chimeric structure of apical meristem of axillary buds, it is necessary to find out the appropriate plant growth regulators to induce axillary branching without affecting the organization of the chimeric structure of apical meristem. Further detailed studies will be needed to clarify the mechanism involved in the dechimerization of the variegated shoots produced from the shoot tips of 'Ukigumo-nishiki'.

In contrast to 'Ukigumo-nishiki', variegation character of 'Temboshi' and 'Kinkan' was highly stable and homogeneous because all plants derived from three kinds of explants exhibited the same variegation patterns as those of the original plants. Thus, it can be considered that 'Temboshi' and 'Kinkan' are not chimeric but genetically stable mutants. Consequently, there are two types of variegated cultivars, chimera and non-chimera, among the cultivars of *F. japonicum* used in the present study.

The boundary in variegation of 'Temboshi' and 'Kinkan', non-chimeric cultivars, is unclear in contrast to clear boundary of 'Ukigumo-nishiki', a chimeric cultivar, as described above. For the micropropagation of variegated cultivars, it is important to know the stability of the variegation during the tissue culture process.

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Therefore, it is interesting to know whether the presence of the clear boundary between variegated and green regions can be used as an indicator to discriminate both types of variegation. Since both types of variegation are found in other cultivars of *F. japonicum*, further tissue culture studies are needed to confirm the stability of the variegation in these cultivars with special reference to the nature of boundary regions.

In the present study, an *in vitro* asexual propagation method was developed for non-chimeric variegated cultivars of *F. japonicum*. In this type of cultivars, leaf blade segments should be suitable as the explants because of the ease to obtain sufficient amount of explants with high shoot regeneration ability and multiplication without loosing the original variegation characteristics. In contrast, the use of shoot tips or rhizome segments should be avoided because of a risk to loose rare cultivars by giving the serious damage of the source plants. Especially, rhizome segments are unfavorable due to the low frequency of shoot formation. As a practical procedure, the number of the shoots induced from leaf blade segments could be efficiently increased by successive culture on propagation medium with approximately 3.2 times proliferation rate in average in both 'Temboshi' and 'Kinkan' after 40 days of culture, followed by transferring each separated shoot onto rooting medium for establishing plantlets. This established method enables to produce ca. 35000 shoots in a year.

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