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Original Paper

The construction of transgenic *Forsythia* plants: comparative study of three *Forsythia* species

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Abstract *Forsythia* spp. are perennial woody plants containing abundant amounts of beneficial lignans, and the transgenic metabolic engineering of *Forsythia* is expected to produce plants with a more efficient production of specific lignans on demand. However, the transgenic methods for *Forsythia* have yet to be fully established. In this study, we have investigated the optimal conditions for the regeneration, growth, and antibiotic-based selection of *Forsythia suspense* (Fs), *F. koreana* (Fk), and *F. intermedia* (Fi), and compared the possibility of the construction of transgenic plants among the three species. Fk, Fi, and Fs explants regenerated more than 100, 36, and 4 shoots per leaf, respectively, revealing that Fk is especially endowed with potent regeneration ability. Fi initiated shoot formation 6 days earlier, but required 6 extra days for the initiation of rooting than the two *Forsythia* spp. Moreover, Fs, Fk, and Fi displayed different preferences for the components of the MS macro elements in the regeneration media. Fk and Fi calli grew to 10-cm long plants for approximately 120 day. We also found that Fk and Fi exhibited prominent dose-dependent sensitivity to hygromycin, but not to kanamycin. We constructed transgenic hygromycin-resistant Fk and Fi via agrobacterium-based transformation with a hygromycin-resistant gene, *hptII*. Polymerase chain reaction analyses confirmed the introduction and expression of *hptII* in the transgenic Fk and Fi. Altogether, these data showed the establishment of the methods for Fk and Fi transgenic plants, and revealed multiple different propensities among *Forsythia* species.

Key words: *Forsythia*, hygromycin-resistance, regeneration, transgenic plant.

The construction of transgenic plants is a key technology for the development of novel plants conferring non-naturally occurring but beneficial phenotypes, such as insect-resistance, pathogen-resistance, drought-resistance, improved growth rate and biomass, and the efficient production of useful secondary metabolites of plants. To date, many transgenic plants have been developed for both academic and industrial purposes. However, a universal methodology for the construction of recombinant plants has yet to be developed. In particular, the experimental procedures for constructing transgenic plants of "model annual plants", including *Arabidopsis thaliana, Nicotiana tabacum,* and *Oryza sativa* L., are frequently useless for the construction of perennial woody plants, although a variety of perennial woody plants are typical farm products and natural resources for the human diet as well as medicinal compounds. Indeed, the practical transformation of woody plants has been limited to very few species: the *Populus, Rosaceae* and Conifers (Aldwinckle and Malnoy 2009; Bradshaw et al. 2000; Malabadi and Nataraja 2007). The woody transformation requires greater time than the annual herbaceous plants, because woody plants generally grow up much more slowly, compared with annual plants. Moreover, universal methods for construction of transgenic woody plants remain to be established, as regeneration, selection, and transformation vary in each plant species due to its different sensitivity to hormones, antibiotic chemicals, and nutrition.

Forsythia (Oleaceae family), commonly known as the golden bell flower, is a perennial woody plant, and consists of a large number of natural and cultivated varieties, including *Forsythia suspense* (Fs)*, F. koreana* (Fk)*, F. viridissima,* and *F. intermedia,* (Fi), a natural hybrid between *F. suspensa* and *F. viridissima* (Rosati et al. 1996). The leaves and fruits are extensively used in Chinese medicines and health diets owing to their rich

Abbreviations: BA, 6-Benzylaminopurine; Fi, *Forsythia intermedia*; Fk, *Forsythia koreana*; Fs, *Forsythia suspense*; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; PCR, polymerase chain reaction; PLR, pinoresinol / lariciresinol reductase; RNA interference, RNAi.

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amounts of lignans: pinoresinol, lariciresinol, and matiresinol as well as their glycosylated forms (Guo et al. 2007, Kitagawa et al. 1988, Nishibe et al. 1988, Piao et al. 2008, Umezawa 2003). Plant lignans are believed to play various pivotal physiological and/or ecological roles in plant-insect interactions due to their antifeedant activity and effects on the endocrine systems, although the precise mode-of-action remains to be elucidated (Harmatha and Dinan 2003; Schroeder et al. 2006). In the digestive tract of humans, plant lignans are metabolized by intestinal microflora into the phytoestrogens, enterodiol and enterolactone (Heinonen et al. 2001). The phytoestrogens are believed to reduce the risk for various cancers and cardiovascular diseases. Furthermore, there is a growing body of reports on diverse biological effects on mammals, including antioxidative, antitumor, and antiviral activities (Adlercreutz 1995; Adlercreutz 2001; Fini et al. 2008; Macrae and Towers 1984). These bioactivities have attracted considerable attention to the potential of lignans as pharmaceutical agents, as well as food supplements for human health, and intensive efforts have been made to establish the metabolic engineering of lignans. In our previous study, transgenic Fk cells, in which endogenous pinoresinol/lariciresinol reductase (PLR) had been suppressed by RNA interference (RNAi), were shown to accumulate 20-fold pinoresinol compared with the wild type Fk suspension cells (Kim et al. 2009). Moreover, we originally produced an exogenous lignan, sesamin, in Fk transgenic cells co-expressing *PLR*-RNAi and *Sesamum* CYP81Q1 (a sesamin biosynthetic enzyme) (Kim et al. 2009; Ono et al. 2006). Furthermore, a *Forsythia* plant is successfully grown from a cut explant, indicating that excellent lignan-producing transgenic lines are easily propagated without a requirement for flowering or seed formation. These findings allowed us to develop transgenic *Forsythia* plants which were competent in the efficient and selective biological lignan production. Nevertheless, neither have general methods for construction of transgenic *Forsythia* plants been established, nor have the advantages and disadvantages been compared in the construction of the transgenic *Forsythia* species.

In this study, the goal is to establish the procedures for the construction of major *Forsythia* species. We investigated the optimal conditions for the cultivation, regeneration, transformation, and antibiotic-directed selection for the calli, shoots, and rooting plants of each *Forsythia* species, and compared the advantages and disadvantages in the methods of constructing the transgenic plants. The present data led to the elucidation of the reproducible methods for transgenic *Forsythia* species and, ultimately, to the construction of hygromycin-resistant Fk and Fi.

Materials and methods

Plant material

Fs plants were obtained from a local gardening shop in Kyoto. Fk plants were kindly provided by Professor T. Umezawa (Research Institute for Sustainable Humanosphere, Kyoto University, Japan). Fi (Linwood) plants were kindly provided by the Niigata Prefectural Botanical Garden.

Plant growth condition

The plants were maintained at 23–25°C under 100 μmol m^{-2} s^{-1} photosynthetic photon flux density with a day length of 16 hours.

Culture media

For the induction and propagation of *in vitro* plants of *Forsythia* spp., the explants, including two buds, were initiated by sterilization. These explants were soaked with 70% ethanol and then 10% NaOCl, and washed 5 times with distilled water. The propagation and rooting medium, the F medium, contains MS (Duchefa Biochemie, Haarlem, The Netherlands) macro- and micro elements at half and full strength, respectively, as well as vitamins, 15 g l^{-1} sucrose, 7 g Phyto-agar (Duchefa Biochemie) and 0.12 mg l^{-1} indole-3-butyric acid (IBA) (Nacalai Tesque, Kyoto, Japan).

For the regeneration of leaf explants, the optimal hormone concentrations of 6-Benzylaminopurine (BA) and 0.5 mg l^{-1} indole-3-acetic acid (IAA) (Nacalai Tesque) were determined (see the Results section). The medium was supplemented with 30 g l^{-1} sucrose, 3 g l^{-1} Gerlite (Duchefa Biochemie), 100 mg l^{-1} myoinositol, 0.5 mg thiamin, 0.75 mg l^{-1} nicotinic acid and 0.75 mg l^{-1} pyridoxine (Nacalai Tesque). We also prepared modified MS mediums as follows: No. 1, half concentration of all macro elements, and No. 3, half concentration of NH_4NO_3 and KNO_3. Thereafter, the No. 1 regeneration medium is called F0, and the No. 3 regeneration medium is called FM0. The pH of all culture media was adjusted to 5.8 before autoclaving at 121°C for 20 min.

Regeneration and growth

To regenerate shoots, cutting leaf discs as explants were plated onto a regeneration medium (F0 or FM0) containing 2 mg l^{-1} BA and 0.5 mg l^{-1} IAA. The medium was refreshed every 10 days. After 60 days, the regenerated shoots were put on the F medium. The formation and elongation of shoots and roots were observed for various days (see the Results section).

Antibiotic and selection agents in Forsythia spp.

Ticarcillin disodium (thereafter ticarcillin) (Duchefa Biochemie) was used for the disinfection of *Agrobacterium tumefaciens*. Various concentrations of kanamycin and hygromycin were tested for selection at the regeneration and rooting stages.

Bacterial strain and culture

A single colony of *A. tumefaciens* strain EHA105 was grown with a binary vector and inoculated into LB (Luria-Bertani) liquid medium containing 50 mg l^{-1} kanamycin and grown at 28°C in a gyratory shaker (180 rpm). The density of the

overnight culture was measured at OD600. The bacteria were spun down (2000 g for 5 min) and resuspended in MS regeneration liquid medium to obtain an inoculum at 10^{-7} cells ml^{-1}.

Binary vector

The empty binary vector pBINPlus (van Engelen et al. 1995), encoding a kanamycin-resistant gene, Nos-*hptII*, and hygromycin-resistant gene, Nos-*hptII*, was used for transformation.

Transformation

Leaf discs of 2-month old plants were excised and cut into 5 mm segments. The cut leaf explants were dipped into the inoculums. Excess suspension was drained on sterile filter paper and the explants were placed onto the regeneration medium for 3 days under the cover of darkness. The regeneration, growth and antibiotic-based selection of transgenic *Forsythia* were performed under the conditions as stated above. The hygromycin resistant rooted plant candidates grew to 10 cm in length (see the Results section).

Genomic polymerase chain reaction (PCR) analysis

Total DNA was isolated from the wild-type and transgenic lines using a DNeasy Plant Mini Kit (Qiagen). PCR was performed using the kanamicin-resistance gene *nptII* and the hygromycin-resistance gene *hptII* with the binary vectors and total DNA (100 ng) as a template. The following PCR primers were used to amplify DNA with ExTaq DNA polymerase (TAKARA BIO INC., Ohtsu, Japan) for 30 cycles of 94°C for 20 s, 58°C for 30 s, and 72°C for 1 min. The endogenous rRNA (accession No. AJ236041) was amplified with the following primer sets rRNA-F (5′-GAA ACC TGC AAA GCA GA-3′) and rRNA-R (5′-CTG ACC TGG GGT CGC TGT CGA-3′). Transformed *nptII* and *hptII* were amplified with the following primer sets: *npt II* -F (5′-TAT GGA TGG ATG AGT CGA CG-3′) and *npt II*-R (5′-ACA GGT TCT TCT CGA GAC TC-3′) for *npt II*; and *hpt II* -F (5′-GCG TGA CCT ATT GCA TCT CC-3′) and *hpt II*-R (5′-TTC TAC ACA GCC ATC GGT CC-3′) for *hptII*. The products were resolved on a 1.5% agarose gel and visualized by ethidium bromide staining.

Reverse transcription (RT)-PCR

Total RNA was isolated from wild type and transgenic lines by the use of an RNeasy Plant Mini Kit (Qiagen). First-strand cDNA synthesis was performed using 1 μg of total RNA with reverse transcriptase under the recommended conditions of SuperScript III (Invitrogen). The endogenous rRNA and transformed *nptII* and *hptII* were amplified with the same primer sets as described for the genomic PCR. The PCR with ExTaq DNA polymerase (TAKARA BIO INC.) was run at 94°C for 1 min followed by 30 cycles at 94°C for 20 s, at 58°C for 30 s, at 72°C for 1 min, and a final extension at 72°C for 7 min (GeneAmp 2400, PerkinElmer, CA, USA). PCR products were visualized with 1.5% agarose gel electrophoresis by ethidium bromide staining.

Results

Regeneration of Forsythia spp

Initially, we examined the regeneration from wild type leaf explants of Fs, Fk and Fi (Figure 1). The regeneration was classified into four major stages: 1, callus formation, 2, adventitious buds formation, 3, multiple shoots formation, 4, shoot elongation and rooting. The callus formation per explants was counted at 20 days of cultivation on the F0 or FM0 medium. The leaf and petiole explants of *Forsythia* spp. displayed a proliferation of calli at the cut end, in particular, at the veins. The callus formation was observed within 6 days of cultivation. The calli of all *F.* spp. were relatively compact and green in color (Figure 1). The formation rate of adventitious buds and multiple shoots per calli were counted at 30 days of cultivation. The maximal and minimal numbers of multiple shoots from one part on a callus were also counted (Table 1).

The adventitious bud formation was observed to a similar degree on the F0 and FM0 medium. The Fs primordial shoots on the callus were observed clearly at 20 days after cultivation (Figure 1). The shoots generated their roots at approximately 45 days of cultivation on the FM0 medium. Subsequently, the regenerated 1 cm Fs shoots were transferred onto the F medium at 60 days of cultivation on the FM0 medium. Finally, only 4 shoots normally elongated to 10 cm long and protruded their roots from one leaf at 60 days of cultivation on the F medium (Figure 1, Table 1).

The Fk adventitious bud formation rate was 78% and 93% of the calli on the F0 and FM0 medium, respectively, showing that the FM0 medium is more effective for the regeneration than the F0 medium (Figure 2). The Fk primordial shoots on the calli were clearly observed at approximately 20 days of cultivation (Figure 1). The largest number of multiple shoots on one callus was 7, and one leaf produced more than 100 regenerated shoots (Table 1), indicating much more effective regeneration of Fk calli than Fs calli. The rooting was observed at 45 days of cultivation on the FM0 medium. The regenerated 1 cm Fk shoots were transferred onto the F medium at 60 days of cultivation on the FM0 medium. Finally, more than 100 shoots normally elongated to 10 cm-long and protruded their roots from one leaf at 60 days of cultivation on the F medium (Figure 1, Table 1).

The Fi adventitious bud formation was 92% and 40% of the calli on the F0 and FM0 medium, respectively, showing that the Fi calli, unlike the Fk calli, were much more effectively regenerated on the F0 medium than on the FM0 medium. Also the Fi primordial shoots on F0 were produced 3 days earlier than on FM0 medium. The primordial shoots on the callus on F0 medium were observed clearly at 14 days after cultivation, which is 6 days faster than for Fs or Fk (Figure 1). 1 to 6 multiple shoots were generated from a single area of a callus on the F0 medium (Table 1). The regenerated 30% shoots rooted at approximately 55 days of cultivation on the F0

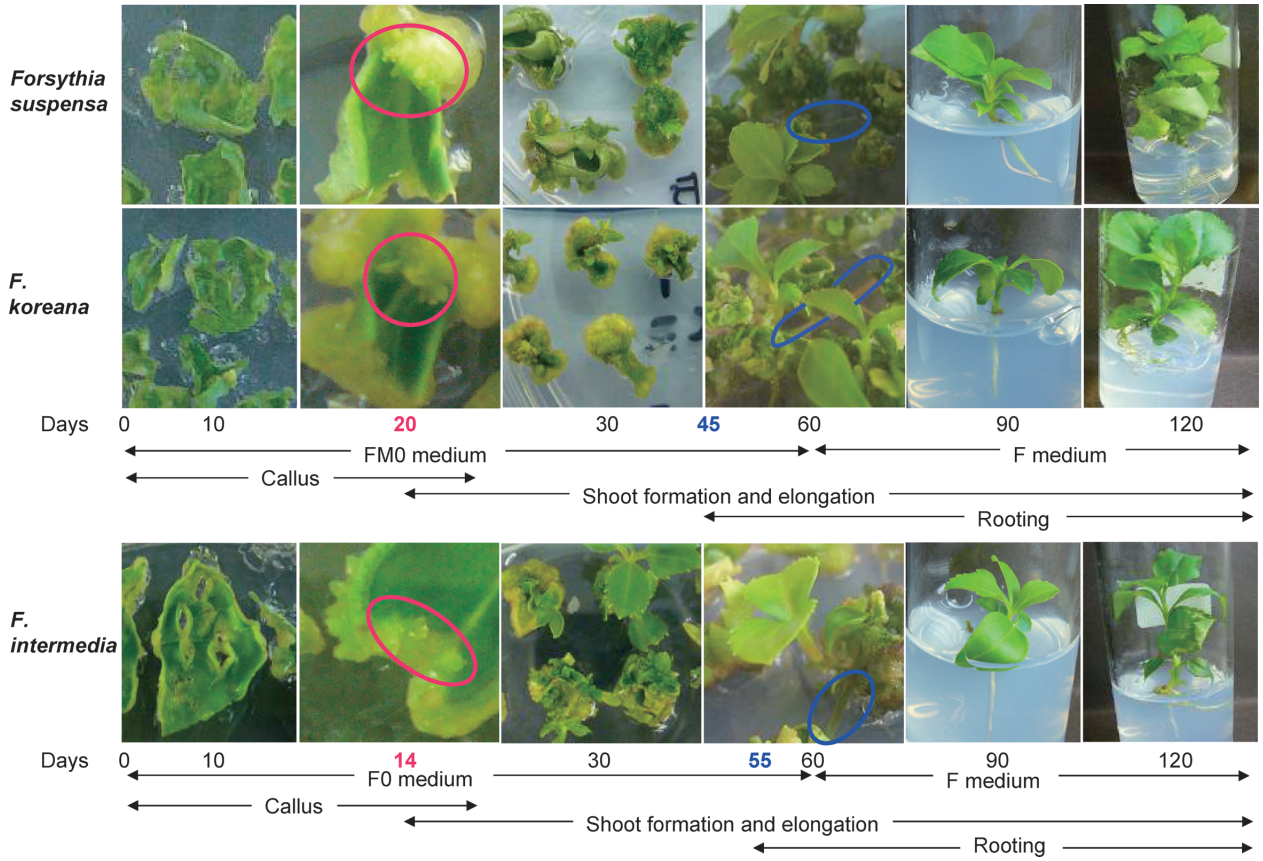

Figure 1. Time course for regeneration and growth of each *Forsythia* spp. from the calli on the respective optimal medium. Red circles and blue circles indicate regenerating adventitious buds formation and rooting, respectively.

Table 1 Frequency of the bud and shoot formation per explant of *Forsythia* spp

	Medium	Callus (%)	Advantitious buds (%)	Multiple shoots (%)	Multiple shoots Max.	Multiple shoots Min.	Regenerated plant number from one leave
F. suspensa	FM0	100	58 ± 14.4	44 ± 11.8	3	1	4 ± 1
F. koreana	FM0	100	93 ± 7.1	78 ± 2.4	7	1	100<
F. intermedia	F0	100	92 ± 0.2	88 ± 5.2	6	1	36 ± 5

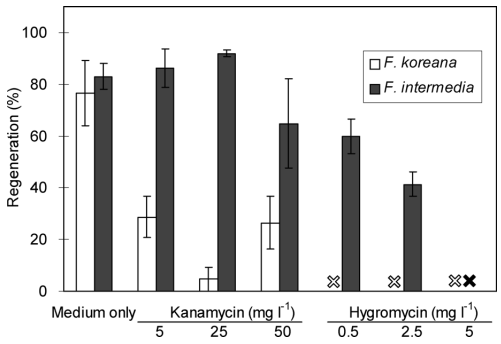

Figure 2. The effects of kanamycin and hygromycin on regeneration frequencies of *Forsythia* spp. Regenerating plants were counted at 30 days of cultivation on the FM0 (for Fk) or F0 (for Fi) containing indicated concentrations (mg l^{-1}) of kanamycin or hygromycin. The number of the initiating calli was taken as 100%. White and black crosses indicate no regenerating Fk and Fi, respectively. Each point represents of the mean±S.E.M. of five preparations.

medium, which is one week slower than the Fk shoots. The regenerated 1-cm Fi shoots were transferred onto the F medium at 60 days of cultivation on the F0 medium. Finally, 36 shoots normally elongated to 10 cm in length and protruded their roots from one leaf at 60 days of cultivation on the F medium (Figure 1, Table 1).

Collectively, these results reveal that Fk calli on the FM0 meduim and Fi calli on the F0 meduim generate a sufficient number of rooted shoots for a subsequent construction of transgenic plants. Moreover, the Fi calli exhibited approximately 6 days faster shoot formation and elongation and 1 week slower rooting, compared with Fk. Accordingly, both Fk and Fi were concluded to require an almost equivalent period (approximately 120 days) for complete regeneration and growth of plants from the callus (Figure 1). In contrast, Fs calli exhibited considerably poor regeneration. Consequently, we

employed these cultivation systems for two *Forsythia* species, Fk and Fi, in the following studies.

Elucidation of effective concentrations of kanamycin and hygromycinfor for the selection of the transformed calli and rooting shoots

Subsequently, we examined the ability of kanamycin and hygromycin to eliminate non-transformants of the Fk and Fi calli. 29, 5, and 27% of the Fk adventitious buds were generated on the FM0 medium in the presence of 5, 25 and 50 mg l^{-1} kanamycin medium, respectively (Figure 2), demonstrating an incomplete dose-dependent sensitivity of Fk calli to kanamycin. In contrast, none of the Fk calli produced adventitious buds in the presence of more than 0.5 mg l^{-1}-hygromycin (Figure 2), confirming the prominent sensitivity of Fk calli to hygromycin.

The effect of kanamycin on the Fi calli was smaller than the Fk calli. 50 mg l^{-1} kanamycin resulted in the decrease in Fi adventitious bud formation to only 63%, demonstrating that Fi calli are potently resistant to kanamycin (Figure 2). The Fi adventitious bud formation rate was 58% and 43% at 0.5 mg l^{-1} and 2.5 mg l^{-1} hygromycin-containing medium, respectively (Figure 2). However, neither proliferation nor the formation of adventitious buds on Fi calli was observed in the presence of 5 mg l^{-1} hygromycin (Figure 2). These results suggested that 5 mg l^{-1} of hygromycin might be effective for the selection of transgenic Fi calli.

Furthermore, we evaluated the resistance of growth and rooting of Fk and Fi shoots to kanamycin or hygromycin. Three shoots of each species were cultivated in the presence of kanamycin or hygromycin at various concentrations (Figure 3). The root development of all of the three Fk shoots were arrested on the F medium in the presence of 50 mg l^{-1} kanamycin, whereas 1–3 shoots protruded roots in the presence of less than 25 mg l^{-1} kanamycin for 12 to 24 days (Figure 3). The root elongation of Fk was strongly inhibited in the presence of 5 mg l^{-1} hygromycin, whereas the Fk shoots rooted in the presence of less than 2.5 mg l^{-1} hygromycin for 12–25 days (Figure 3), proving the potent inhibitory effect of 5 mg l^{-1} hygromycin on the root elongation of Fk shoots.

One of three Fi shoots rooted at more than 25 days on the 5 mg l^{-1} kanamycin containing medium. More than 25 mg l^{-1} kanamycin resulted in the complete inhibition of root elongation of all Fi shoots. The rooting and growth of Fi shoots were inhibited in the presence of more than 0.5 mg l^{-1} hygromycin, demonstrating the higher and more stable sensitivity of the root elongation of Fi shoots to hygromycin than to kanamycin (Figure 3).

Taken together, these data allowed the formulation of an experimental strategy for the selection of hygromycin-resistant transgenic Fk and Fi at two stages: shoot formation and root generation. Finally, 5 mg l^{-1} of hygromycin was employed to select Fk and Fi transgenic plants at both of the stages to simplify the preparation of medium and antibiotic solutions.

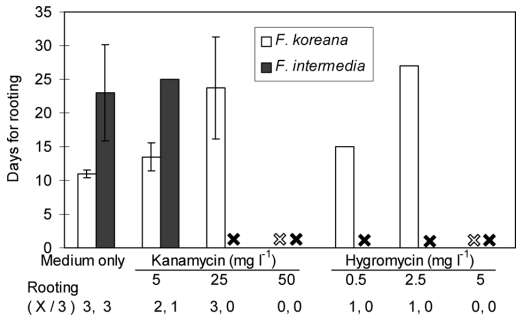

Figure 3. The effects of kanamycin and hygromycin on rooting of *Forsythia* spp. 'Rooting' indicates the number of rooting Fk and Fi per three. White and black crosses indicate no rooting Fk and Fi, respectively. Each point represents of the mean±S.E.M. of five preparations.

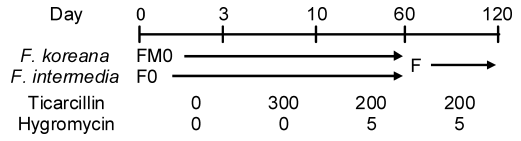

Figure 4. Time course of Fk and Fk transgenic plants on the indicated medium supplemented with ticarcillin and hygromycin (mg l^{-1}), respectively.

Resistance to the disinfection of agrobacterium

To introduce exogenous genes into *Forsythia* spp, we employed the agrobacterium transgenic method, and ticarcillin disodium was used for agro bacterium disinfection (Kim et al. 2009). We thus evaluated the effect of 300 mg l^{-1} or 200 mg l^{-1} ticarcillin on the regeneration and rooting of *Forsythia* spp. The adventitious bud formation in *Forsythia* spp. was not affected by the treatment with ticarcillin (results not shown), confirming that the agrobacterium-mediated transgenic method is applicable to the construction of transgenic *Forsythia* explants.

Construction of hygromycin-resitant Fk and Fi

Based on the experimental strategies stated above, we attempted to construct transgenic Fk and Fi plants. Three Fk and Fi leaf discs were transformed by an agrobacterium-mediated method with a binary vector for constitutive expression of the kanamicin-resistance gene *nptII* and the hygromycin-resistance gene *hptII* conjugated with the NOS constitutive expression promoter (Kim et al. 2009). After co-cultivation with the agrobacterium for 3 days, the explants of Fk and Fi were disinfected on FM0 and F0 medium containing 300 mg ticarcillin for 7 days, respectively (Figure 4). Subsequently, these Fk and Fi explants were further

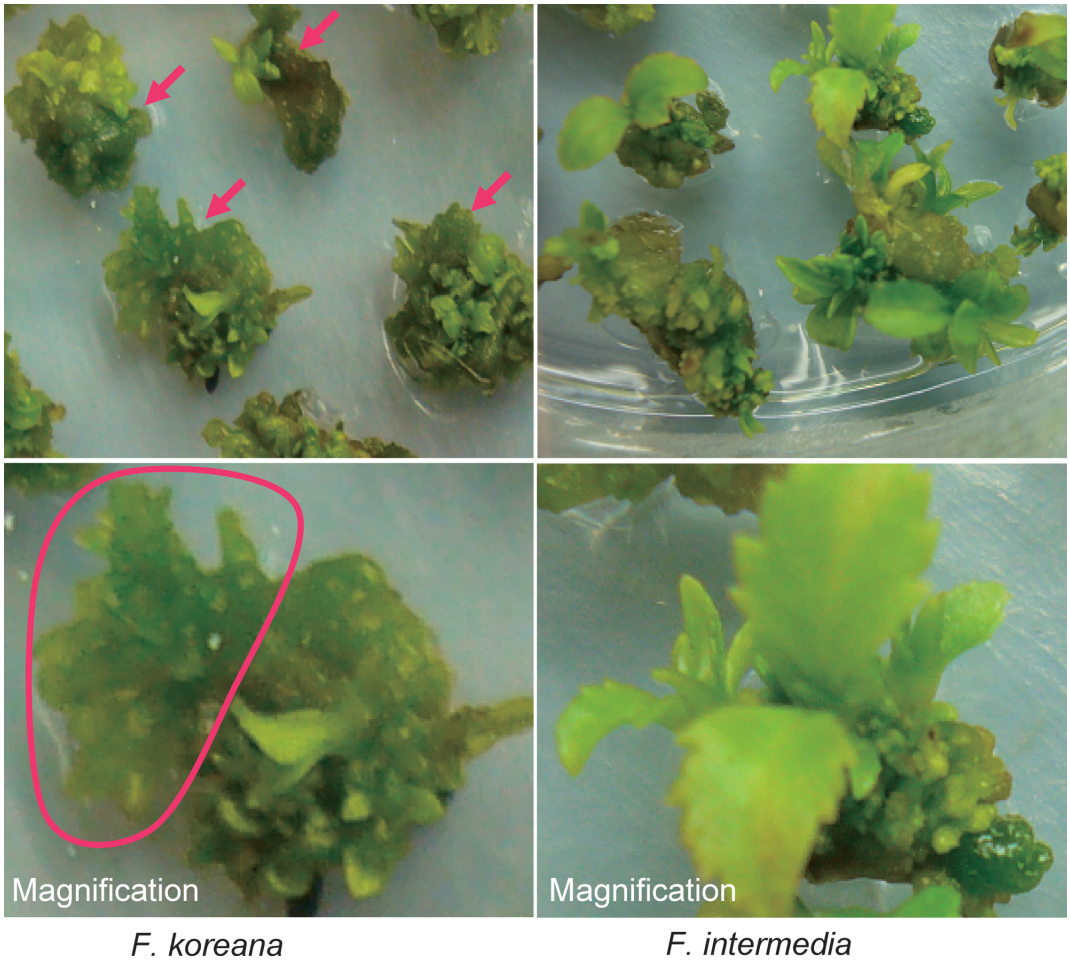

Figure 5. Vitrification of Fk and Fk transgenic plants regenerating from leaf explants at 45 days of cultivation. Allows and a circle indicate the point of vitrification. The lower panel shows the magnified images.

cultivated for 50 days on FM0 and F0 medium, respectively, containing 5 mg l^{-1} hygromycin and 200 mg ticarcillin for shoot formation from calli (Figure 4). Moreover, the Fk and Fi shoots were further planted for rooting on the F medium containing 5 mg l^{-1} hygromycin and 200 mg ticarcillin for 60 days (Figure 4). Nevertheless, almost 90% of the infected Fk shoots was found to vitrify and their growth was arrested during the cultivation (Figure 5), whereas the vitrification of agrobacterium-free Fk was not detected in the presence or absence of hygromycin (Figures 1, 5). In contrast, such a drastic vitrification was not seen in the Fi shoots (Figure 5). Eventually, one Fk and two Fi shoots rooted and normally grew on the F medium containing 5 mg l^{-1} hygromycin. The morphology of the transgenic plants was apparently normal, compared with the control wild-type plants. In addition, the regeneration, rooting, and growth of transgenic Fk and Fi were comparable to those of the wild types (Figure 1).

Finally, we examined introduction of the *nptII* and *hptII* genes into the Fk and Fi genomes and the expression of these genes. As shown in Figure 6, we

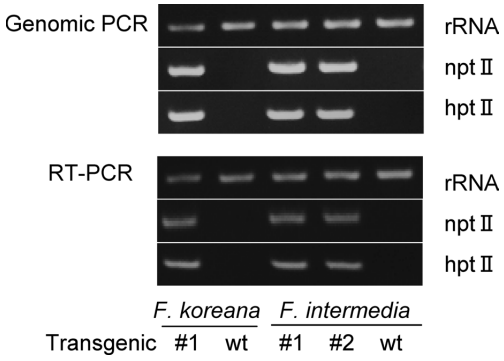

Figure 6. Genomic (upper panel) and RT-PCR (lower panel)of the ribosome RNA (rRNA), *nptII*, and *hptII* genes, and in transgenic (#1, 2) and wildtype (wt) plantsof Fk and Fi.

confirmed that the *nptII* and *hptII* genes were stably integrated into the Fk and Fi genomes. Figure 6 also shows the expression of the *nptII* and *hptII* genes in one Fk and two Fi plants, revealing that the transgenes were functionally expressed. Collectively, these results provided evidence that our transgenic procedures led to

the construction of hygromycin-resistant Fk and Fi plants. Additionally, these transgenic *Forsythia* plants were observed to grow well and preserve the hygromycin-resistance after propagation by cutting.

Discussion

In this study, the aim was the construction of the transgenic plants of three *Forsythia* spp, and evaluation of the utility of the methods for each *Forsythia* spp, given that the efficiency in construction of transgenic plants frequently differs among plant species. We ultimately constructed transgenic hygromycin-resistant Fk and Fi plants, in which the exogenous *hptII* gene is expressed (Figure 6). To our knowledge, this is the first report on the construction of transgenic plants of multiple *Forsythia* species, even though the transgenic efficacy of Fk and Fi is not so high as that of herbaceous model plants including *A. thaliana, N. tabacum* and *O. sativa* L.

We first elucidated the conditions for regeneration, and then performed selection using wild type Fs, Fk, and Fi. Of particular significance is that these three *Forsythia* species showed different medium preferences, regeneration frequencies, and growth rates (Figures 1–3). Previously, the regeneration of Fi calli was performed using standard MS medium (Rosati et al. 1996, Rosati et al. 2003). However, in our study, all of the *Forsythia* calli exhibited consistently poor regeneration, given that bud formation of *Forsythia* spp was inhibited, and thus, the generation of excess vigorous callus formation on standard MS medium was observed (results not shown). This poor regeneration efficiency on standard MS medium in all *Forsythia* spp. was highly likely to be due to a difference in the nitrogen source; both F0 and FM0 contain half as much nitrogen source (KNO_3 and NH_4NO_3) as MS standard. This view is compatible with the finding that the amounts of KNO_3 and NH_4NO_3 in an *in vitro* medium were shown to have a significant effect on the rate of cell growth, differentiation and cell topipotency of various plants (Kirby et al. 1987).

The Fs and Fk calli regenerated more frequently on the FM0 medium, whereas the F0 medium conferred a 2-fold greater regeneration of Fi calli compared with the FM0 medium. The F0 and FM0 medium differ in all macro-elements such as $CaCl_2$ and KH_2PO_4, except KNO_3 and NH_4NO_3; the FM0 medium contains 2-fold more of these elements. In *Hordeum vulgare* L., increased amounts of KH_2PO_4 enhanced shoot bud induction and plant regeneration (Chauhan and Kothari 2004). In *Torenia* stem segments, application of the calcium ionophore, A23187, which induces the influx of calcium ion, significantly upregulated the adventitious bud formation (Tanimoto and Harada 1986). These findings suggest that the concentrations and ratio of macro elements affected the efficiency of adventitious bud formation of *Forsythia* spp, although the molecular mechanisms remain to be elucidated.

Only 4 Fs shoots were obtained from one leaf, and Fs calli produced many adventitious buds, but almost none of the Fs buds grew shoots (Table 1). Medium type did not exert a powerfully positive effect on the regeneration frequency of Fs, suggesting that Fs calli possess much lower regeneration ability than Fk and Fi calli, and there is the problem that Fs is at present not available as a transgenic host plant. Fk and Fi calli regenerated and elongated sufficient shoots followed by protrusion of the roots for 120 days of cultivation, although Fk and Fi differed in their rates of shoot formation, elongation and rooting (Figure 1, Table 1).

Most of the agrobacterium-treated Fk shoots specifically vitrified and ceased to grow (Figure 5). Such vitrification have resulted in the growth of only one transgenic Fk despite the regeneration of more than 100 Fk shoots per leaf disk. *In vitro* explants are believed to be caused by exogenous or endogenous stress including the hormone balance and/or total amount, infection and physiological conditions (Benson et al. 2007; Kevers et al. 1984). For instance, Rosati et al. (2003, 1996) pointed out that more vitrified shoots of Fi were generated by 2 $mg\,l^{-1}$ BA than by 1 $mg\,l^{-1}$ BA. However, no serious vitrification was observed in agrobacterium-free Fk shoots under our condition (2 $mg\,l^{-1}$ BA). Furthermore, Fk was not subjected to the vitrification even in the presence of hygromycin (Figure 3), suggesting that the Fk vitrification is not attributed to hygromycin at the concentrations in this study. Hence, the agrobacterium treatment, in concert with the potent toxicity of hygromycin, if any, may cause serious vitrification of Fk (Figure 5). In other words, combined with the high regeneration and rooting efficiency of Fk (Table 1), the reduction in the vitrification of agrobacterium-treated Fk is expected to lead to the efficient production of Fk transgenic plants. Alternatively, an agrobacterium-free transfection using a particle gun may be effective for reduction of the vitrification.

Forsythia is the most major biological source of lignans such as pinoresinol, phyllegenin, secoisolarisiresinol, and matiresinol (Nishibe et al. 2001). These lignans are known for their beneficial biological activities, including antioxidative, antibacterial, antiviral, and anti-tumor effects (Heinonen et al. 2001, Milder et al. 2005, Milder et al. 2006, Saarinen et al, 2007). Schmitt and Petersen (2002) reported a high level of pinoresinol accumulation in Fi leaves and cell culture. In our previous study, PLR-RNAi-transgenic Fk cells accumulated approximately 20-fold increased (+)-pinoresinol glucoside, compared with the wild type (Kim et al. 2009), and Fk transgenic cells expressing PLR-RNAi and an exogenous sesamin-

synthesizing enzymes, CYP81Q, produced sesamin, a major lignan in sesame seed, which is completely absent in *Forsythia* spp (Kim et al. 2009). Combined with these findings, our present study will pave the way for the construction of novel transgenic *Forsythia* plants with high efficiency and/or on-demand lignan production. Construction of lignan biosynthetic enzyme-engineered transgenic *Forsythia* plants is currently in progress.

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