第17卷第4期 西北农业大学学报 Vol.17.No.4
1989年12月 Acta Univ. Septentrionali Occident.Agric. Dec.,1989

High Transformation Frequency of Brassica Crops with Agrobacterium rhizogenes

HE YUKE

(Department of Horticulture, Northwestern Agricultural University, Yang-Ling, Shaanxi, China)

ROEIAND E. VOORRPS

(Institute for Horticultural Plant Breeding, Wageningen, The Netherlands)

ABSTRACT

Genetic transformation by *Agrobacterium rhizogenes* was carried out using hypocotyl segments of cabbage, cauliflower, Brussels sprouts and oilseed rape. Among these plant genotypes, cauliflower exhibited the highest root induction frequency whereas cabbage showed the highest transformation frequency. Bacterial strain LBA 9402 induced more roots than strains LBA 9365 and LBA 8490. The culture of sequential segments along the full length of the hypocotyl showed that there was a gradient of increasing root induction frequency from cotyledons to the root.

Transformation frequency was remarkably raised when 1-2 mm top parts of segments with all roots still attached were transferred onto the solid medium before individual roots on segments were transferred.

Key words: *agrobacterium rhizogenes*; *brassica*; root induction frequency(RIF); transformation frequency(TF)

1 INTRODUCTION

Genetic transformation of T-DNA fragments from *Agrobacterium rhizogenes* to dicotyledonous plants has been developed into routine procedures in which hypocotyl segments of plant seedlings are widely used. With these procedures, roots are induced on the inoculated segments and then individually

本文于1989年3月13日收到。

explanted to soild medium for hairy root growth (Shahin 1986) .Usually,only long roots (1-2 cm in length) from segments are transferred as root initials onto soild medium (David and tempe 1988) , and most of induced roots which are too short are difficult to explant or fail to grow further after explanting. In this way, many short induced roots including transformed roots are abandoned or lost in subculture, which makes it impossible to evaluate TF under different conditions and methodically reduces TF. For estimation of TF, it is necessary to modify the routine procedures to maintain as many transformed roots as possible to grow further at the following stage.

In recent years, Brassica crops such as cabbage, cauliflower, oilseed rape and turnip have been transformed with *A.rhizogenes* to study the morphological responses of T-DNA expression in plants (Guerche *et al* 1987, Jouanin *et el* 1987, Ooms *et al* 1985, Tanaka *et al* 1985) and infection of club root (*Plasmodiophora brassicae*) to hairy roots *in vitro* (Mugnier 1986) . In these experiments, different strains of *A.rhizogenes* were used to inoculate seedlings of different ages. No measurements of RIF or TF are made under the conditions used. Therefore, it is difficult to know which bacterial strain or what kind of hypocotyl segments is most suitable for obtaining high TF.

The objective of the present study was to find the reliable way to evaluate RIF and TF, and simultaneously to deal with some important aspects of high frequency of transformation with *A. rhizogenes*.

2 MATERIALS AND METHODS

2.1 Bacterial strains *Agrobacterium rhizogenes* wide type strains LBA 9402 (agropine type) , LBA 9365 (mannopine type) and LBA 8490 (cucumopine type) were used. Bacteria from fresh colonies were transferred to YMB liquid medium (Ooms *et ai* 1985) and cultured for two days at 28℃.

2.2 plant genotypes Four cultivars were selected for transformation. Septa (cabbage, *Brassica oleracea L.var.capitata*) ,Cervina(cauliflower, *Brassica oleracea L. var. botrytis*) , Vrosa (Brussels sprouts, *Brassica oleracea var. gemmifera*, and Toria (oilseed rape, *Brassica napus* L.) .

2.3 Seedling management The seeds of four cultivars were sterilized in 2% w/v sodium hypochlorite solution for 20 minutes by three rinses in sterilized water and sown on MS medium without vitamins, inositol and growth regulators. Hypocotyl segments were cut off from the seedings after 7,14, 21 and 28 days.

2.4 segment origin Hypocotyls of two-week-old seedlings were cut into 3-5 segments in sequence. Each segment was 10-15 mm in length. In this way, three types (for cabbage) and five types (for rape) of segments were used; S1 segments were nearest to hypocotyl/root junctions and S3 segments for cabbage or S5 segments for oilseed rape were nearest to the cotyledons.

2.5 Segment incubation All segments from the seedlings of different ages were placed upside down on MS medium in glass pots and incubated at 25°C after the attachment of a droplet of *A. rhizogenes* suspension (2μl) on the top of segments. After two days the segments were transplanted on MS medium supplemented with 250mm/l vancomycine and incubated at 25 °C in the light (4000 lux) with 16 h daylength.

2.6 Root transfer 20 days after inoculation, the induced roots were transferred from the segments to petri dishes in two ways: (1) direct transfer: Individual roots (5mm or longer) were directly dissected from the segments, transferred into petri dishes and cultured on MS medium containing 100 mg/l cefotaxime and 100 mg/l vancomycine; (2) top transfer: The top parts (1-2mm in length) of rooted segments with all induced roots attached were cut off and transferred into petri dishes, and then cultured on MS medium supplemented with 100mg/l vancomycine for 10 days. Subsequently the roots were cultured separately as described for roots directly transferred.

2.7 Transformation freguency Number and length of induced roots were determined every five days. Agropine and mannopine were measured (Jung and Tepfer, 1987) to identify transformed roots after the fourth transfer.

In all experiments except where indicated otherwise, the bacterial strain used was LBA 9402, the original transformation method was top transfer, and the seedling age was two weeks.

3 RESULTS AND DISCUSSIONS

The first roots developed on the top surface of hypocotyl segments of cauliflower five days after bacterial inoculation, on explants from cabbage and Brussels sprouts after seven days, and of oilseed rape after 10 days. After their first appearance of roots, the number and length of induced roots increased rapidly on segments.

When the segments were cultured longer, top parts of most segments turned brownish and tips of some induced roots wilted. Also, growth of Agrobacteria occurred on the medium around segments.

3.1 Root Transfer

Top transfer involved transfer of a part of the segment where the indu-

ced roots were attached. These top parts gave rise to the remarkable increase in RIF (number of induced roots per segment) and TF (number of transformed roots per segment)(table 1) . TF in the case of top transfer was 3-6 times higher in all tested genotypes. In order to compare top transfer with direct transfer, every root longer than 5 mm was counted.

Individual roots (root initials) quickly resumed growth on new medium when they were longer than 10mm. The roots shorter than 5mm needed more time to resume growth or ceased growth. On the average, the roots available for direct transfer were shorter than 5mm so that most of them did not grow well. The roots which had grown for another 10 days after top transfer were much longer (10mm or longer) and so exhibited better growth in subculture. From this point top transfer just rescued those short roots not available for transfer and made them grow longer enough to transfer.

Table 1. Transformation frequency of segments after direct and top transfer of induced roots on segments *

genotype	first transfer	root induction frequency	transformation frequency	mean root length (mm)
cabbage	direct	3.8	0.40	4.39
	top	3.6	2.50	16.49
Brussels sprouts	direct	2.5	0.22	8.09
cauliflower	direct	4.2	0.25	4.70
	top	4.4	1.30	14.25
rape	direct	0.8	0.09	2.91
	top	0.5	0.30	14.02

* 1. A minimum of segment number per treatment is 36 for cabbage and 15 for any of other genotypes respectively.

2. Mean root length is the average value of all inbuced roots (1mm or longer) on segments for direct transfer or of all roots on top parts of segments for top transfer.

The roots differentiated during culture of the whole segments were used to determine TF. With top transfer the original roots formed on whole segments could grow longer before they were transferred. In practice, besides those original roots, many new roots appeared during the subsequent culture of segment top. If these newly formed roots are considered, actual number of induced roots or transformed roots will be larger.

After top transfer, the roots attached on segment top continued to grow on the fresh medium while individual roots need several days to resume their growth. Meanwhile, because the cut surface of segment top and the roots on

it were both in contact with the medium, the roots were easy to absorb more nutrients for their growth. So, these roots exhibited faster growth on fresh medium.

Using top transfer,shoots were easily formed on the segment top, particularly after 10 days of culture. On the medium containing high concentration of antibiotics (500 mg/l cefotaximum), more shoot buds were observed but root growth was highly inhibited. In contrast, roots grew faster on the medium containing low concentration of antibiotics (100 mg/l vancomycine) , but growth of *A. rhizogenes* became visible after 10 days of culture. Therefore, the period for the culture of segment top was defined to be 10 days. At second transfer, high concentration of antibiotics was required in the medium to eliminate the bacteria inside root cultures.

3.2 Plant genotypes

Of the different plant genotypes used, the segments of cauliflower produced the most roots. In contrast to this, the segments of cabbage exhibited a lower RIF,but TF was much higher. The untransformed roots grew slowly or stopped growing in subculture as David and Tempe(1988) observed.

Segments of oilseed rape reacted slowly and produced the lowest number of induced roots. Top transfer or direct transfer was performed 20 days after bacterial inoculation. The segments of oilseed rape were still inducing roots. So, if the whole segments had been cultured longer before root transfer, RIF and TF of oilseed rape would have been slightly higher.

Transformed roots from all four genotypes showed fast growth duing the following culture. However, the difference in hairy root phenotype were observed between plant genotypes. Hairy roots from cabbage and oilseed rape displayed a pronounced and typical phenotype: increased lateral root formation and partial lose of geotropism. Lateral roots from cabbage roots were much thinner than those from hairy roots of oilseed rape. On the other hand, transformed roots from cauliflower failed to show such hairy root phenotype. Usually, these roots elongated, and differentiated less lateral roots. David and Tempe(1988) also noticed the disappearance of hairy root phenotypes in transformed roots of cauliflower.

3.3 Bacterial strains

Among three wild type bacterial strains used, LAB 9402 (agropine type) incited the most roots on infected segments of cabbage (table 2) . This result suggested that agropine strain was much more virulent on *Brassica oleracea*. The roots initiated by LBA 9365 or LBA 8490, even if transformed, displayed a lower growth rate and poorer lateral root formation during subcu-

ltures. Partial loss of geotropism was observed on the transformed roots incited by LBA 9365.

In their experiment, Petit and co-workers (1983) found that agropine strains (A4, 15834) were the most virulent ones on some Brassica crops. The result of the present work supports this conclusion. Auxin genes, which play an important role in root induction, have been located on agropine plasmids. but not on mannopine plasmids (White *et al*. 1987). Cardarelli and his colleagues (1987) noticed that mannopine and cucumopine type *A.rhizogenes* strains are naturally devoid of auxin genes. In our present experiment, high RIF of segments infected by agropine strain LBA 9402 may be associated with the involvement of auxin genes existing on the plasmids of this strain. Meanwhile, low growth rate and poor lateral root formation as well as low RIF of segments infected by mannopine strain LBA 9369 and cucumopine strain LBA 8490 may be related to the defects of auxin genes in the plasmids of these two strains.

Table 2. The effects of bacterial strains on transformation frequency of cabbage segments *

strains	percentage of rooted segments (%)	root induction frequency	transformation frequency
LBA 9402	92	3.6	2.5
LBA 0365	40	1.5	1.1
LBA 8490	36	1.2	0.8

* 1. A minimum of number of segments infected by each strain were 36.
2. Cucumopine was not measured, and the transformed roots induced with LBA 8490 was determined according to hairy root phegotpye.

3.4 Seedling age

RIF of cabbage segments decreased with seedling age from 2 to 5 weeks(figure 1). However, the segments from the seedlings aged 1-3 weeks exhibited nearly the same potential of root differentiation. Like cabbage, the segments from cauliflower and oilseed rape showed higher RIF at seedling age of 1-3 weeks.

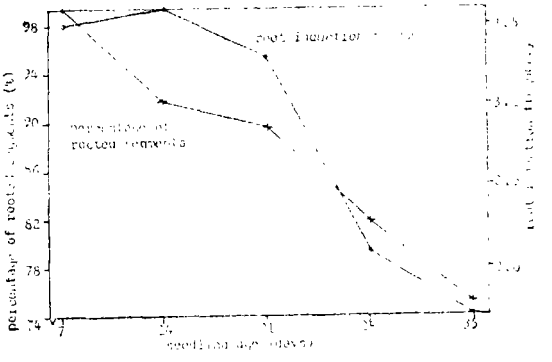

Figure 1 Root induction of hypocotyl segments from cabbage seedlings of different age

Root induction of infected segments from differently aged seedlings was associated with bacterial concentration or applied volume of inoculum suspension. The segments from old seedlings (older than 4 weeks) differentiated

fewer roots, particularly when high concentration of bacterial suspension was used for inoculation. Occasionally, the infection sites of these segments turned brown or total segments became watery. These changes apparently prevented root formation and root growth. It is possible for the segments from old seedlings to differentiate more roots by modifying bacterial concentration.

Comparatively, the segments from young seedlings (three weeks or younger) were not so sensitive to concentration of bacterial suspension to some certain extent. Therefore, efficient transformation is easily obtained working with young seedlings.

3.5 Segment origin

Root induction was generally favoured by using the segments nearest to the root / hypocotyl junction(figure 2) . But this does not mean that the segments nearest to the junction produced more transformed roots than those away.

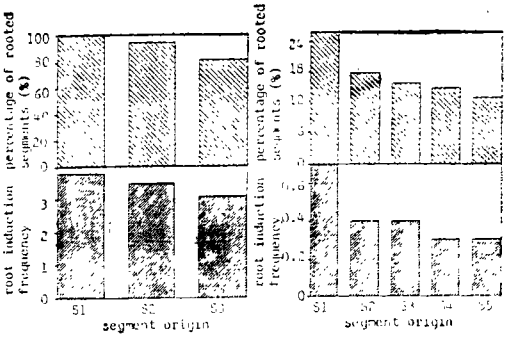

Figure 2. Root induction of hypocotyl segments of different origin from cabbage (left) and oilseed rape (right)

4 CONCLUSION

Top transfer has offered the opportunity to increase number of transformed roots and to determine TF under various conditions.

For transformation of Brassica crops with *Agrobacterium rhizogenes*, high transformation efficiency of hypocotyl segments can be obtained using the top transfer procedure described in this paper. Agropine strains of bacteria appear to be more efficient in this respect than mannopine or cucumopine strains. And the segments for transformation can be dissected from 1--3 week-old seedlings.

Acknowledgement

we thank Dr Th Kramer and Dr. F. van der Mark for their helpful suggestions in the accomplishment of the experiment. we also thank Dr. J. J. W. Dons for his critical remarks to the manuscript.

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发根土壤杆菌 (*Agrobacterium rhizogenes*)在芸薹属作物上的高效遗传转化

何玉科

(西北农业大学园艺系)

R. E. Voorrips

(荷兰园艺植物育种研究所)

摘 要

利用发根土壤杆菌 (*Agrobacterium rhizogenes*) 在甘蓝、花椰菜、抱子甘蓝和油菜的下胚轴切段上进行遗传转化。在这些植物基因型中，花椰菜的转化培养获得了最高的根诱导频率，而甘蓝的转化培养得到了最高的转化频率。同菌株LBA9365和LBA8490相比，发根土壤杆菌LBA9402诱导的根数较多。沿着下胚轴依次截取切段进行转化培养，结果发现从子叶下切段到根系上面的切段，其根诱导频率呈阶梯状上升趋势。在切段上的单根转移培养之前，截取下胚轴切段（此切段仍保留有所有的根）上1～2mm 长的顶端部分，转移到固体培养基上培养，结果大大提高了转化频率。

关键词：发根农杆菌；芸薹属；根诱导频率；转化频率