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Short Communication

Long-term maintenance of high regeneration ability of switchgrass embryogenic callus

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Abstract Switchgrass (*Panicum virgatum* L.) is one of the most important crops for forage and bioenergy, and embryogenic callus is an important material for molecular breeding of this species. In this study, the longevity of caryopsis-derived compact Type I callus of switchgrass was investigated during 24 months. The regeneration ability of the callus was gradually reduced after 18 months of subculture, but remained at a relatively high level after 24 months. In addition, albino formation was not induced throughout the 24-month subculture. Casamino acids improved the regeneration ability of embryogenic calli without apparent morphological change or albino induction, while proline induced friable Type II calli as well as albino shoots. Cell straining treatment coupled with medium containing casamino acids led to 4-fold higher regeneration ability. The ploidy levels of 24-month-old calli were similar to seedling explants. The present results indicate that caryopsis-derived Type I callus is stable and could be maintained long-term, and thus would be a useful source for genetic transformation of switchgrass.

Key words: Embryogenic callus, long-term culture, regeneration, somaclonal variation, switchgrass.

Embryogenic callus is an important material for molecular breeding of grass species including switchgrass (*Panicum virgatum* L.), which is one of the most important crops for forage and bioenergy. The typical embryogenic calli of grasses are classified into two types according to defined characteristics as follows: Type I, which is compact, highly organized, slow growing, pale white to light yellow in color, and regenerable; Type II, which is soft, friable, fast growing, and highly regenerable (Vasil and Vasil 1994). Prolonged subculture of callus, especially using the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D), turns embryogenic cells into non-embryogenic cells resulting in the loss of regeneration ability, and also leads to somaclonal variations, such as albinism (Karp 1994; Vasil and Vasil 1994). Although both Type I and Type II embryogenic callus cultures of switchgrass have been established from various explant sources, i.e., mature caryopsis, young leaf, immature inflorescence and young seedling (Alexandrova et al. 1996; Denchev and Conger 1994; Song et al. 2012), reports on the longevity of embryogenic cells and the occurrence of somaclonal variations are limited. Burris et al. (2009) reported that inflorescence-derived Type II callus could be maintained for more than 6 months, while the embryo viability of Type I callus was limited to less than 2 months.

In this report, the shoot regeneration ability of caryopsis-derived Type I callus of switchgrass was periodically investigated (the scheme is summarized in Supplemental Figure S1). Embryogenic calli were induced from caryopses of switchgrass cultivar Alamo according to Denchev and Conger (1994) using PVC medium (Murashige and Skoog [MS] salts and vitamins (Murashige and Skoog 1962), 30 g l^{-1} maltose, 22.6 μM 2,4-dichlorophenoxyacetic acid [2,4-D], 4.4 μM 6-benzylaminopurine [BAP], 2.5 mM 2-(*N*-morpholino)-ethanesulfonic acid [MES], 8 g l^{-1} agar, pH 5.7). Callus lines induced from different caryopses were maintained independently by standard subculture as follows; cell clumps showing Type I morphology were visually selected, divided into pieces 3–5 mm in diameter using forceps, and then transferred to fresh medium. Three callus lines, 10-01, 10-04 and 10-05, showing high regeneration abilities (250–400 shoots g wet weight [WW]$^{-1}$) were screened using PVS medium (MS salts and vitamins, 30 g l^{-1} maltose, 1.4 μM gibberellic acid [GA_3], 2.5 mM MES, 8 g l^{-1} agar, pH 5.7), and were used for periodic investigation of regeneration ability over 24 months. All cultivations were performed at 28°C either in the dark for callus culture or under 16-h light for shoot regeneration.

The ability to regenerate green shoots for the three cell lines was constant during 12 months of standard subculture on PVC medium (Figure 1). It was gradually

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Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; Type I callus, compact embryogenic callus; Type II callus, friable embryogenic callus.
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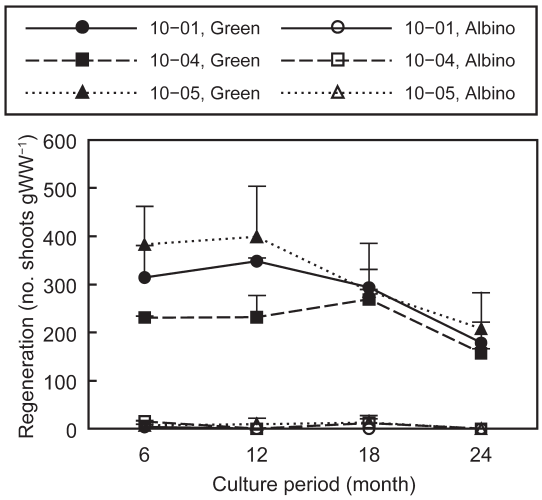

Figure 1. The regeneration abilities of calli maintained long-term by standard subcultures on PVC medium. Data represents a mean±standard deviation of three replications (15 calli each).

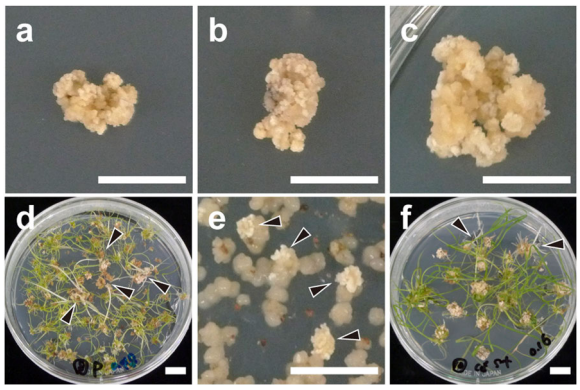

Figure 2. Growth and shoot regeneration of caryopsis-derived Type I callus of switchgrass. Callus cultured on (A) PVC medium without additive, (B) PVCc medium containing 5 g l^{-1} casamino acids and (C) PVCp medium containing 5 mM proline for 4 weeks. (D) Shoot regeneration from calli cultured on PVCp medium. (E) Growth of calli strained using 1000-μm sieve on PVCc medium and (F) shoot regeneration of these transferred onto PVS medium. Arrowheads indicate albino shoots and compact calli in (D, F) and (E), respectively. All bars indicate 10 mm.

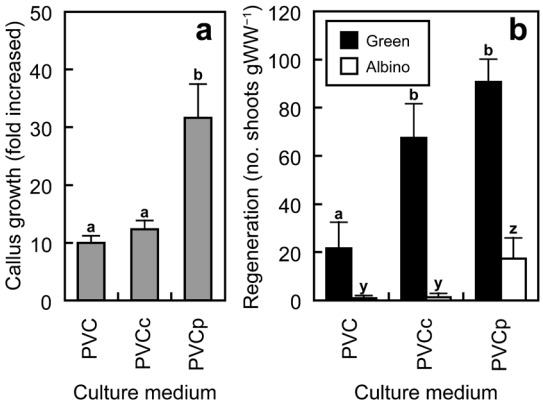

Figure 3. The effects of additives in the culture medium on embryogenic callus of switchgrass Cell line 09-01 (6- to 12-month-old) was used for experiments. (A) Callus growth after 4 weeks of culture. (B) Green and albino shoot regeneration after 4 weeks of culture. Seven calli (ca. 0.15 g WW per plate) were used for callus growth and all calli grown after 4 weeks were transferred to PVS medium for shoot regeneration (up to 0.5 g WW per plate). Data represents a mean±standard deviation of three independent experiments and data with the same letters are not significantly different according to the Tukey–Kramer method at the 5% level.

reduced after 18–24 months of subculture, but remained at a relatively high level. Moreover, albino shoots were formed occasionally (0–15 shoots g WW^{-1}, less than 6.3% of total shoots) throughout 24 months of subculture.

To enhance and recover regeneration ability, two experiments were performed using another callus line, 09-01, which showing high regeneration ability and typical Type I morphology (Figure 2A). The effects of two organic additives, casamino acids (acid hydrolyzed casein; BD, Franklin Lakes, NJ, USA) and L-proline (Sigma-Aldrich, St. Louis, MO, USA), on caryopsis-derived calli were examined first. When calli were cultured on PVCc medium (PVC medium supplemented with 5 g l^{-1} casamino acids) for 4 weeks, they generally maintained typical Type I callus morphology but turned slightly dark in color and became partially friable (Figure 2B). The growth rate of calli cultured on PVCc medium was similar to that on PVC medium (Figure 3A). These results were in agreement with Xi et al. (2009). However, calli grown on PVCc medium formed more than 3-fold as many shoots as calli grown on PVC medium (Figure 3B). Calli cultured on PVCp medium (PVC medium supplemented with 50 mM proline) morphologically changed to Type II after 4 weeks of culture (Figure 2C). Although they showed drastically higher growth rate and regeneration ability than calli cultured on PVC medium, they produced albino shoots in significantly higher numbers than the other two conditions (Figures 2D, 3). These effects of casamino acids and proline were similar in the ranges of 0.5–10 g l^{-1} and 5–100 mM, respectively (data not shown). Proline has been used at low concentrations (100–500 mg l^{-1}) in combination with casein hydrolysates (Burris et al. 2009; Ramamoorthy and Kumar 2012) to induce Type II calli in switchgrass. Li and Qu (2011) used proline alone at high concentration (2 g l^{-1}) for friable embryogenic callus induction, and reported that all regenerated plants were albino after calli of cultivar Alamo were subcultured for 14 months. These results suggested that PVCc medium containing casamino acids would be a better medium than proline-containing medium to improve shoot regeneration without albino induction.

It has been reported that older callus cultures have a reduced ratio of embryogenic cells to non-embryogenic cells and lose their capacity for plant regeneration (Vasil and Vasil 1994). Since Type I callus of switchgrass comprises large, tightly-packed cell clumps (Figure 2A), it is difficult to inspect and select cells possessing

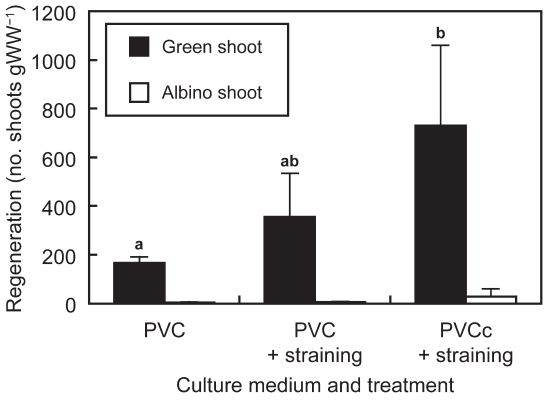

Figure 4. The effects of cell straining and additives in culture medium on shoot regeneration. Cell line 09-01 (6- to 12-month-old) was used for experiments. Data represents a mean±standard deviation of three independent experiments with 15 calli (ca. 0.2 g WW) in each replication and data with the same letters are not significantly different according to the Tukey–Kramer method at the 5% level. A significant difference was not observed in albino shoot formation by ANOVA (p=0.204).

high regeneration ability within a clump. Therefore, a cell straining treatment was subsequently attempted to overcome this limitation. Clumps of Type I calli were collected on a 1000-μm stainless steel sieve, physically strained with a spatula, and then placed onto either PVC or PVCc medium. After 4 weeks of culture, 2–3 mm compact cell clumps were formed on a lawn of friable and mucoid cells (Figure 2E). Compared to calli manually divided into similar sizes using forceps, the small compact clumps formed on PVC and PVCc media after straining treatment regenerated 2.1- and 4.4-fold higher numbers of green shoots, respectively (Figure 4). In addition, an increase of albino shoots was not observed after cell straining (Figures 2F, 4). These results indicate that cell straining treatment would be useful to select embryogenic cells possessing high regeneration ability without the need for skillful judgment.

In the periodic investigations, PVCc medium and cell straining treatment also improved the regeneration ability of 12- to 24-month-old calli by 2- to 3-fold, but improvement was not observed in line 10-01 (Supplemental Figure S2). When straining-treated calli were subcultured by standard methods on either PVC or PVCc medium, the positive effect of this treatment on lines 10-04 and 10-05 was lost after 3 months (Supplemental Figure S3). In addition, the regeneration ability of line 10-04 was almost lost after 6 months of subculture on PVCc medium. Therefore, PVCc medium and cell straining would be useful before regeneration and transformation as a short-period treatment.

Since 2,4-D potentially increases chromosome instability at high concentrations (Karp 1994), the ploidy levels of 24-month-old calli were determined by flow cytometry according to Mishiba and Mii (2000). Long-term-maintained calli showed a dominant 2C peak

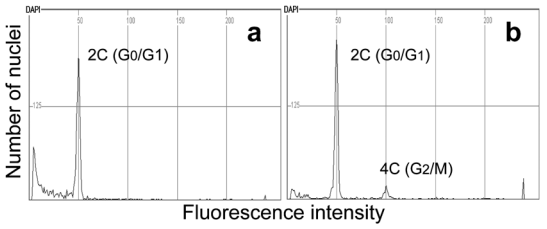

Figure 5. Ploidy analysis by flow cytometry. (A) Leaf of a seedling as a control. (B) 24-month-old callus of line 10-01.

corresponding to G_0/G_1 nuclei, which was similar in fluorescence intensity to the single peak of seedling leaf explants (Figure 5). A small 4C peak was also observed in long-term-maintained calli. This was possibly derived from G2/M nuclei of the 2C cell cycle, because callus contains actively dividing cells. These results showed that a change of ploidy level did not occur in callus subcultured long-term in the presence of 2,4-D.

Caryopsis is the most readily available explant in switchgrass, and Type I callus is the most prevalent type of callus induced from caryopsis (Denchev and Conger 1994; Vasil and Vasil 1994). In fact, we have successfully obtained transgenic switchgrass plants from 18-month-old calli (Ogawa et al. 2014). These findings indicate that caryopsis-derived Type I callus is useful source for genetic transformation of switchgrass. Further studies into the establishment of an efficient transformation method using this explant are in progress.

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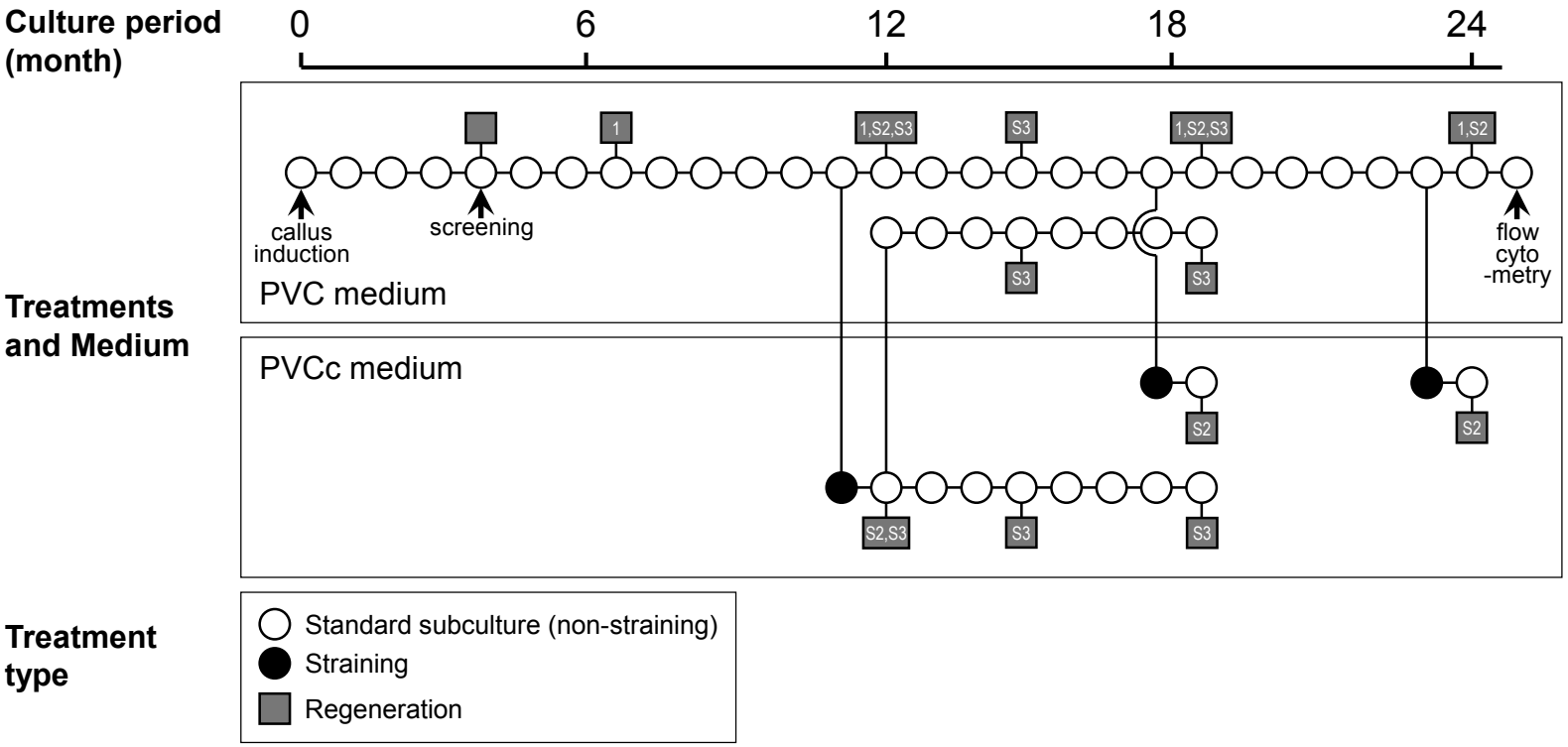
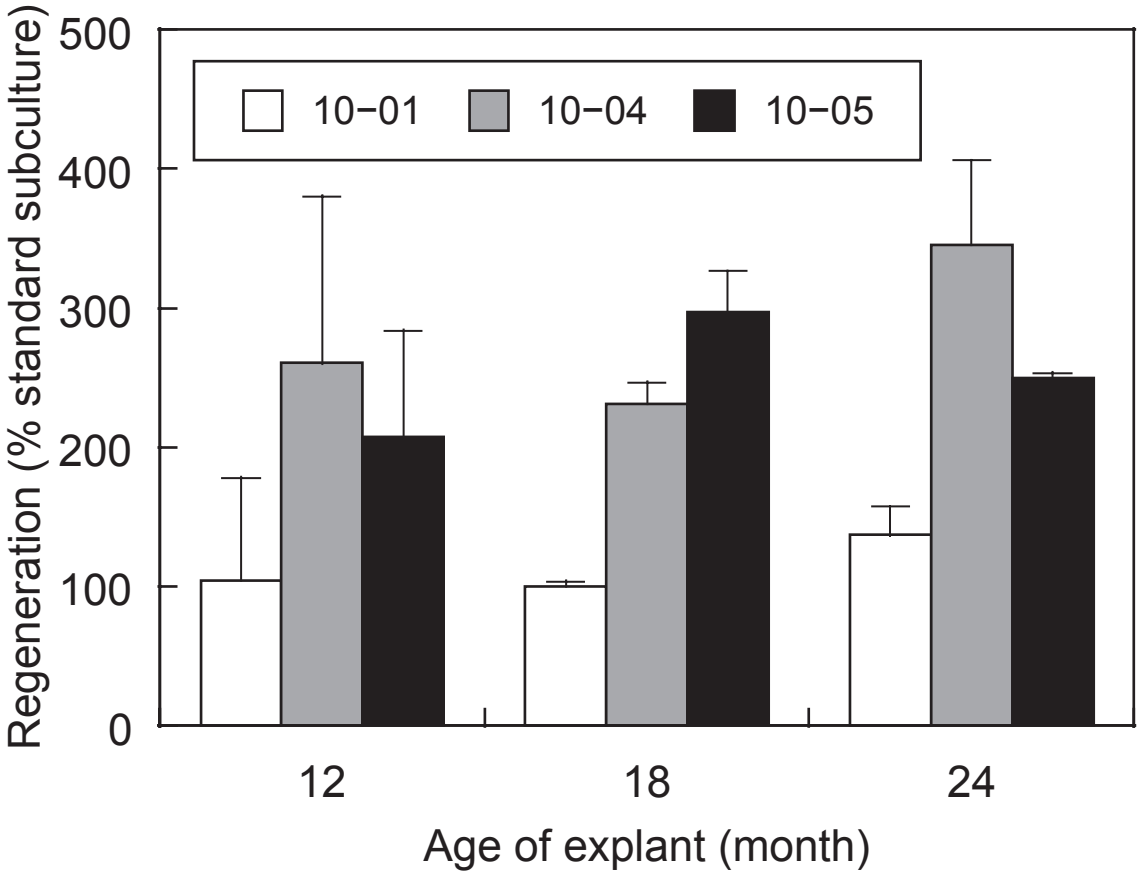
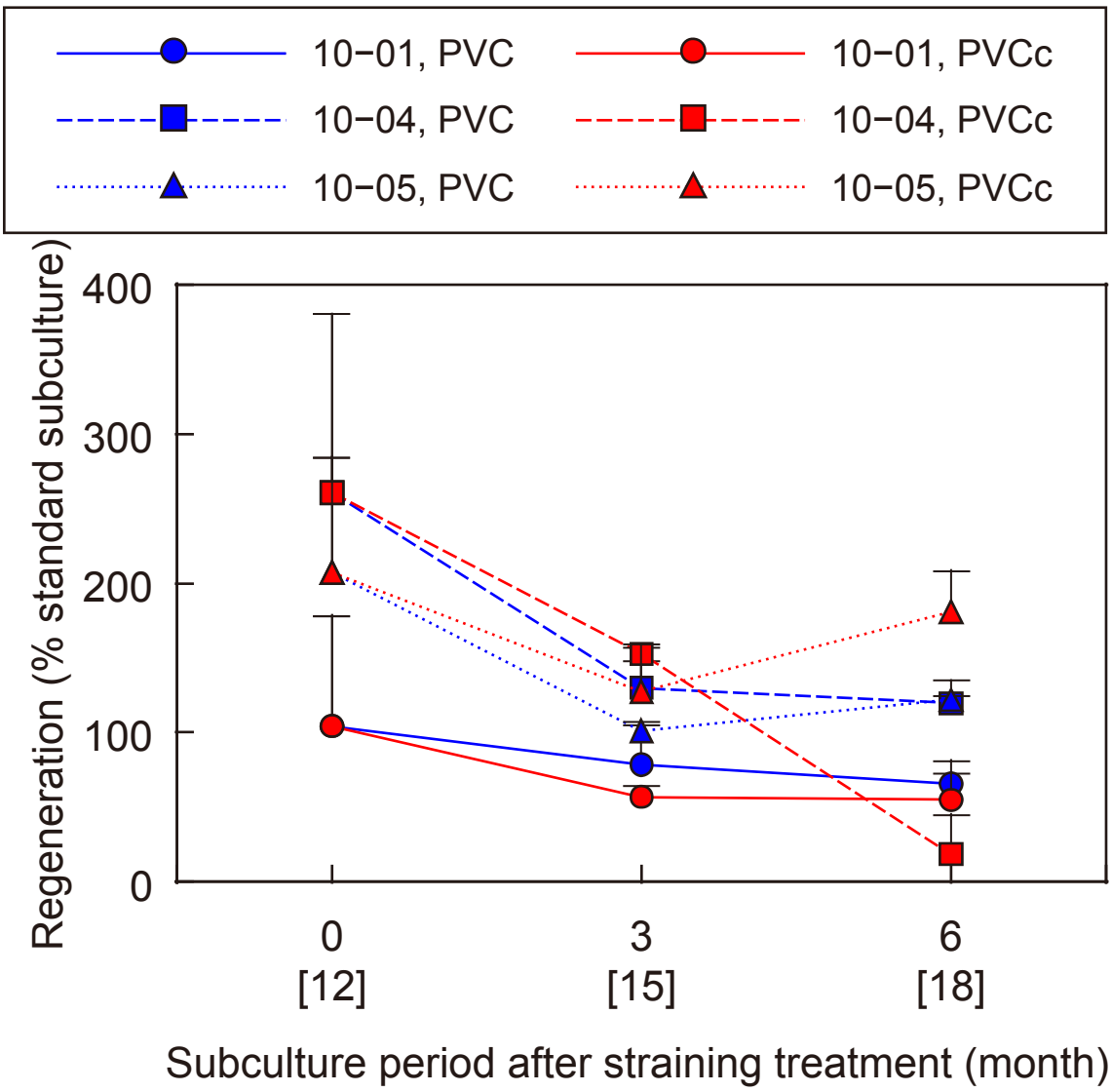


Supplemental Figure S1. Scheme of periodic subculture and regeneration experiments. Figure numbers corresponding to regeneration experiments are given in each rectangle.



Supplemental Figure S2. Improvement of the regeneration ability of long-term subcultured calli by PVCc medium and cell straining. Data represents a mean ± standard deviation of three replications (15 calli each).



Supplemental Figure S3. Durability of the enhanced regeneration ability induced by PVCc medium and cell straining. Cell straining-treated calli were subcultured on either PVC medium (blue) or PVCc medium (red) for various periods before transfer to regeneration medium. Total subculture periods from callus induction are represented in an x-axis in parentheses. Data represents a mean ± standard deviation of three replications (15 calli each).