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Factors influencing the regeneration capacity of oilseed rape and cauliflower in transformation experiments

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Abstract

The efficiency of *Agrobacterium*-based transformation technique in oilseed rape and cauliflower was influenced by cultivar specificity, donor plant age and explant type. Marked differences in demands for plant hormone contents in the regeneration medium were recorded already among different types of nontransformed explants. The highest regeneration capacity was recorded with stem and leaf segments isolated from one-month-old aseptically grown plants. The regeneration was markedly species-dependent. Regeneration of transformed plants from stem segments and thin layers isolated from field-grown oilseed rape plants (at the most 2 % of regenerating explants) and from oilseed rape hypocotyls (0.8 % of regenerating explants) and cauliflower (1.2 % of explant regenerated transformed shoots) was achieved after disarmed *Agrobacterium* treatment. Hypersensitive reaction of explants could be prevented by using prolonged *in vitro* precultivation and delayed application of the selective agent.

Introduction

Brassica plants are very susceptible under natural conditions to the *Agrobacterium* infection. The formation of crown galls is induced in most cases by both nopaline and octopine *A. tumefaciens* strains (Holbrook and Miki 1985); but oilseed rape (*B. napus*) is susceptible only to nopaline strains. *A. rhizogenes* causes the formation of numerous hairy roots in infected plants. Both *A. tumefaciens* and *A. rhizogenes* were successfully used for the preparartion of transgenic plants in various *Brassica* species (Guerche *et al.* 1987, Pua *et al.* 1987, Radtke *et al.* 1988, De Block *et al.* 1989, Pechan 1990).

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Abbreviations: BAP - 6-benzylaminopurine; IBA - indole-3-butyric acid; KIN - 6-furfylaminopurine; NAA - 1-nafthalene acetic acid; MS - medium of Murashige and Skoog (1962); ZEA - trans-6-(hydroxy-3-metylbut-2-enylamino) purine; 2,4-D - 2,4-dichloroacetic acid; Claforan-cefotaxime-Na-salt

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Intact plants of various age as well as primary explants taken from various organs could be employed. However the effectivity of transformation techniques was to a considerable extent dependent, beside others, on both the genotype and the actual physiological status of the plant material.

The number of *Brassica* species cultivars which have so far been used in transformation experiments is relatively limited. As a rule, the protocols reported in the literature have been of lesser relevance for our domestic cultivars. Thus the first aim of our experiments was to compare the regeneration capacity of different cultivars and to select materials with the highest regeneration rates. For the preparation of transformed plants various transformation procedures were used to overcome hypersensitive reaction and to encourage subsequent selection of Kan^r regenerants.

Material and methods

Plants of oilseed rape (*Brassica napus* L. var. *napus* cv. Loras, Ceres, Arabela, Darmor, OP08) and cauliflower (*Brassica oleracea* L. var. *botrytis* cv. Bora, Regent, Fortuna, Biflop and Diplomat) were used as experimental material. The explants were isolated from plants of different age, *i.e.*: (*1*) 3-d-old seedlings (hypocotyls, cotyledons, leaves), (*2*) 1-month-old aseptically grown plants (stem and leaf segments), (*3*) mature field-grown plants (stem segments and "thin layers" composed of epidermis and adjoining layers of the primary cortex; these primary explants were isolated from oilseed rape stems before the opening of the first blossom, from the cauliflower inflorescence stem in three different phases: at the beginning of the inflorescence differentiation differentiation, just before opening the first flower and at the time of full flowering). Plant material detached from field cultures was surface sterilized by washing with 70 % ethanol and with soaking in 10 to 20 % Savo solution (commercial preparation of NaOCl) for 5 to 10 min. The explants were then cultured on MS medium (Murashige and Skoog 1962) supplemented with 20 g l^{-1} of sucrose and with growth regulators NAA (0.1 - 1 mg l^{-1}); IBA (0 - 1 mg l^{-1}); BAP (1 - 5 mg l^{-1}); kinetin (0 - 5 mg l^{-1}), zeatin (0 - 1 mg l^{-1}); 2,4-D (0 - 1 mg l^{-1}); in continual light (irradiance 146 μmol m^{-2} s^{-1}), at 25 °C. Suspension of *Agrobacterium tumefaciens* C58, C58Cl (pGVL 2260; pGV 941) (Deblaere *et al.* 1987) and *Agrobacterium rhizogenes* A4, precultivated in minA medium overnight to the stationary phase, were employed for transformation of plant material. For incubation (cocultivation), either freshly isolated explants or explants precultivated *in vitro* for 24 h to 7 d were used. Two systems of precultivation were tested - either direct laying of explants on the surface of agar medium or their insertion on the feeder layer of tobacco cell culture, strain VBI-0, precultivated two weeks on the V4 medium (Opatrný 1972). To eliminate *Agrobacterium* Claforan in concentration 500 mg l^{-1} was added to the medium. For the selection of transformants kanamycin sulphate at a concentration of 10 to 50 mg l^{-1} was used. Regenerated shoots were excised and rooted on MS hormone-free medium T-DNA presence in plant material was detected by Southern hybridization (Sambrook *et al.* 1989).

Results and discussion

Organogenesis in nontransformed explants: All the explant types examined showed at least a minimum bud regeneration capacity *in vitro*. The hormonal composition of the medium was the most important morphogenetic foctor inducing bud formation. The results obtained were in agreement with the general experience on the morphogenetic effect of the auxin:cytokinin ratio (Skoog and Miller 1957). But the organ origin and especially the species and cultivar specificities of the explants also participated in the actual determination of the regeneration efficiency.

Table 1. Recomended regeneration protocols for different rape and cauliflower primary explants.

Explant type	Growth regulators [mg l^{-1}]	Best regenerating cultivar of rape and percentage of regenerating explants	Best regenerating cultivar of cauliflower and percentage of regenerating explants
Cotyledons*	0.1 IBA + 5.0 BAP	Darmor 23 % Arabella 20 %	Bora 38 %
Primary leaves*	0.1 IBA + 5.0 BAP	Arabella 14 %	Bora 21 %
Hypocotyls*	(1d 1.0 2,4-D + 1.0 KIN) 3.0 BAP + 1.0 ZEA	Ceres 26 % Arabella 21 %	Bora 62 %
Stem segments**	4.0 BAP + 0 5 NAA	Arabella 85 % Ceres 82 %	Bora 89 % Fortuna 80 %
Leaves**	4.0 BAP + 0.5 NAA	Arabella 49 % Darmor 50 %	Bora 31 %
Stem segments***	1.0 BAP + 0.5 NAA	OP.08 23 %	Bora 80 %
Thin layer***	1.0 BAP + 0.5 NAA	OP.08 32 %	Bora 37 %

* of seedlings
** of 30-d-old aseptically cultivated plantlets
***of field plants

Oilseed rape and cauliflower seedlings (1 to 2 weeks-old) represent a suitable experimental material, because they can be obtained at any time throughout the whole year. Explants derived from *Brassica* cotyledons and hypocotyls can regenerate well according to literature data (Singh 1981, Jain *et al.* 1988). However, we achieved a fair degree of regeneration with the cultivars employed by us only after a two-step cultivation of hypocotyl segments (1 mg l^{-1} of 2,4-D + 1 mg l^{-1} of kinetin for one week and thereafter 1 mg l^{-1} of *Zea* + 3 mg l^{-1} of BAP). Cotyledon segments and primary leaf segments regenerated sporadically, similarly as hypocotyl segments cultivated only on media with BAP + IBA or BAP + NAA.

A very good regeneration capacity exhibited stem and leaf segments, isolated from one-month-old (*in vitro* cultivated) cauliflower and oilseed rape plants. The optimum concentrations of growth regulators were as follows: 0.5 mg l^{-1} of NAA and 4 mg l^{-1} of BAP.

Bud regeneration often occurred directly from surface tissues of stem cuttings without the interphase of callus formation.

A good regeneration capacity was also observed in primary culture of segments isolated from mature oilseed rape plants. But the regeneration from "thin layers" was very variable and was markedly cultivar-dependent. The optimum concentrations of growth regulators were 0.1 mg l^{-1} of NAA and 5 mg l^{-1} of BAP.

The regeneration capacity of cauliflower inflorescence stem segments proved to be considerably dependent on inflorescence development. Explants taken from young developmental stages regenerated a higher number of shoots, whereas only sporadic shoots were recorded on older ones, and beside that their viability rapidly decreased. The regeneration capacity could not be enhanced even by high cytokinin contents in the cultivation medium. The optimum concentration of growth regulators was 0.1 mg l^{-1} of NAA and 5 mg l^{-1} of BAP (Table 1).

Table 2. The natural sensitivity of hypocotyl explants to different concentrations of kanamycin sulphate (precultivation 0, 1 or 7 d). Hypocotyls were cultivated on MS medium supplemented with 1 mg l^{-1} 2,4-D + 1.0 mg l^{-1} KIN and later with 3.0 mg l^{-1} BAP + 1.0 mg l^{-1} ZEA.

[d]	Cultivar	Concentration of kanamycin [mg l^{-1}]							
		0		5		12.5		25	
		CF	BR	CF	BR	CF	BR	CF	BR
	rape								
0	Arabella	93	25	90	22	76	0	20	0
0	Ceres	95	32	91	22	89	2	11	0
0	Loras	81	45	86	13	60	0	10	0
1	Arabella	92	26	-	-	-	-	30	0
1	Ceres	95	31	-	-	-	-	35	0
1	Loras	80	42	-	-	-	-	42	0
7	Arabella	91	27	-	-	-	-	87	0
7	Ceres	97	40	-	-	-	-	91	0
7	Loras	90	38	-	-	-	-	85	0
	cauliflower								
0	Bora	98	62	12	0	5	0	0	0
0	Regent	96	63	18	0	7	0	0	0
0	Diplomat	90	59	11	0	8	0	0	0
1	Bora	98	68	-	-	10	0	-	-
1	Regent	96	61	-	-	12	0	-	-
1	Diplomat	93	62	-	-	9	0	-	-
7	Bora	89	53	-	-	49	0	-	-
7	Regent	88	59	-	-	51	0	-	-
7	Diplomat	91	57	-	-	46	0	-	-

CF - callus forming explants in percentage of total number (= 60 samples *per* variant)
BR - buds regenerating explants in percentage of used explants

A marked cultivar specificity also manifested itself in our experiments. Good regeneration capacities were recorded with explants derived from oilseed rape cv. Ceres and cv. OP.08 and cauliflower cv. Regent and cv. Bora.

Regeneration of transformants: All the explant types with good regeneration capacity were employed for transformation experiments. After the infection of stem segments isolated from field-grown plants with wild-type *A. tumefaciens* (strains C 58 and A4), either crown gall tissues (C58) or callus and hairy roots (A4) were formed, in agreement with our anticipations (Guerche *et al.* 1987, Tempe and Casse Delbart 1989).

In experiments with disarmed *Agrobacterium* C58C1 (pGVL 2260; pGV 941), such kanamycin sulphate concentrations were applied which did not yet fully inhibit the formation of callus tissues, *i.e.* 10 mg l^{-1} in case of cauliflower and 25 mg l^{-1} in case of oilseed rape. Higher concentrations inhibited the development of not only normal but also transformed shoots (see Radtke *et al.* 1988). (Table 2).

Stem segments isolated from field-grown plants, similarly as thin layers from the same material, could be successfully transformed only after their precultivation *in vitro*. No difference was observed between the precultivation with or without feeder layer. The yield of transformed shoots was low even in this case, at the most 2 % of explanted cuttings regenerated 1 to 2 shoots giving positive blott reaction. Most explants taken from one-month-old *in vitro* plantlets died owing to hypersensitive reaction. In this case, neither precultivation or delayed kanamycin application helped to overcome lethal effect of the combination of two stress factors excission shock and bacterial toxins (elicitors) (Table 3).

Table 3. The effect of the *in vitro* precultivation of primary explants on the regeneration of transformed buds. Approximately 150 explants were used for each variant. Results are presented in percentage of used explants.

Type of explant	Cultivar	Without precultivation			24 h precultivation			24 h precultivation on feeder layer of VBI 0		
		V	CF	BR	V	CF	BR	V	CF	BR
Stem segments	OP.08	11	8	0	35	27	2	39	50	0
	Darmor	12	10	0	23	24.5	0	21	18	0
Thin layer	OP.08	7	22	0	14	5	0	28	21	0.7
	Darmor	0	O	0	33	2	0	13	11	0

V - percentage of surviving explants; CF - percentage of explants proliferating callus tissue; BR - percentage of explents producing buds

Paradoxically better results obtained with seedling hypocotyls did not allow to accept hypothesis: the younger plant tissue - the higher probability of hypersensitive reaction. Hypocotyl explants cocultivated with *Agrobacterium* immediately after isolation, mostly necrotized. Callus formation occurred only sporadically on the initiation medium, and the callus tissue was not able to survive longer than 3 weeks its subcultivation on cultivation medium. But already one-day precultivation of segments increased the frequency of explants forming callus. In callus tissue transfered on the regeneration medium with BAP + zeatin, green sectors developed (in max. 5 % of the colonies) but no bud regeneration occurred. Seven-day-long

precultivation of explants on the initiation medium with 2,4-D and kinetin resulted in the formation of compact callus tissue. The explants were thereafter cocultivated with *Agrobacterium* for 48 h (maximally longer cocultivation again induced total necrosis). Cultivated for another week on fresh initiation medium containing Claforan but without kanamycin explants profitenated new callus tissues; on the other hand the presence of kanamycin induced necrotization. The explants were only thereafter transferred onto the regeneration medium containing kanamycin (concentration 25 resp. 12.5 mg l^{-1}) inhibiting the formation of buds in non transformed tissues. In this way, transformants obtained with up to 0.8 % of oilseed rape cv. Ceres explants and 1.2 % of cauliflower cv. Regent explants.

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