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Influence of Day-length on Flowering of Ri-transformed *Cichorium intybus* L. Plants

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Introduction

The soil bacterium *Agrobacterium rhizogenes* induces the development of hairy roots on many dicotyledonous plant species. The infectious bacterium introduces the T-DNA region of its Ri plasmid into the host plant cell to stably transform it (see review[1]). Plants can usually be regenerated from hairy roots with the ordinary culture conditions for the given plant species. Many transgenic plants with the T-DNA(s) from Ri plasmids have been established in various species so far[1]. Such transgenic plants were observed to exhibit certain characteristics different from untransformed plants. Some of the commonly observed characteristics among the Ri-transformants are: rapidly growing and branching roots, wrinkled leaves, dwarfism, and reduced apical dominance[2]. Flowering response has also been shown to be modified in the Ri-transformants. For example, Oono *et al.* reported that Ri-transformants of a day-neutral tobacco showed early flowering when compared to the untransformed plants[3]. Thus the Ri-transformant can be a good material to study mechanisms regulating flowering. Any deviation or modification of flowering behavior could be attributed to the introduced T-DNA.

Witloof chicory (*Cichorium intybus* L. var. Witloof) is a biennial plant that requires vernalization and subsequent exposure to long-days for flowering (see review[4]). Recently, Kamada *et al.* reported that Ri-transformed chicory plants did not require vernalization for flowering[5]. In this report, we examined the influence of day-length on flowering of Ri-transformed chicory plants.

1. *Requirements for flowering of normal chicory plants in vitro*

We first studied the requirements for flowering of an untransformed chicory plant grown *in vitro*. Seeds of Witloof chicory cv. Vilmorin No. 5 (Vilmorin, France) were surface sterilized by a brief soaking in 70% ethanol followed by soaking in 1% sodium hypochlorite solution for 15 min. The

Table 1. Influence of vernalization on bolting of normal chicory seedlings *in vitro*.

Experiment	Light regime	No. tested	No. bolted*1
without vernalization			
1	continuous LD	5	1
2	continuous SD	5	0
3	SD 4 weeks→LD	5	1
with vernalization			
4	SD 2 weeks→V*2→LD	5	0
5	SD 4 weeks→V*2→LD	5	3
6	SD 6 weeks→V*2→LD	5	3
7	SD 8 weeks→V*2→LD	5	4

*1 scored at month 4 after sowing.
*2 vernalization (2 weeks at 4°C in darkness).

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seeds were then cultured on Murashige and Skoog's (MS) medium[6] solidified with 0.2% gellan gum in a 9 cm petri dish for germination. Two-week-old seedlings were then transferred to a 900 m*l* culture bottle containing 200 m*l* of MS solid medium. Chicory seedlings germinated in the short-day condition (8 h photoperiods, 20,000 lx, 25°C) were exposed to 4°C for two weeks in darkness at various times after sowing, then transferred into the long-day condition (16 h photoperiods, 20,000 lx, 25°C). Bolting was observed four months after sowing from seedlings vernalized when they were at least two weeks old (experiments 4 to 7 in **Table 1**). These stalks flowered after subculturing them to a new culture bottle. Although the number of seedlings used and replication were insufficient and bolting was observed from few seedlings that were not vernalized (experiments 1 to 3 in **Table 1**), these results suggest that chicory requires vernalization for flowering even *in vitro*. However, occasional bolting was observed more frequently from unvernalized plants when the culture period was prolonged beyond four months (data not shown).

2. *Flowering of LD-transformant*

To determine the effect of the long-day condition on flowering of Ri-transformed chicory, we established Ri-transformants either under long-day or short-day conditions.

Hairy roots were induced with *A. rhizogenes* strain A4[7] by conventional leaf disk method[8]. Plants were regenarated from hairy roots by culturing them on MS solid medium containing 0.01 mg/*l* NAA and 2 mg/*l* BA. The regenerants were maintained in 900 m*l* culture bottles containing 200 m*l* of MS solid medium. Whenever a transformant was established under long-day condition

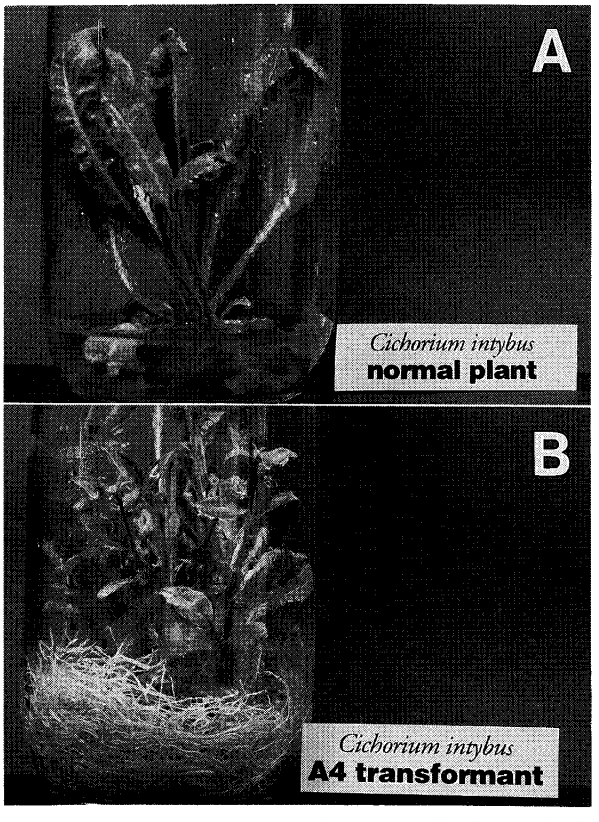

Fig. 1 Flowering of LD-transformed chicory without vernalization under long-day condition. A: An untransformed chicory plant grown under long-day condition without vernalization. B: A LD-transformed chicory plant grown under the same condition as A. Note flower stalks with bracts and well-developed roots on the LD-transformed plant.

Table 2. Influence of day-length during plant regeneration from leaves of flowering chicory on bolting of regenerants.

Mother plant	Light regime	No. tested	No. bolted*1
LD-18*2	LD	17	11
LD-18*2	SD	14	4
Normal*3	LD	12	0
Normal*3	SD	12	0

*1 scored at week 8 after the induction of regeneration.
*2 LD-transformant # 18.
*3 flowering untransformed plant.

(LD-transformant), the flowering process was initiated when the plantlet was regenerated from a hairy root (**Fig. 1**), confirming the report of Kamada *et al.*[5].

We further examined the effect of day-length on flowering of regenerants from leaves of the LD-transformant. To induce adventitious buds, leaf disks (*ca.* 1 cm square) were cultured on MS solid medium containing 0.01 mg/*l* NAA and 2 mg/*l* BA. Regenerants from a flowering LD-transformant (LD-18) were induced and grown either under long-day or short-day conditions without vernalization, and bolting of the regenerants was scored at week 8 after the induction of regeneration. There were more plants bolted to flower under the long-day condition than under the short-day condition (**Table 2**). When regenerants were induced from leaves of a flowering, untransformed plant, however, no bolting was observed under either light regime (**Table 2**). On the other hand, Harada reported that plants regenerated from flower stalks of untransformed chicory flowered under long-day condition without vernalization[9]. These imply that the flowering stimulus in the untransformed plant did not remain in the leaves but remain in the flower stalks, whereas the stimulus in the LD-transformant remained or was produced even in leaf segments.

3. *Flowering of SD-transformant*

We then established a Ri-transformed chicory plant that had never experienced a long-day condition nor vernalization. To achieve this, we cultivated a mother plant and induced a hairy root on it; a Ri-transformed plant was then regenerated from the hairy root as described in the last section. All these were done under short-day condition at 25°C. For unknown reasons, however, only one hairy root line could be isolated under the short-day condition after repeated infection experiments.

The short-day transformant (SD-transformant, SD-1) did not flower for at least four months after regeneration (**Fig. 2**) and this duration was sufficient for the LD-transformants to form flowers. When the SD-transformant was placed under the long-day condition, bolting occurred within 7 weeks (**Fig. 2**) and flowers were observed. The plant continued to flower even when it was transferred back to the short-day condition (data not shown).

4. *PCR and Southern analyses*

Finally, genomic DNA extracted from the LD-transformants, the SD-transformant and an untransformed plant were analyzed by PCR amplification for the TL- and the TR-DNA sequences. Genomic DNA was extracted from 10 g fresh weight of leaf tissue of a transformed or a control plant according to the method of Paszkowski *et al.*[10]. One hundred ng of the DNA were used as the template for a PCR reaction. Primers (rolBAS and rolCAS) for the amplification of an internal part of the TL-DNA of pRiA4b were prepared according to Handa[11]. For the amplification of the TR-DNA, primers (Com-ags and Com-mas 1') with sequences 5'-CTCTGGGCCTGCAAGGATGC-3' and 5'-ACGAACATCGGTCTCAATG-3', respectively, were designed and synthesized in this

Fig. 2 Effect of day length on flowering of SD-transformed chicory plants.
Two shoots separated from a SD-transformant and cultured under short-day (left) or long-day (right) conditions for 24 days. Note the flower stalk (indicated by open arrowhead) on the LD-grown plant.

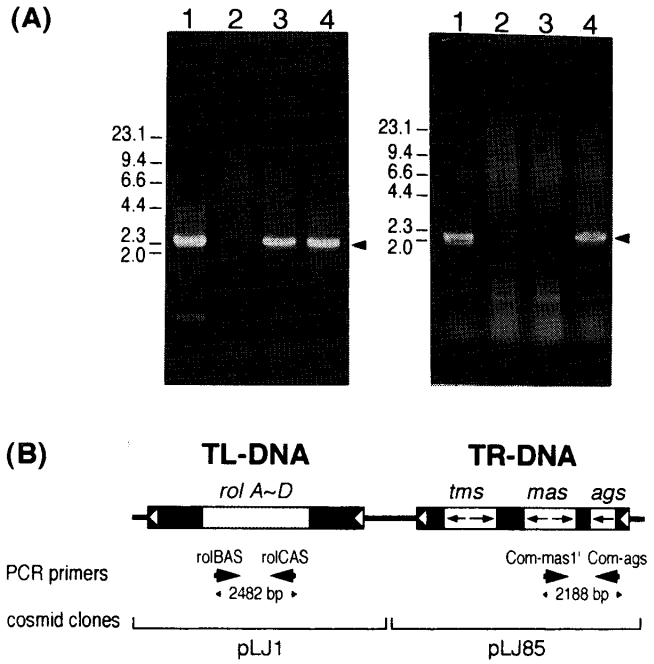

Fig. 3 PCR amplification of T-DNA sequences in Ri-transformed chicory plants.
A: Ethidium bromide-stained gels for the TL- (left panel) and the TR- (right panel) DNA fragments. For both panels, lane 1, cosmid clones containing the TL- and the TR -DNA, respectively; lane 2, control (untransformed) plant; lane 3, SD-transformant SD -1; lane 4, LD-transformant LD-18. B: Schematic diagram for the amplification of a 2382 bp TL- and a 2188 bp TR- DNA sequences. Shaded area indicates the core T-DNA region containing *rolA~D* loci. *tms, mas* and *ags* are genetic loci coding for auxin, mannopine and agropine synthase, respectively. Arrows in the T-DNA diagram indicate genes in each locus. rolBAS and rolCAS, and Com-mas 1′ and Com-ags are primer pairs to amplify the TL- and the TR-DNA fragments, respectively. pLJ 1 and pLJ 85 are cosmid clones containing entire TL-DNA and TR-DNA, respectively, of pRiHRI[12].

work. The PCR reaction was carried out with the following program; preheating at 94°C for 10 min., 30 cycles of 94°C for 1 min., 55°C for 1 min. and 72°C for 2 min., and final elongation at 72°C for 7 min. A portion of the amplified DNA was separated by the agarose gel electrophoresis and stained with ethidium bromide to be photographed.

Both the TL- and the TR-DNA sequences were detected in all LD-transformants tested, whereas only the TL-DNA sequence was detected in the SD-transformant SD-1 (**Fig. 3**). Those amplified sequences were further confirmed to be the T-DNA sequences by Southern hybridization (data not shown).

As the SD-transformant carried the Ri TL-DNA was able to flower under the long-day condition without vernalization, it implies that gene(s) on the TL-DNA was(were) responsible for the altered flowering response of the Ri-transformants. Earlier report by Kamada *et al.*[5] revealed that a transformed chicory with only the *rolC* gene could flower without vernalization.

Conclusion

In this study, we first demonstrated that Ri-transformed chicory established under long-day condition could undergo the complete flowering process with no need of vernalization, which was required by untransformed seedlings *in vitro* as known for field-grown plants. The result is consistent with that reported by Kamada *et al.*[5]. Furthermore, our results demonstrated that flowering of Ri-transformed chicory was, to certain degree, influenced by the long-day condition based on the following observations: 1) transformant established completely under the short-day condition did not flower; and 2) short-day reduced the flowering response of shoots regenerated from LD-transformant when compared to that of the long-day.

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