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Optimal Conditions for Transformation of Brassica Crops with *Agrobacterium rhizogenes**

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Abstract Light and temperature have had an important effect on *in vitro* genetic transformation of cabbage, cauliflower and oilseed rape by *A.rhizogenes*. In the light more transformed roots were induced on hypocotyl segments than in dark. In contrast to this, the *hairy root* initials in the dark produced more, longer lateral roots and larger hairy root lines than those in the Light. With cabbage segments, the highest transformation frequency was attained at 25°C whereas the highest relative growth rate of hairy roots was reached at 28°C. Growth rate of hairy roots was not affected by pH in the range 5.2.-6.7 but some outgrowth of *A.rhizogenes* occurred when pH was above 6.2. This pattern of environmental responses makes it possible to divide such transformation procedures into two stages: a root induction stage and a root growth stage.

Subject words *Agrobacterium*, *Brassica*, temperature/*Agrobacterium rhizogenes*, genetic transformation

0 Introduction

Agrobacterium rhizogenes is able to insert a fragment of special DNA (T-DNA) into the genome of dicotyledonous plants, giving rise to the initiation and rapid growth of hairy roots. Despite a considerable amount of work on biochemical and genetic aspects of transformation, little is known about the effects of environmental factors such as temperature, light and pH, which certainly contribute to the integration and expression of T-DNA in plant cells.

Hairy roots induced in this way can be used to study plant resistance to obligate pathogens[1-2]. In recent years, transformed roots have been established from Brassica crops such as cabbage, oilseed rape, cauliflower and turnip[2-5]. An attempt was made to infect transformed roots of cabbage with *plasmodiophora brassicae* which causes clubroot disease[6]. All the transformations of Brassica

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crops were carried out in the light and no mention was made of the effects of a range of environmental factors on transformation frequency, as seen in many experiments of tobacco and tomato transformation.

In an effort to establish hairy root lines for the study of clubroot disease resistance, we used hypocotyl segments of some Brassica crops to perform genetic transformation mediated by *A.rhizogenes* under different conditions.

1 Materials and Methods

Wild type *A.rhizogenes* LBA 9402(agropine type)was cultured at 28℃ for 2 days on YMB liquid medium[2].

Three cultivars were used in the experiment: Septa (cabbage, *Brassica oleracea* L.*var.capitata*), Toria (oilseed rape, *B.napus L.*)and Cervina (cauliflower, *B.oleracea L.var.botrytis*).Seeds were sterilized in sodium hypochlorite by three rinses in sterilized water and sown on MS medium (Murashige and Skoog 1962)without vitamins, inositol and growth regulators. Segments were excised from hypocotyls of 2-week-old seedlings and placed upside down on MS20 medium(MS with 20 g/L sucrose)in glass pots. Inoculation with *A.rhizogenes* was performed by applying 2 μL bacterial suspension on the cut surface of each segment. The inoculated segments were cocultivated first on MS20 medium for two days and then on MS medium supplemented with 250 mg/L vancomycine. Some of glass pots containing the inoculated segments were placed in the light and the others in the dark. Temperatures for the cocultivation were 20, 25 and 28℃. Segments which were not inoculated were taken as controls.

Twenty days after inoculation, 1-2 mm of the top part of the segment, with all induced roots on it, was cut off carefully with scissors and explanted onto MS solid medium supplemented with 100 mg/L vancomycine. This is regarded as the first transfer. After 10 days, the second transfer was carried out by explanting all individual roots separately onto MS medium containing 250 mg/L vancomycine. From the first transfer on, the roots were continuously cultured in various combinations of environmental factors: the total dark or light of 2.7w/m^2 with 16 h daylength; temperature of 20, 25, 28 or 30℃; medium pH of 4.7, 5.2, 5.7, 6.2, 6.7 or 7.5 which was measured after agar was dissolved in the medium.

The number and length of roots arising from segments or root initials were determined every five days. Relative growth rate of hairy roots was assayed using length of roots instead of the weight as is usual[7]. Agropine and mannopine in hairy roots were determined to identify transformed roots[8].

2 Results

It is useful to divide the transformation procedures into two stages: (1)root induction stage during which the inoculation with *A. rhizogenes* and incubation of segments are carried out, and (2)root growth stage during which the induced roots are explanted and transferred for subculture.

2.1 Root Induction Stage

Although the first roots were seen on the infected sites of segments at different time, the whole root induction stage for the varieties of *Brassica oleracea* was about 20 days. During this stage, exposure to light and different temperatures influenced the number of rooted segments of induced roots and of transformed roots(table 1). Segments from cabbage and cauliflower gave rise to more roots in the light than segments in the dark. In the light were a higher percentage of rooted segments, higher root induction frequecy (number of induced roots per segment) and higher transformation frequencny (the number of transformed roots per segment). This was not the case with oilseed rape.

Table 1. Root induction frequency of segments from some Brassica crops when incubated in the light or in the dark

genotype	treatments	percentage of rooted segments (%)		root induction frequency		transformation frequency	
		light	dark	light	dark	light	dark
cabbage	inocula.	92	86	3.6	2.9	2.5	0.7
	control	2	33	0.1	0.1	0	0
rape	inocula.	17	58	0.5	1.5	0.3	0.2
	control	14	50	0.2	1.3	0	0
cauli-	inocula.	100	41	4.4	0.8	1.3	0.5
flower	control	13	33	0.1	0.3	0	0

1. Segments were incubated at 25℃.
2. A minimum of number of segments per treatment was 36 in the light, 26 in the dark and 15 for controls respectively.
3. Measurement was made 20 days after inoculation with *A. rhizogenes*.

It should be noted that some control segments were also able to differentiate roots. The control segments from cauliflower and oilseed rape differentiate more roots than those from cabbage. Such root formation of control segments from all genotypes was apparently prohibited in the light.

Usually, only one or two roots were observed on one control segment

if this segment differentiated root(s). In contrast to this, segments inoculated with *A.rhizogenes* usually differentiated more than three roots if they were induced. Among these inoculated segments, some exhibited a high capacity of root differentiation, with more than 30 roots per segment. Inoculated segments also produced much more untransformed roots than control segments. The untransformed roots differed from transformed roots in growth behaviour. They grew rather slowly or even stopped growing, and seldom differentiated lateral roots and lost geotropic responses.

When temperature was raised from 20 to 25℃, there was a large increase in percentage of rooted segments, in root induction frequency and in transformation frequency(tabale 2). A further increase in temperature only had a minor influence. If the segments were incubated at 30℃ in the dark, only 15% produced roots at very low root induction frequency (0.3). In the dark, the optimum temperature for root induction of cabbage was about 25℃. The highest transformation frequency was obtained in the light.

Table 2. Root induction frequency of cabbage segments when incubated at different temperatures.

temperature (℃)	percentage of rooted segment (%)		root induction frequency		transformation frequency	
	light	dark	light	dark	light	dark
20	45	50	1.0	1.0	0.5	0.1
25	92	86	3.6	2.9	2.5	0.7
28	82	45	2.5	1.0	0.9	0.2
30		15		0.3		0.1

•Measurement was made 20 days after inoculation with *A·rhizognes*.

In the dark, all inoculated segments lost their green color and progressively turned brownish during root induction stage. This became more pronounced at higher temperature. However, control segemets lost their green color, but did not turn brownish. The inoculated segments lost green color faster than control segments.

2.2 Root Growth Stage

Transformed roots grew faster in the dark than in the light (figure 1). Hairy root initials, when cultured in the dark, tended to produce more lateral roots than untransformed roots.

When temperature wes raised from 20 to 28℃, the significant increases

(Duncan's Multiple Range Test , data not shown)were observed in number and length of hairy roots respectively both in the dark and in the light.

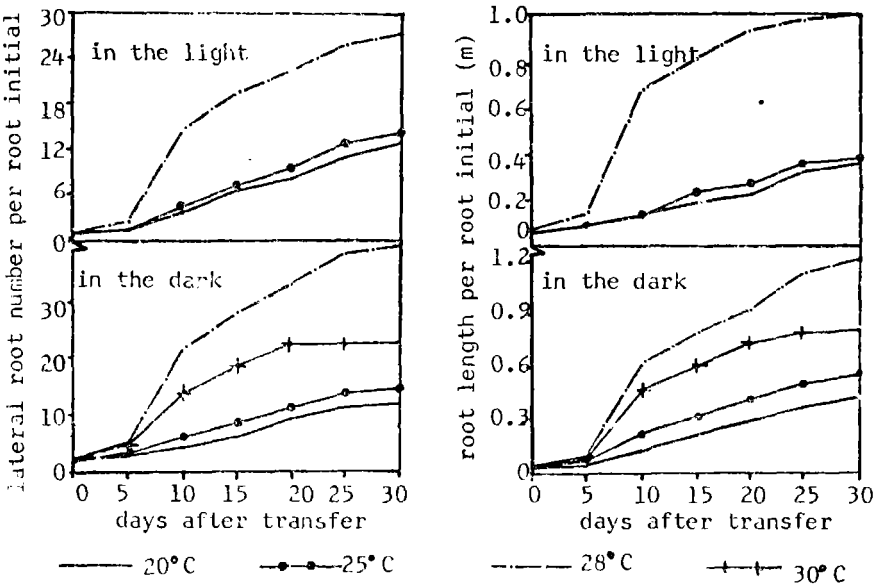

Figure 1. Growth responses of hairy roots to light and temperature during root growth period. The hairy roots used were derived from cabbage segments.

During the first 5 days, hairy roots grew very slowly. Then there wasa rapid increase both in number and length of hairy roots, particularly at higher temperature and in the dark. Growth was optimal around 10 days after transfer, and after 20 days growth of hairy roots cultured at higher temperature slowed down. Hairy roots cultured at 20℃ maintained the steady,but slow increase in number and length.

Hairy roots of cabbage reached the highest relative growth rate at 28℃ while those of oilseed rape and cauliflower did this either at 25℃ in the light or at 28℃ in the dark (figure 2).

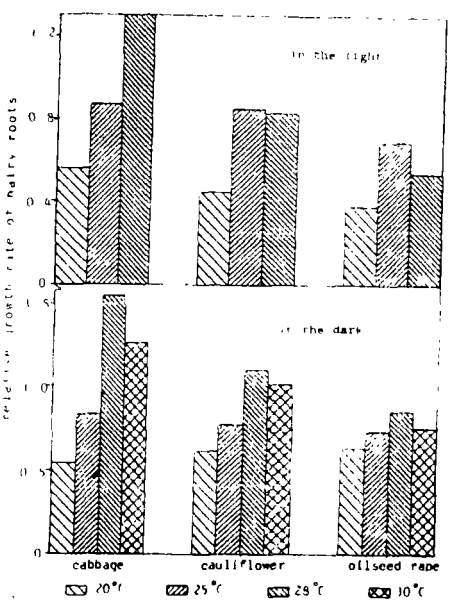

Figure 2. Relative growth rate (RGR) of hairy roots derived from three cultivars at different temperatures. It is calculated using the equation $R=(lnL_1-lnL_0)/t$ where initial root length (L_0) and final root length (L_1) are taken at time interval (t) of 10 days

No significant difference was observed in number and length of hairy roots when medium pH was changed from 5.2 to 6.7. But, hairy roots grew slowly if pH was above 7.2 or below

4.7. On the medium without antibiotics, some outgrowth of *A.rhizogenes* was found around hairy roots at pH higher than 6.2, even after third transfer. Outgrowth of bacteria was faster in the dark and with rise in temperature.

Some transformed roots exhibited extremely high relative growth rate at different temperatures. Root line B12212, cultured for 20 days at 28℃ in the dark, gave rise to 120 lateral roos with 3075 mm of total length. Root line B11235, cultured for the same period at 20℃ in the dark, produced 64 lateral roots with 1823 mm of total length. On the other hand, some root initials formed long roots with few lateral roots. Root line B14334, for example, produced only 12 lateral roots, but with 2236 mm of total length. These specific features of different lines did not change after several generations of subculture.

3 Discussion

The various procedures used to transform plants with *A.rhizogenes* are different, but they all have the same two stages in common. The first step (root induction stage) involves the infection of *A.rhizogenes* and the integration of T-DNA into plant cells which in turn give rise to initiation of transformed roots. On the other hand, the exprssion of T-DNA which causes fast growth of hairy roots is the characteristic event during root growth stage. The results in our present experiment indicate that these two stages have different requrements for temperature and light conditions.

3.1 Root Induction

Compared with control segments, inoculated segments differentiated more untransformed roots. This fact shows that the infection of *A.rhizogenes* stimulates the differentiation of both transformed and untransformed roots. Obviously, the roots from inoculated segments are a mixture of transformed and untransformed roots.

Control segments produced fewer roots in the light than in the dark. In contrast to this, exposure to light stimulates the induction of transformed roots on inoculated segments.

In the dark, the inoculated segments gradually lost their green color and turned brownish, concomitant with decreasing frequency of root induction and transformation. These changes became more visible and faster with a rise in temperature. This observation forms an indication that light plays an important role in induction of transformed roots. Possibly, the heavier infection of *A. rhizogenes*, usually at high temperature (30℃) in the dark, terminated photosynthesis of segments and damaged the original tissues to the extent which

made the segments not suitable for root induction. However, direct effects of light on the transformation process can not be excluded.

The previous research workers found that at temperature of 30℃ or higher, there was a thermosensitive phenomenon on plant tissues infected wfth *Agrobacterium tumefaciens*[9-11]. More recently, Alt-Moerbe and his collegues studied temperature-sensitive step in Ti plasmid *vir*-region induction and observed that tumour induction was temperature-dependent and induction of *vir*D2 was significantly reduced at 28℃[12]. In our present experiment, hairy root induction on segments, took the form of similar sensitivity.

3.2 Root Growth

Among transformed roots, there were the "long" type hairy root lines which had fewer, long lateral roots, and "bushy" type hairy root line which differentiated many short lateral roots. Also, large differences in relative growth rate were observed among transformed root lines. Unlike the hairy roots which had extremely high potentials in root growth, some hairy roots grew rather slowly although they were confirmed by opine measurement. These differences appeared to be stable through several transfers. One possible explanation for this diversity in root growth might be the heterogeneity among plants. If this were the case, then it would imply that all the hairy roots from the same seedling or the same segment behave similarly to each other. However, the fact is that the transformed roots from the same seedling or the same segment behaved differently, and only a few of them exhibited high potentials in root growth, while the others did not. It is clear that plant genotypic difference was not main reason for the diversity in growth behaviours between these root lines. Rather, these difference might be explained by variation in the integration and expression of the T-DNA in the plant genome. Some analysis of experiments implicate abnormal T-DNA insertoin and minus changes in the border ends of T-DNA[13, 14]. These transgenic abnormalities may affect morphorlogical features of transformed roots. Therefore, it is reasonable to deduce that the diversity in growth behaviour of transformed roots is mainly caused by the variations in expression of T-DNA. Nevertherless, temperature and light influence such variations.

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芸薹属作物在发根农杆菌介导下的最适转化条件

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摘　要　光照和温度对甘蓝、花椰菜和油菜在发根农杆菌介导下的离体遗传转化具有很大的影响。光下培养的下胚轴切段比暗中培养的下轴胚切段诱发出较多的转化根，而暗中培养的毛状根比光下培养的毛状根具有较强的侧根分生和伸长生长能力。在25°C的温度条件下，甘蓝切段的转化频率最高，然而在28°C的温度条件下，甘蓝毛状根的相对生长速度最高。在5.2～6.7的范围内，pH值对毛状根的生长速率无明显影响。根据环境条件的不同反应，可以将转化过程分为两个阶段：根诱导阶段和根生长阶段。

主题词　土壤杆菌属，芸薹属，温度/发根农杆菌，遗传转化