Genotype and procedure dependence of *Agrobacterium*-mediated transformation of *Brassica napus*

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Abstract

Various experiments to optimize shoot regeneration and transformation efficiency were carried out with the oilseed rape cvs. 'Drakkar' and 'RS306', varying phytohormone combination and concentration and *Agrobacterium* strain. Compared to the slightly modified standard protocol for hypocotyl segments (De Block et al., 1989) no higher regeneration or transformation efficiency was achieved with alternative procedures or phytohormone combinations. But differences were obtained with different cultivars in the regeneration and transformation response after co-cultivation with *A. tumefaciens*. In cv. 'RS306' a transformation rate of 12 % was obtained in comparison to cv. 'Drakkar' with 1 % by using the same gene construct. In another study in which the *Agrobacterium* strain GV3101 pMP90RK and ATHV C58C1 were compared in terms of their transformation efficiency the best results could obtained with the nopaline strain ATHV C58C1.

Key words: Genetic transformation, oilseed rape, *Agrobacterium tumefaciens*

Introduction

The amphidiploid oilseed rape (*Brassica napus*) is one of the important plant source worldwide of oil and protein, both for human consumption and for feeding livestock. It ranking third only to soybean and palm oil in global production. Thus *B. napus* is a frequent target for crop improvements, mainly focused on improving oil quality or making it herbicide tolerant. The use of genetic transformation to efficiently transfer foreign DNA into plant nuclear genome enable the breeders to overcome crossing barriers and provide valuable alternatives to those time consuming techniques available through conventional breeding. Various methods have been used for transformation, however, most transformation procedure have been carried out using *Agrobacterium* because of its relatively easy and coupled with minimal equipment costs. Moreover, the transgenic plants obtained by this method often contain a limited number of integrated transgenes (De Block, 1993), in the most cases a single copy of the transferred DNA. These advantages were the reason that *Agrobacterium*-mediated transformation has generated the establishment of a number of well-documented gene-transfer protocols using different explants. It is known that transformation efficiency varies amongst different genotypes and only a few genotypes/varieties regenerate *in vitro* at high frequency. So it is nessecary to improve the transformation procedure still further.

The factors that influence transformation efficiency can be divided into those that influence the gene transfer and those that influence the regeneration of transgenic plants from transformed cells. Amongst them, the genotype of plants and the *A. tumefaciens* strain have been found to be crucial.

In order to investigate differences to transformation efficiency we have genetically transformed the resynthesised *B. napus* line 'RS306' and the spring rapeseed cultivar 'Drakkar' with different *A. tumefaciens* strains harbouring genes for oil modification.

Material and methods

Plant material: 'RS 306' is a resynthesised high erucic and high glycosinolate line originating from reciprocal cross of *B. rapa* ssp. trilocularis ('Yellow Sarson') × *B. oleracea* conv. *botrytis* var. *botrytis* ('Super Regama'). Seeds were provided by Justus-Liebig-University of Gießen. Seeds of cv. 'Drakkar' were obtained from Norddeutsche Pflanzenzucht Hans-Georg-Lembke KG (Hohenlieth, Germany).

Bacterial strains and vectors: The *A. tumefaciens* strains GV3101 pMP90RK and ATHV C58C1 were transformed with chimeric LPAAT and KCS genes and FAD2-desaturase genes (Spiekermann, 2005) with the aim to increase the erucic-acid and the oleic-acid content in rapeseed. The binary vector is pRE1, which providing the neomycin-phosphotransferase (NPTII) gene as selectable marker. Other experiments used *A. tumefaciens* strain GV3101 pMP90RK with chitinase (Chi-VdW) and glucanase (Glu-VdW) gene for the development of resistant plants.

Transformation procedure and regeneration of transgenic plants: For genetic transformation the method of De Block et al. (1989) was applied with minor modifications. Seeds were surface-sterilized in 70 % ethanol for 2 min and further 30 min in a 3 % $NaOCl_3$ solution. The seedlings were germinated on hormone-free MS medium (Murashige and Skoog, 1962) by 25 °C in darkness and etiolated hypocotyl segments of 5-7 mm in length were cut after 6-7 days. The hypocotyl explants were co-cultivated with *A. tumefaciens* for 3 days in A3 solution. Then the hypocotyls were transferred to a callus inducing A5-medium with kanamycin (50 mg/l) as selective agent, 5 mg/l $AgNO_3$ and 6 g/l agarose. Further the medium contained carbenicillin and triacillin each of them 250 mg/l to inhibit the bacterial growth. The subculturing of hypocotyls was done in

intervals of two weeks. The regenerated shoots were removed from the hypocotyl explants and transferred to A6 medium until they appeared normal. After development of roots they were transferred to the greenhouse for further analysis.

Confirmation of transformation: The transgenic status of putative transformed plants was confirmed for studying accumulation of protein by an NPTII-ELISA assay (Agdia Incorporated, Elkhardt, Indiana, USA. Distribution: Linaris GmbH, Wertheim-Bettingen, Germany). This assay provided a clear response to the presence of the NPTII enzyme. Leaf tissues were used for homogenisation with extraction buffer to preparate the plant extract.

Results

Initial experiments on transformed rapeseed cv. 'Drakkar' with varying phytohormones in comparison to the method of De Block (1989) revealed no increase of frequency of regeneration (Table 1). The highest regeneration frequency, with up to 13 % regenerating hypocotyls, was obtained when De Block-A5 media were supplied with $AgNO_3$. Explants on medium without $AgNO_3$ turned brown or yellow and finally died.

Table 1. Regeneration and transformation efficiency of rapeseed cv. 'Drakkar' transformed with chitinase (Chi-VdW) and glucanase (Glu-VdW) gene in *Agrobacterium* strain GV3101 pMP90RK in four different MS (Murashige-Skoog, 1992) media

Gene	Medium	Number of explants	Regeneration efficiency (%)	Transformation efficiency (%)
Chi-VdW	1	500	0.0	0.0
	2	300	10.4	1.0
	3	300	6.7	2.3
	4	300	6.3	1.0
Glu-VdW	1	100	0.0	0.0
	2	675	12.6	1.9
	3	750	10.7	2.0
	4	400	12.2	1.8

1 = MS + 1.0 BAP + 0.01 GA_3 + 0.1 NAA
2 = MS + 1.0 BAP + 0.01 GA_3 + 0.1 NAA + 5.0 $AgNO_3$ (A5, De Block 1989)
3 = MS + 0.2 NES + 1.0 TDZ + 5.0 $AgNO_3$
4 = MS + 3.0 BAP + 0.2 NES + 5.0 $AgNO_3$
additions in mg/l

Following the modified protocol of De Block et al. (1989) we have co-cultivated hypocotyl segments of the *B. napus* genotypes cv. 'Drakkar' and the resynthesised line 'RS306' with the *A. tumefaciens* strain GV3101 pMP90RK. The results of the experiments revealed a difference between the two genotype regarding their regeneration response after co-cultivation and the efficiency of transgenic shoots (Table 2). In 'RS306', a mean regeneration rate of 14.1 % was obtained in comparison to cv. 'Drakkar' with 10.1 %. The highest frequency of transformation in 'RS306' was 20.4 % to 1.2 % in cv. 'Drakkar'. These results show once again, that for each genotype favourable conditions must be found.

Table 2. Rate of regeneration and transformation of *B. napus* cv. 'Drakkar' and the resynthesised line 'RS306' after co-cultivation with chimeric LPAAT and KCS genes in the *A. tumefaciens* strain GV3101 pMP90RK

B.napus-cultivar	'Drakkar'			'RS306'		
	Number of hypocotyls	Rate of regeneration (%)	Rate of transformation (%)	Number of hypocotyls	Rate of regeneration (%)	Rate of transformation (%)
GV3101 (pRE1-kan)	250	13.6	1.2	250	8.0	6.0
	175	7.4	0.0	225	11.1	8.0
	250	8.4	0.8	250	22.8	20.4
Average	675	10.1	0.7	725	14.1	11.6

Large differences in regeneration and transformation efficiency was found within the genotype 'RS306'. The regeneration efficiency ranging from 8.0 % to 22.8 % in this three experiments and transformation efficiency ranged from 6.0 % up to 20.4 %.

The use of *Agrobacterium* strain was another important factor in increasing of the transformation efficiency. Explants co-cultivated with *A. tumefaciens* strain ATHV C58C1 yielded higher efficiencies than those co-cultivated with GV3101 pMP90RK (Fig.1).

Discussion

We found that the addition of silver nitrate to the nutrient medium for control of ethylene levels was a pre-requisite for efficient shoot regeneration in all transformation experiments. The stimulating effect of silver nitrate on shoot regeneration is also reported by Zhang et al. (1998), Kuvshinov et al (1999) and Khan et al. (2003). Zhang et al. (1998) showed that $AgNO_3$ enhanced both shoot regeneration and ethylene production in *B. campestris*.

It is a well-documented phenomenon that the genotype has a strong influence on the regeneration frequencies of various tissue-culture techniques. This is also true for transformation. We reported above the high regeneration response in the line 'RS306' using *Agrobacterium*-mediated transformation. Weier et al. (1997) and Zarhloul et al. (1999) have also shown an excellent regeneration response in 'RS306'.

Special attention was given to the effect of $AgNO_3$ and genotypic variation by Akasaka-Kennedy et al. (2005) where leaf explants were examined for shoot regeneration of rapeseed.

Further we observed that transformation of shoots after co-cultivation with *A. tumefaciens* strain GV3101 pMP90RK was to be less effective compared to the nopaline strain ATHV C58C1. Charest et al. (1989) reported that the most virulent agrobacteria for *B. napus* were nopaline strains.

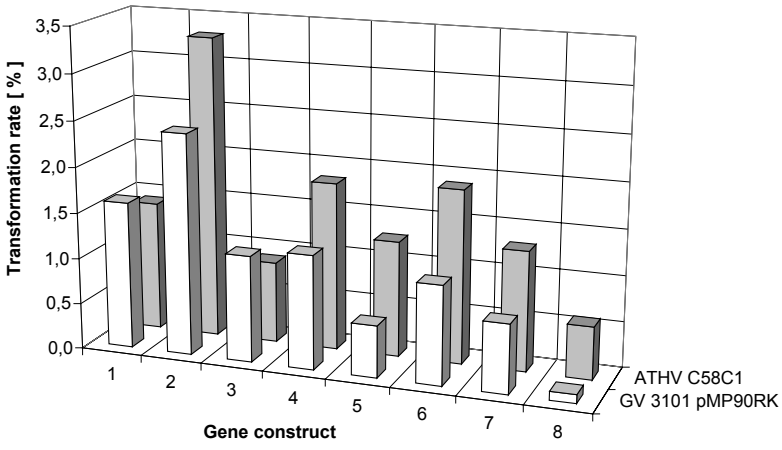

Fig.1. Influence of the *A. tumefaciens* strains GV3101 pMP90RK and ATHV C58C1 on the transformation of the spring type *B. napus* cv. 'Drakkar' co-cultivated with eight FAD2-desaturase genes

Conclusions

The results of Table1 and 2 and Fig.1 indicate the importance of the media supplements, the genotype and the virulence of different *A. tumefaciens* strains. Consequently, every given transformation protocol must be adapted to the genotype to allow the regeneration of a sufficient number of transgenic plants.

In further experiments the use of explants of haploid plants could be an alternative approach for efficient transformation of oilseed rape because with this way the selection of a good tissue culture ability is realized and at the same time homozygous plants were produced.

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