

Note

Generation of transgenic *Linum perenne* by *Agrobacterium*-mediated transformation

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Abstract Here we report new methods for tissue culture, plant regeneration, and *Agrobacterium*-mediated transformation of perennial flax (*Linum perenne*). To generate transgenic plants, *L. perenne* hypocotyls were co-cultivated with *Agrobacterium* harboring a binary vector (pBI121 or pRI909) for 2 days. The transgenic calli and shoots were induced on Murashige–Skoog medium supplemented with 10 μ M 6-benzylaminopurine and 0.3 μ M 1-naphthalenacetic acid under selection with 100 mg l⁻¹ kanamycin. For root induction from the shoots, 0.1 mg l⁻¹ indole-3-butyric acid was found to be most efficient. We transformed four types of binary vectors encoding a florigen gene under the control of 35S or phloem-specific promoters. Transgenic plants were obtained with high efficiency (4–24%) and exhibited early flowering phenotypes. Our transformation method will contribute to future studies of *L. perenne* that require transgenic plants.

Key words: *Linum perenne*, tissue culture, transgenic plant.

Floral heterostyly is a reproductive system promoting outcrossing through the formation of two morphological types of flowers. Heterostyly has been widely observed in 135 genera of 24 families in angiosperm (Vuilleumier 1967). In many cases, one flower type has a long style, short stamen, and small pollen, and is called “pin”, while another has short style, long stamen, and large pollen, and is called “thrum”. These plants often show self-incompatibility, also a system for outbreeding, by rejecting self-pollen. In heterostylous plants, pollen from the different morph is accepted on the pistil, but pollen from the same morph is rejected. These complex characteristics of heterostylous plants are controlled by a single locus consisting of two alleles, *S* and *s* (Bateson and Gregory 1905). *S* allele behaves dominantly against *s*, with the genotype *Ss* showing the thrum phenotype and *ss* the pin. At least five genes controlling style length, stamen height, pollen size and shape, and pollen or pistil determination of self-incompatibility are thought to be encoded in the *S*-locus (Lewis 1954).

Heterostyly and self-incompatibility in *Linum perenne* were initially described by Charles Darwin (1863). *L. perenne* also has differences in the exine sculpture of pollen between the morphs (Ockendon 1968). *L. perenne* is one of the best candidates for a model plant to study heterostyly because of its short life cycle

(about 4 months), small size of plants (30–40 cm), and ready availability on the market. However, transgenic experiments are important for the investigation of gene functions, and thus far there has been no report on transgenic research using *L. perenne*. Some transgenic studies have been reported in *L. usitatissimum*, a related species (Basiran et al. 1987; Jordan and McHughen 1988; Mlynárová et al. 1994). Unfortunately, *L. usitatissimum* is homostylous and self-compatible, and thus unsuitable for the study of floral heterostyly. Therefore, in this study, we aimed to develop a protocol for *Agrobacterium*-mediated transformation of *L. perenne*.

First, we determined tissue culture and plant regeneration conditions. Sterilized seeds of *L. perenne* (Fujita Seed, Osaka) were placed on Murashige–Skoog (MS) medium with 1% sucrose and 0.7% agar (Murashige and Skoog 1962). Following the previous study in *L. usitatissimum* (Basiran et al. 1987), 1–2 mm sections of hypocotyl were placed on callus and shoot regeneration medium of MS with 3% sucrose, 0.7% agar, 1.0 mg l⁻¹ 6-benzylaminopurine (BAP), and 0.1 mg l⁻¹ 1-naphthalenacetic acid (NAA). After 4 weeks, efficiencies of callus formation and shoot regeneration were 100 and 70%, respectively. These results suggest that these conditions would also be suitable for the shoot regeneration of *L. perenne*. Next, we tried root induction

Abbreviations: BAP, 6-benzylaminopurine; 2,4-D, 2,4-dichlorophenoxyacetic acid; IBA, indole-3-butyric acid; MS, Murashige–Skoog; NAA, 1-naphthalenacetic acid; PCR, polymerase chain reaction.

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from the shoots. Under the condition of previous study (hormone free MS medium; Basiran et al. 1987), only 23.3% of shoots induced roots within four weeks (Table 1). Therefore, we investigated root induction conditions with 0.1–2.0 mg l⁻¹ indole-3-butyric acid (IBA). After 4 weeks, the best condition for root induction was found to be 0.3 mg l⁻¹ IBA-containing medium (80%), but callus formation (13.3%) was also observed (Table 1). On 0.1 mg l⁻¹ IBA medium, 63.3% of root induction were observed without callus formation. Based on these results, we adopted 0.1 mg l⁻¹ IBA for the root induction medium. Because multiple shoots could be obtained from a single callus, this efficiency is sufficient to obtain perfect plants from every callus.

We next tried to establish *Agrobacterium*-mediated transformation of *L. perenne*. Following the method of *L. usitatissimum* (Basiran et al. 1987), hypocotyl sections from 2-week seedlings of *L. perenne* were co-cultured for 2 h with *Agrobacterium* (pMP90) harboring vector pBI121 that had been cultured overnight and diluted 1:20 in MS medium with 3% sucrose. After 2 days of incubation on a sterile filter paper over the MS medium with 3% sucrose and 0.7% agar in the dark at 25°C, the hypocotyls were transferred to MS medium containing 3% sucrose, 0.7% agar, 1 mg l⁻¹ BAP, 0.1 mg l⁻¹ NAA, 100 mg l⁻¹ kanamycin, and 200 mg l⁻¹ carbenicillin. Although several kanamycin resistant calli were obtained within a month, no transgenic shoots were regenerated from the calli. A further two-month incubation on selection medium showed no positive effect for shoot regeneration. Therefore, we reanalyzed the selection medium. The kanamycin resistant calli were cut into small pieces and transferred to MS medium with 3% sucrose, 0.7% agar, 0.1 mg l⁻¹ 2,4-dichlorophenoxyacetic acid (2,4-D) for removing BAP and NAA from the selection medium. Two week-cultured calli were transferred to 36 kinds of selection media with combinations of 0–1.0 μM NAA and 0–10 μM BAP. The

medium containing 10 μM BAP and 0.3 μM NAA showed the best score for shoot induction (46%) after 4 weeks of incubation (Figure 1). Thus, MS medium with 3% sucrose, 0.7% agar, 10 μM BAP, 0.3 μM NAA, 100 mg l⁻¹ kanamycin, and 200 mg l⁻¹ carbenicillin was chosen for the selection medium.

To confirm whether the protocol developed for *L. perenne* transformation was reproducible, we tested four constructs encoding the florigen gene. Because *L. perenne* requires a two-month vernalization for flowering, early flowering plants through the induction of florigen would speed up the study of heterostyly and self-incompatibility. We prepared constructs encoding Arabidopsis (*FT*, Abe et al. 2005; Corbesier et al. 2007) and rice (*Hd3a*, Tamaki et al. 2007) florigen genes under control of a strong 35S promoter. Two phloem-

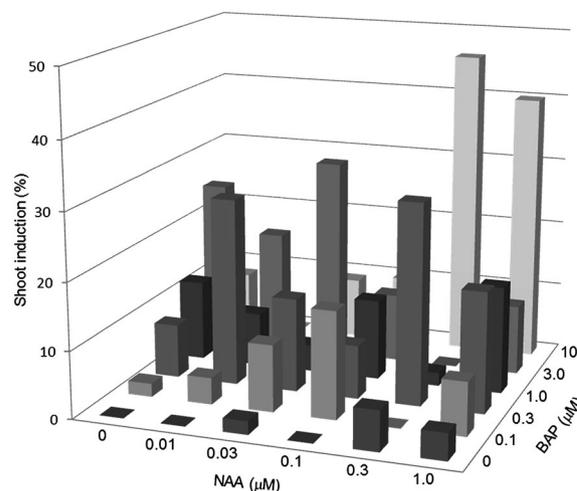


Figure 1. Efficiency of shoot induction from transgenic calli. Transgenic calli infected by *Agrobacterium* harboring pBI121 were grown on MS agar medium containing 0.1 mg l⁻¹ 2,4-D for two weeks before this experiment. Fifty calli were transferred into each medium and cultured in the growth chamber at 25°C. After 4 weeks, shoot induction was observed and is shown as percentage induced.

Table 1. Root induction of *L. perenne* from shoots.

IBA (mg l ⁻¹)	No. of explants	Root formation (%)	Callus formation (%)
0	30	23.3	0
0.1	30	63.3	0
0.3	30	80.0	13.3
1.0	30	76.6	73.3
2.0	30	40.0	96.7

Table 2. Efficiency of *L. perenne* transformation.

Construct	No. of explants	No. of shoots	Efficiency (%)
35S: <i>FT</i>	50	7	14
35S: <i>Hd3a</i>	50	10	20
<i>rolC</i> : <i>Hd3a</i>	50	2	4
<i>Rpp16</i> : <i>Hd3a</i>	50	12	24

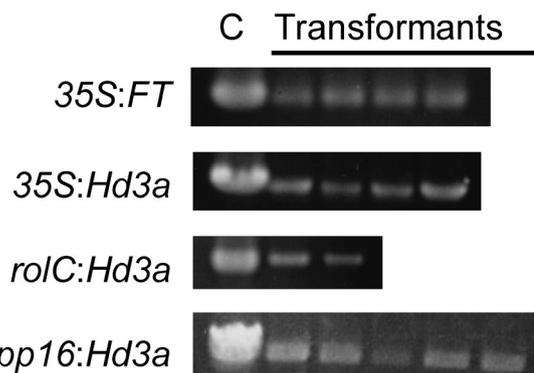


Figure 2. PCR analysis of kanamycin resistant shoots. Transgenes were amplified from genomic DNA with primers for amplifying the multiple cloning site of the binary vectors. This primer set did not amplify the band from wild type plants (data not shown). C means positive controls amplified from binary vectors.

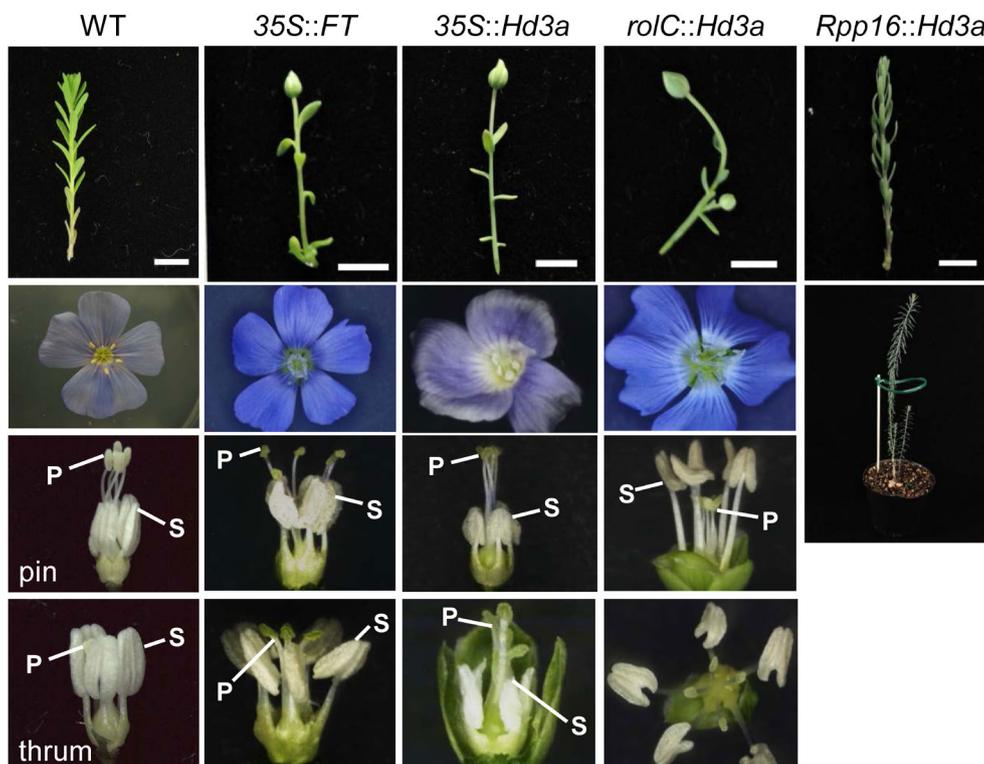


Figure 3. Phenotypes of transgenic plants. WT means control shoot and flowers from soil-grown plants. Pin flowers have long pistils and short stamens, and thrum flowers have short pistils and long stamens. An early-flowering phenotype was observed in *35S::FT*, *35S::Hd3a*, and *rolC::Hd3a* transgenic plants. P, pistil; S, stamen. Bars indicate 5.0 mm.

specific promoters, *rolC* (An et al. 2004) and *Rpp16* (Asano et al. 2002), were also used for *Hd3a* expression. These fragments were subcloned into binary vector pBI121 (*35S::FT*) or pRI909 (*35S::Hd3a*, *rolC::Hd3a*, and *Rpp16::Hd3a*). *L. perenne* hypocotyl sections were infected by *Agrobacterium* harboring these vectors and cultured on the selection medium described above. The hypocotyls were subcultured on fresh selection medium every two weeks. For each construct, two to twelve independent kanamycin resistant shoots were obtained with an efficiency of 4–24% (Table 2). The difference in transgenic efficiency between these constructs and those used in the shoot induction experiments (Figure 1) might be due to the genetic diversity of the seeds, because *L. perenne* is an out-crossing plant. To confirm the presence of transgenes in the kanamycin resistant shoots, polymerase chain reaction (PCR) was performed using DNA extracted from leaves. The transgenes were amplified in every kanamycin resistant shoot tested, suggesting that these kanamycin resistant shoots are transgenic plants (Figure 2). All transgenic plants of *35S::FT*, *35S::Hd3a*, and *rolC::Hd3a* showed a very early flowering phenotype (Figure 3). The transgenic shoots regenerated from calli formed a bud on the top of shoots with a height of only 2–3 cm (generally 30–40 cm height on soil). This phenotype is similar to *35S::FT* transgenic lines of *Arabidopsis* (Kobayashi et al. 1999). The flowers of these transgenic plants often showed abnormal

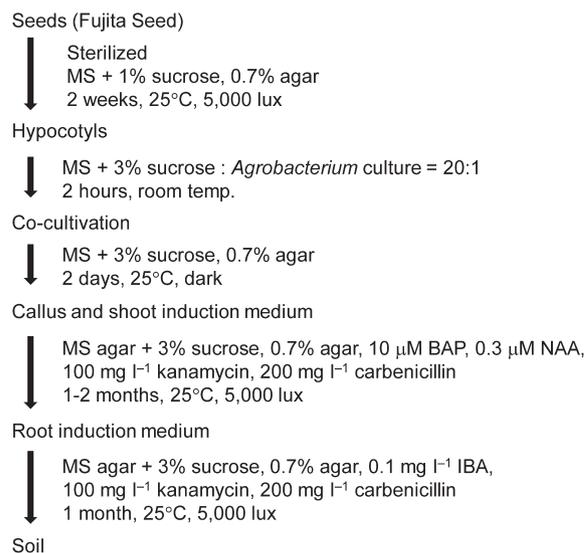


Figure 4. Summary of the method for generating *L. perenne* transgenic plants.

development such as smaller flowers, reduced number of organs, and opening of developing buds. Despite this, the pin or thrum flower type could be discriminated (Figure 3). On the other hand, *Rpp16::Hd3a* transgenic plants did not flower on the selection medium. The transgenic plants were transferred to the soil and kept in growth chamber under long day conditions at 23°C. However, only one branch of one plant showed any flowering

within three months. These results reflect the weaker promoter activity of *Rpp16*.

Here, we developed methods for tissue culture, plant regeneration, and *Agrobacterium*-mediated transformation of *L. perenne*. We show the final protocol for generation of transgenic *L. perenne* in Figure 4. In this protocol, the time of transformation from *Agrobacterium* infection to soil is 3–4 months. Three more months are needed for the transgenic plants to flower. Although florigen induction leads to abnormal flowering, pin or thrum phenotypes can be observed within 2–3 months, indicating possible application for fast screening of candidate genes involved in heterostyly. In conclusion, our transgenic protocol enables analysis of gene functions of *L. perenne* and contributes to understanding of the molecular mechanism of heterostyly in plants.

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References

- Abe M, Kobayashi Y, Yamamoto S, Daimon Y, Yamaguchi A, Ikeda Y, Ichinoki H, Notaguchi M, Goto K, Araki T (2005) FD, a bZIP protein mediating signals from the floral pathway integrator FT at the shoot apex. *Science* 309: 1052–1056
- An H, Roussot C, Suárez-López P, Corbesier L, Vincent C, Piñeiro M, Hepworth S, Mouradov A, Justin S, Turnbull C, et al. (2004) CONSTANS acts in the phloem to regulate a systemic signal that induces photoperiodic flowering of *Arabidopsis*. *Development* 131: 3615–3626
- Asano T, Kusano H, Okuda T, Kubo N, Shimada H, Kadowaki K (2002) *Rpp16* and *Rpp17*, from a common origin, have different protein characteristics but both genes are predominantly expressed in rice phloem tissues. *Plant Cell Physiol* 43: 668–674
- Basiran N, Armitage P, Scott RJ, Draper J (1987) Genetic transformation of flax (*Linum usitatissimum*) by *Agrobacterium tumefaciens*: Regeneration of transformed shoots via a callus phase. *Plant Cell Rep* 6: 396–399
- Bateson W, Gregory RP (1905) On the inheritance of heterostyly in *Primula*. *Proc R Soc Lond, B* 76: 581–586
- Corbesier L, Vincent C, Jang S, Fornara F, Fan Q, Searle I, Giakountis A, Farrona S, Gissot L, Turnbull C, et al. (2007) FT protein movement contributes to long-distance signaling in floral induction of *Arabidopsis*. *Science* 316: 1030–1033
- Darwin C (1863) On the existence of two forms, and on their reciprocal sexual relation, in the several species of the genus *Linum*. *J Pro Linn Soc Bot* 7: 69–83
- Jordan MC, McHughen A (1988) Glyphosate tolerant flax plants from *Agrobacterium* mediated gene transfer. *Plant Cell Rep* 7: 281–284
- Kobayashi Y, Kaya H, Goto K, Iwabuchi M, Araki T (1999) A pair of related genes with antagonistic roles in mediating flowering signals. *Science* 286: 1960–1962
- Lewis D (1954) Comparative incompatibility in angiosperms and fungi. *Adv Genet* 6: 235–285
- Mlynárová L, Bauer M, Nap JP, Pretová A (1994) High efficiency *Agrobacterium*-mediated gene transfer to flax. *Plant Cell Rep* 13: 282–285
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15: 473–497
- Ockendon DJ (1968) Biosystematic studies in the *Linum perenne* group. *New Phytol* 67: 787–813
- Tamaki S, Matsuo S, Wong HL, Yokoi S, Shimamoto K (2007) Hd3a protein is a mobile flowering signal in rice. *Science* 316: 1033–1036
- Vuilleumier BS (1967) The origin and evolutionary development of heterostyly in the angiosperms. *Evolution* 21: 210–226