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Tissue culture and transformation of *Oenothera biennis*

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Abstract

Five cultivars of *Oenothera biennis* have been tested for callogenesis and organogenesis on different media. The cultivar CV3 has been transformed by *Agrobacterium tumefaciens* strain which introduces into the plant genome kanamycin resistance gene and the T-DNA *ipt* gene which causes increased levels of cytokinins. Transformed tissues showed elevated levels of cytokinins and grew as teratomas forming clumps of short, branched shoots with small modified leaves. Roots appeared rarely in later subcultivations of some teratomous clones.

Additional key words: *Agrobacterium tumefaciens*, cytokinins, regeneration.

Introduction

Evening primrose is becoming increasingly important for the production of seed oils with high proportions of unsaturated fatty acids (Hudson 1984), for the treatment of ectopic exeme and for decreasing of the level of cholesterol in the bloodstream (Horrobiun and Manku 1984).

New breeding of this plant is expected to progress rapidly with maximum exploitation of tissue cultures, transgenosis and molecular mapping. One of the necessary prerequisites is the knowledge of the basic properties of this object. Tissue cultures of *Oenothera* were first described by Stubbe and Hermann (1982), but no data are given on *Oenothera* transgenosis.

The aim of the experiments presented here was to check the possibilities of transformation of *Oenothera biennis* by *Agrobacterium* vectors. The necessary prerequisite was to set up a satisfactory method of plant tissue cultures of this object.

Materials and methods

Material: Five cultivars of *Oenothera biennis* have been used: VNK, NC-1, NC-2, PL-1, CV-3. The *A. tumefaciens* strain used was LBA4404 carrying disarmed helper

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plasmid pAL4404 (Hoekema *et al.* 1983) and vector plasmid pCB1334 (Vlasák and Ondřej 1992) *ipt* gene. This vector brings two important genes into the plant genome: one of the pTi C58 T-DNA genes, *ipt* gene with own promotor, the expression of which leads to the elevated levels of cytokinins and in plants expressed kanamycin resistance (*nptII*) gene.

Chemicals: Indole-3-acetic acid (IAA), 2,4-dichlorophenoxyacetic acid (2,4-D), N^6-(2-isopentenyl)adenosine (2iP), kinetin and kanamycin were purchased from *Sigma* (St. Louis, USA), ticarcillin from *Beecham Laboratories* (Brentford, England) and polyvinylpyrrolidone K30 (PVP) from *Fluka* (New-Ulm, Germany).

***In vitro* cultivation:** Plants have been grown *in vitro* from seedling stage on MS medium (Murashige and Skoog 1962). Callogenesis and regeneration of plants have been tested on MS medium with different growth regulators:
MS-1 (MS with 1 mg dm^{-3} IAA and 8 mg dm^{-3} 2iP),
MS-2 (MS with 0.1 mg dm^{-3} IAA and 2 mg dm^{-3} 2iP),
MS-3 (MS with 2 mg dm^{-3} 2,4-D, 6 mg dm^{-3} IAA and 1.5 mg dm^{-3} kinetin),
MS-4 (MS with 1 mg dm^{-3} 2,4-D and 0.05 mg dm^{-3} kinetin).

Transformation of explants by *A. tumefaciens* was performed as described by Ondřej *et al.* (1991). *A. tumefaciens* treated explants were cultivated on MS-1 supplemented with kanamycin 50 mg dm^{-3} and ticarcillin 500 mg dm^{-3}. Transformed tissues were subcultured at one month intervals. Antibiotics were omitted after the sixth subculture.

Analysis of cytokinins: For cytokinin analysis, plant material (leaves and stems) was collected and stored at -70 °C until measurement. The material was freeze-fractured and grounded in liquid nitrogen before being extracted in 80 % methanol. Internal standard (^{3}H zeatin) was added up to 0.5 kBq per sample. After centrifugation (25 000 *g*) supernatants were passed through *C 18 Sep-Pak* cartridge (*Waters*, Milford, USA) and evaporated to water phase at 35 °C. Samples were diluted in 0.04 M ammonium acetate (pH 6.5) and loaded to dual *DEAE Sephadex A25-C18* cartridge columns. Retained cytokinins were eluted from the *C18* column with 80 % methanol and evaporated to dryness. A *Separon SGX C18* column (5 μm, 250 × 4.6 mm i.d., *Tessek*, Prague, Czech Republic) was used with a flow rate 0.8 cm^3 per min for fractionation of the extracted cytokinins. The solvent system was changed from 100 % *A* [*A*: 10 % methanol in 0.1 M acetic acid (buffered to pH 3.35 with triethylamine)] to 100 % *B* (*B*: 100 % methanol) in a specific gradient. Collected fractions of zeatin (Z), zeatin riboside (ZR), isopentenyladenine (IPA) and isopentenyladenosine (IPE) were evaporated and subsequently measured with standard *ELISA* method (antibodies have been produced and kindly provided by Dr. M. Strnad, Institute of Experimental Botany, ASCR in Olomouc). The results were recalculated per fresh mass unit and 100 % added internal standard.

Results

Callogenesis and regeneration: Callus initiation has been obtained in all five cultivars of *O. biennis* (Fig. 1*a*) in leaf segments and roots and in VNK cultivar also in hypocotyls. The medium MS-4 was shown to be most efficient for callogenesis of

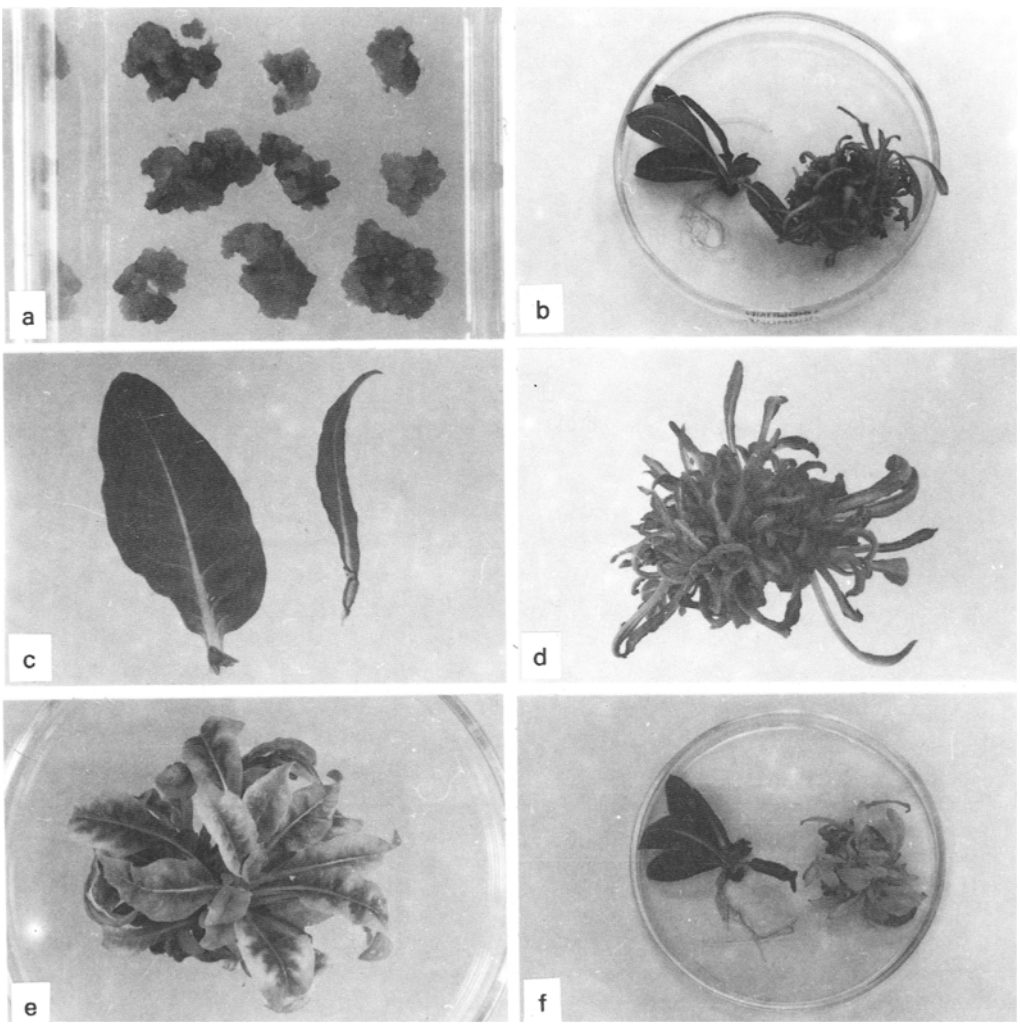

Fig. 1. Callus tissues of *Oenothera biennis* cv. CV3 (*a*); control untransformed plant (*left*) and teratoma tissue transformed by pCB1334 vector (*right*) (*b*); leaf of untransformed plant (*left*) and teratoma tissue (*right*) (*c*); green teratoma tissue (*d*); teratoma tissue with yellow and green sectors (*e*); control plant (*left*) and yellow teratoma (*right*) (*f*).

both leaf segments and roots (Table 1). Some of the calli blackened due to the formation of polyphenolic compounds. PVP (2 mg dm^{-3}) was sometimes added into the medium and it decreased these effects.

Table 1. Callogenesis in different cultivars of *O. biennis* on media with different levels of growth regulators (see Materials and methods for detail).

Cultivar	Leaves	Roots	Hypocotyls	Leaves	Roots	Hypocotyls
	MS-1			MS-2		
VNK	+	++	++	+	++	+
NC-1	–	–	N	–	–	N
NC-2	–	–	N	–	+	N
PL-1	–	+	N	+	+	N
CV-3	+	–	N	+	–	N
	MS-3			MS-4		
VNK	+++	++	+	+++	++	–
NC-1	–	–	N	+++	+++	N
NC-2	+++	++	N	+++	+++	N
PL-1	+	+	N	+++	+++	N
CV-3	+++	+++	N	++	+++	N

N - not tested; – - no regeneration; +, ++, +++ - relative size of calli

Intense callogenesis was found in cultivars VNK, NC-2 and CV-3 also on the medium MS-3. Light stimulated the callus growth. Regeneration was induced on media MS-1 and MS-2 and the regeneration process was rather efficient (Table 2). Calli derived from leaf segments regenerated mostly to plants or shoots, less frequently to roots only. Root derived calli regenerated roots only.

Table 2. Regeration of plants from calli on medium MS-1 or MS-2.

Cultivar	MS-1 leaves	roots	hypocotyls	MS-2 leaves	roots	hypocotyls
VNK	++	–	+	++	–	–
NC-1	–	–	N	–	–	N
NC-2	+	+	N	–	++	N
PL-1	+	+	N	++	+++	N
CV-3	++	+	N	++	+	N

N - not tested; – - no regeneration; + - weak regeneration; ++ - good regeneration; +++ - abundant regeneration

Degree of kanamycin resistance: The spontaneous level of kanamycin resistance was tested in seedlings and total growth inhibition was observed starting from 20 mg dm^{-3} kanamycin in the medium.

Tissues transformed by pCB1334 vector tolerated concentrations 200 - 1000 mg dm^{-3}, depending on the transgenic clone used.

Transformation by *A. tumefaciens*: The CV3 cultivar was used for transformation. Mostly calli appeared after treatment of leaf or hypocotyl segments by *A. tumefaciens* in the first phase of transformation, and regenerated buds appeared soon on some of them. The undifferentiated tissues darkened with age, probably in the consequence of synthesis and accumulation of polyphenolic compounds, and died. PVP decreased these effects as mentioned above.

Teratomous tissues appeared together with most of the undifferentiated tissues, as dense clumps of extremely shortened, branched shoots with very short internodes and small, very narrow leaves with distinct central veins (Fig. 1*b,c,d*). There were only minor phenotypic differences among individual clones. Shoots with different morphology derived from a common callus were observed in later subcultures. Part of them was distinctly of more normal appearance, with longer internodes and wider and larger leaves.

A distinct feature of some subclones, even if subcloned in larger clumps including a part of basal callus and several shoots, was light green or completly yellow colour of teratoma leaves (Figs. 1*e,f*). These subclones could be propagated without loss of vitality.

Levels of cytokinins in transgenic clones: Levels of cytokinins have been determined in five clones of transformed plants and control plants (Table 3). The overall levels of cytokinins were elevated in all five teratomous clones. While the levels of zeatin and zeatin riboside were dramaticaly increased, those of IPA showed only slight increase and the levels of IPE were even decreased.

Table 3. Content of cytokinins [total, zeatin (Z), zeatinriboside (ZR), isopentenyladenine (IPA), isopentenyladenosine (IPE)] in control (cv. CV3) and transgenic clones [pmol g^{-1}(f.m.)].

	Total	Z	ZR	IPA	IPE
control	95.4	22.0	12.2	2.2	59.0
1334/2	309.5	131.7	152.1	6.3	19.4
1334/6	273.2	106.5	119.7	6.0	41.0
1334/7	1361.7	557.0	786.0	3.2	15.5
1334/11	151.9	45.7	78.9	2.4	24.9

Discussion

From results presented here we can conclude that *O. biennis* is highly susceptible to *Agrobacterium*-mediated transformation.

The *O. biennis* plants carrying the *ipt* gene show the general properties of plants with this transgene, as observed in tobacco, potato and *Arabidopsis thaliana* (Ondřej *et al.* 1989). The shooty phenotype in all clones studied was correlated with the

endogenous levels of cytokinins. In agreement with other results published (Ainey *et al.* 1993), our *ipt* transformants also exhibited the most pronounced increase in zeatin riboside and zeatin contents, but the separate forms of cytokinins could change rapidly due to complex cytokinin metabolism. Large proportion of the endogenously or exogenously supplied hormone was transformed into conjugated forms which are considered to be less active or completely inactive (Van Staden *et al.* 1994). Then the obtained phenotype of *Oenothera* transgenic plants results from the *ipt* expression and interaction with the endogenous inactivation capacity of the plant material. This equilibrium was also important for callogenesis and shoot induction.

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