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Hygromycin B - an alternative in flax transformant selection

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Abstract

The *in vitro* regeneration of three flax (*Linum usitatissimum* L.) breeding lines (cv. Jitka, cv. Areco and NLN 245) and selection of transgenic plants were studied. *A. tumefaciens* derived binary vector GV3101 (pPM90RK)(pPCVRN4) bearing tetramer of 35S promoter enhancer was used in transformation experiments. Following 3 weeks of cultivation on shoot inducing Murashige and Skoog agar medium containing BAP (0.1 μM) and NAA (0.005 μM) from 82.6 % to 98 % of hypocotyl segments formed shoots. While ticarcillin (500 mg dm^{-3}) used to eliminate *Agrobacterium* following the transformation decreased the organogenic response by about 10 % only, the addition of 20 mg dm^{-3} hygromycin to ticarcillin efficiently suppressed the regeneration of untransformed control plants. To look up for genomic mutations caused by T-DNA insertion from *Agrobacterium* transformation or originated from somaclonal variation over 500 regenerated plants have been cloned, transferred into soil and evaluated especially for their morphological characteristics. Up to now among plants of cv. Areco-background at least 8 genotypes showed changes either in flower or filament and stigma colour and one clone of plants with pollen sterility was identified. Among fifty four plant clones evaluated in 7 clones the presence of transgene specific sequence *hpt* was detected and simultaneously *Agrobacterium* contamination of tissues was firmly excluded.

Additional key words: *Agrobacterium tumefaciens*, mutant induction, nested PCR, plant transformation, T-DNA.

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Abbreviations: BAP - 6-benzylaminopurine; NAA - naphthalene acetic acid; tic - ticarcillin; tim - timentin; hyg - hygromycin B; *hpt* - gene coding for hygromycin B phosphotransferase; PCR - polymerase chain reaction; T-DNA - transferred DNA.

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Introduction

Flax (*Linum usitatissimum* L.) is an important oilseed crop (linseed) as well as a traditional source of natural fibres. Conventional breeding has been successful in improving fibre and grain yield, fibre and oil content including their quality, plant resistance to pathogens, *etc*. Recently, flax breeding has being made more efficient by use of *in vitro* techniques (doubled haploids), molecular markers and plant transformation. Flax is also an excellent alternative for genetic manipulations and gene tagging due to its small nuclear genome.

Flax is susceptible to *Agrobacterium* infection (Hepburn *et al.* 1983). Based on it most of various techniques of gene transfer were developed (Basiran *et al.* 1987, Zhan *et al.* 1988, Dong and McHughen 1993, Bretagne-Sagnard and Chupeau 1996, Ling and Binding 1997) and successfully applied to establish transgenic flax lines and cultivars resistant to various herbicides, *e.g.*, glufosinate, glyphosate, sulfonylurea (Jordan and McHughen 1988a, McHughen 1989, McHughen and Holm 1995, McHughen *et al.* 1997).

Generation of transgenic plants is an integrated process which involves careful choice of vector system, regenerable explants, culture conditions, transformation technique and selection scheme for transformants (Dong and McHughen 1993). Common selection of transformed flax individuals is based on their resistance to antibiotic kanamycin (enzyme neomycin phosphotransferase coded by gene *nptII*). The disadvantage of kanamycin used as a selective agent is a great number of escapes what represents serious problem in flax transformation. Many attempts were done to improve the transformation protocol. Techniques used so far are laborious requiring either preculture period prior to inoculation and extra wounding by slicing of hypocotyl segments or by epidermis removal (Jordan and McHughen 1988b, McHughen *et al.* 1989, Dong and McHughen 1993) or introduction of an additional step of resistance selection (Mlynárová *et al.* 1994).

This work is aimed at development of an alternative protocol for selection of transgenic flax useful for routine evaluation of broad sample numbers.

Materials and methods

Plants: Seeds of three flax (*Linum usitatissimum* L.) breeding lines (Jitka, Areco and NLN 245) were obtained from *AGRITEC Ltd.* (Šumperk, Czech Republic). While cv. Jitka is a newly introduced fiber type flax, cv. Areco represent traditional, and NLN 245 modern type of oil seed (linseed) cultivars. Dry seeds were treated by 70 % ethanol for 2 min and then surface sterilized for 45 - 65 min in diluted solution of commercial bleach *Savo* (*Bochemie*, Bohumín, Czech Republic) in distilled water (1:9) supplemented by few droplets of detergent. Following washing in sterile distilled water the seeds were blotted dry on a filter paper and sown onto cellulose pads wetted by 15 cm^3 of sterile distilled water in 100 cm^3 Erlenmayer flasks (7 - 10 seeds per flask). Cultures were kept for 4 d in the dark and then transferred onto light (growth chamber, 22 ± 2 °C, 16-h photoperiod, irradiance of 40 - 70 μmol(PAR) m^{-2} s^{-1}

supplied by white fluorescent tubes). Hypocotyl slices (3 - 4 mm long) of 7-d-old seedlings were used both in regeneration and transformation experiments.

Bacterial strain: *Agrobacterium tumefaciens* derived binary vector GV3101 (pPM90RK)(pPCVRN4) was used throughout the experiments. Its T-DNA carries tetramer of 35S promoter enhancer to activate silent genes in the vicinity of the sites of integration (Koncz *et al.* 1994). Stable integration of its T-DNA into plant gene results either in mutational inactivation or constitutive gene activation and dominant gene expression. The lost of gene function is manifested as recessive mutation, while the switch on gene function is a dominant change. T-DNA contains also a selectable gene for plant resistance to hygromycin B (*hpt*).

Bacteria were grown in liquid media according to Langley and Kado (1972) supplemented with rifampicin, carbenicillin and kanamycin (50, 100 and 200 mg dm^{-3}, respectively) overnight at 28 °C.

Regeneration experiments and hygromycin resistance tests: Segments of hypocotyls were cultured on surface of modified L media (MS agar medium supplemented with 0.1 μM BAP and 0.005 μM NAA - shoot induction medium according to Tejklová 1992) (Fig. 1). Sterile antibiotics used either to eliminate vector bacteria - ticarcillin (500 mg dm^{-3}) or to select transformed plants - hygromycin B at concentrations 0, 15 and 20 mg dm^{-3} were added to media after autoclaving. In each variant about 70 explants were treated and 3 independent experiments were performed. Cultures were evaluated following 3 and 5 weeks of cultivation. Later, in some experiments the effect of higher hygromycin B concentrations (20, 25 and 30 mg dm^{-3}) in the presence of timentin was followed. In such experiments number of explants per variant was approximately 40. Timentin is thought to be more effective in treatments against bacteria because it contains along with ticarcillin an another active substance, clavulanic acid. All cultures were grown in the chamber under conditions mentioned above.

Fig. 1. Shoot regeneration of linseed cv. Areco on L culture medium.

Plant transformations: Modified method of Dong and McHughen (1993) was used. Hypocotyl segments of *in vitro* grown flax seedlings were directly placed into Petri dishes with bacterial suspensions (19 cm^3 of 10 mM $MgSO_4$ and 1 cm^3 of bacterial culture) and incubated for 2 h. Explants were blotted dry with sterile filter paper and placed onto solidified (0.8 % agar) shoot induction medium devoid of antibiotics. Cultures were kept under dim light for 2 d to enable T-DNA transfer from bacteria into plant cells. Then the bacteria were washed out in 10 mM $MgSO_4$ solution with 1000 mg dm^{-3} ticarcillin. Dry hypocotyls were cultured on L medium supplemented with 500 mg dm^{-3} ticarcillin (later substituted by timentin) and 20 mg dm^{-3} hygromycin B under conditions favourable for plant regeneration.

Cultures of regenerated shoots and transfer of plants into soil: Dark green regenerated buds were excised out of explants and clonaly propagated on L_C medium (L medium with 0.05 μM BAP and 0.005 μM NAA). 15 mm long stems were rooted in L_R medium (L medium without BAP) (Fig. 2). Rooted plants were transferred into soil and cultured in glasshouse under controlled conditions (15 - 25 °C, 16-h photoperiod).

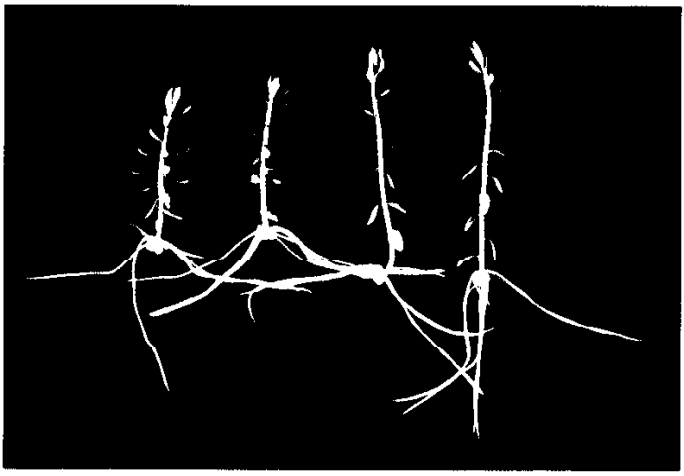

Fig. 2. Rooted shoots of NLN215 transformants, genotype 5A16 after 2 weeks culture on L_R medium.

Molecular detection of transformants: Flax transformants were detected in purified total DNA (*Nucleon Phytopure Plant DNA Extraction* kit) by nested *hpt*-specific PCR (Fig. 3). Nested PCR was chosen as an advanced method to achieve higher sensitivity and specificity in testing for transformant status. The primers for the first run of nested PCR were:
hpt1: 5'-GTC CTGCGGGTAAATAGCTGCGCC (Scheid *et al.* 1994) and
hpt4: 5' ATGTTGGCGACCTCGTATTGG (Aragao *et al.* 1996) which specifically amplify region of 556 bp.

The primers for the second run of nested PCR were:
hpt2: 5`-GTGTATTGACCGATTCCTTGCGG (Scheid *et al.* 1994) and
hpt3: 5`-TCCGGAAGTGCTTGACATTGG (Aragao *et al.* 1996) which were used to specifically amplify region of 212 bp from *hpt* gene.

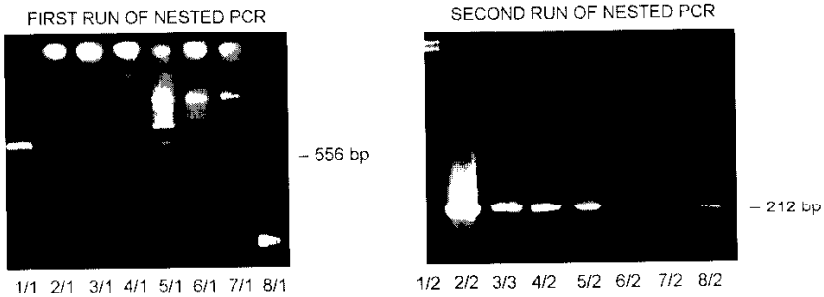

Fig. 3. Gel electrophoresis of nested PCR (1.5 % agarose gel). Effect of various template concentrations is demonstrated. 1/1 - λ/*Pst*I size marker; 2/1 - Areco 7F1 (concentrated template); 3/1 - Areco 7F1 (10 × diluted template); 4/1 - Areco 28A3 (concentrated template); 5/1 - Areco 28A3 (10 × diluted template); 6/1 - NI.N245 4D4; 7/1 - NI.N245 14A1; 8/1 - positive *hpt* control (plasmid TAC 1/1); 1/2 - λ/*Pst*I size marker; 2/2 - Areco 7F1; 3/2 - Areco 28A3 (concentrated template); 4/2 - Areco 28A3 (10 × diluted template); 5/2 - Areco 28A3 (100 × diluted template); 6/2 - NI.N245 4D4; 7/2 - NI.N245 14A1; 8/2 - positive *hpt* control (plasmid TAC 1/1).

PCR reactions were performed in 0.025 cm^3 volume using Taq DNA polymerase from *Promega* (Madison, USA). DNA samples were denatured at 94 °C for 4 min. and amplified using 40 cycles (94 °C for 2 min., 52 °C for 1 min., 72 °C for 2 min) followed by final elongation at 72 °C for 10 min. In order to detect eventual false positive PCR signal caused by contamination of tissues by *Agrobacterium*, nested PCR specific to *picA* gene from *Agrobacterium* chromosome (Kononov *et al.* 1997) was performed with reaction mixture and temperature profile identical with *hpt*-specific nested PCR. The sequence of primers used for the first run of nested PCR were:
picA1: 5`-ATGCGCATGAGGCTCGTCTTCGAG and
picA2: 5`-GACGCAACGCATCCTCGATCAGCT which specifically amplify region of 568 bp (Kononov *et al.* 1997).

The primers for the second run of nested PCR were:
picA3: 5`-GAGGATGGTTGTGTCTATCGCG and
picA4: 5`-CTTCTTGTGCCAATAATTGCCG were used which specifically amplify region of 283 bp (Wiesner and Wiesnerová 1998, unpublished).

Chemicals: Hygromycin B was purchased from *Duchefa Biochemie BV* (Haarlen, The Netherlands) and *Boehringer Mannheim GmbH* (Mannheim, Germany), ticarpen and timentin from *Beecham Pharmaceuticals (Pte)* (Brentford, UK), Nucleon Phytopure Plant DNA Extraction kit from *Amersham Life Science* (Little Chalfont, England).

Results

The hypocotyls of all three tested flax lines express very high degree of regeneration ability *in vitro*. Following 3 weeks of cultivation on shoot inducing L medium containing 0.1 μM BAP and 0.005 μM NAA from 82.6 % (cv. Jitka) up to 98 % (cv. Areco, NLN 245) of segments formed shoots (Table 1). Addition of ticarcillin (500 mg dm^{-3}) to eliminate *Agrobacterium* following the transformation decreased

Table 1. Effect of antibiotics on regeneration efficiency of flax hypocotyl segments (h.s.) determined after 35 d of cultivation. Control was evaluated on 21st day before intensive regeneration and shoot branching took place (* shoots mostly do not elongate, reaching max. 20 mm, frequently yellowish or necrotic).

Cultivar	Antibiotic	Concentration [mg dm^{-3}]	Cultured h.s.	Regenerating h.s. [number]	[%]	Total shoot [number]	Shoots [h.s.$^{-1}$]	Shoots [reg. h.s.$^{-1}$]
cv. Jitka	Control	0	121	100	(82.6)	205	1.69	1.72
	Ticarcillin	500	190	138	(72.6)	295	1.55	2.13
	Tic.+Hyg.	500 + 15	218	39	(17.9)	*93	0.43	2.38
	Tic.+Hyg.	500 + 20	235	19	(8.1)	*29	0.12	1.53
cv. Areco	Control	0	254	250	(98.4)	601	2.37	2.40
	Ticarcillin	500	205	197	(91.6)	702	3.42	3.56
	Tic.+Hyg.	500 + 15	244	64	(26.2)	137	0.56	2.14
	Tic.+Hyg.	500 + 20	222	35	(15.8)	*87	0.39	2.49
line NLN 245	Control	0	207	203	(98.1)	411	1.99	2.02
	Ticarcillin	500	241	205	(84.0)	552	2.28	2.69
	Tic.+Hyg.	500 + 15	229	32	(14.0)	*90	0.83	2.81
	Tic.+Hyg.	500 + 20	227	26	(11.5)	*69	0.30	2.65

the organogenic response by about 10 % only. Hygromycin B at concentration of 20 mg dm^{-3} in the presence of ticarcillin efficiently suppressed the regeneration of untransformed control plants. The frequency of responsive explants varied (8 - 16 %) together with number of shoots produced per explant depending on material used. Fibre type flax cv. Jitka gave in both respects the lowest response. Under selective conditions mentioned above the suppression or complete inhibitions of chlorophyll synthesis along with the necrosis of shoots were the most common features in both control and transformation experiments. Development of untransformed dark green shoots was substantially reduced, only rare stem elongation up to 20 mm was seen. This data were confirmed in a second run of experiments where elevated concentrations (20 - 30 mg dm^{-3}) of hygromycin were used together with timentin (Table 2).

Plant regeneration of bacteria-treated hypocotyl segments (both frequencies of responding explants and shoot formation) was strongly influenced by cultivar used. Comparing to the untransformed control the beginning of shoot formation was by one week delayed. In 1998, over 500 plants from transformation experiments regenerated under selective conditions have been cloned, transferred into soil and cultured in a glasshouse. The regenerated plants were evaluated especially for their morphological

characteristics (*e.g.* colour of petals, stamens, filaments, carpels and seeds). Up to now among plants of cv. Areco-background at least 8 genotypes showed changes either in flower or filament and stigma colour and one clone of plants with pollen sterility was identified. Plants showing differences or abnormalities to the control are characterized further using methods of genetic and molecular analysis, scanning microscopy and image analysis. Seed progeny of selected selfed plants will be evaluated in field conditions this year for changes in morphological characteristics and traits of plant breeders interest (resistance to pathogens and wilting, fatty acids content, *etc.*).

Table 2. Effect of higher doses of hygromycin B in combination with timentin on regeneration efficiency of flax hypocotyl segments (h.s.) determined after 35 d of cultivation; control was evaluated on 21st day (* - shoots mostly do not elongate, reaching max. 20 mm, frequently yellowish or necrotic, ** - shoots reaching max. 3 mm, frequently yellowish or necrotic).

Cultivar	Antibiotic	Concentration [mg dm^{-3}]	Cultured h.s. [number]	Regenerating h.s. [number]	[%]	Total shoot [number]	Shoots [h.s.$^{-1}$]	Shoots [reg. h.s.$^{-1}$]
cv. Jitka	Control	0	121	100	(82.6)	205	1.69	1.72
	Tim.+Hyg.	500 + 20	135	7	(5.2)	**12	0.09	1.71
	Tim.+Hyg.	500 + 25	139	7	(5.0)	**18	0.13	2.57
	Tim.+Hyg.	500 + 30	235	19	(8.1)	**29	0.12	1.53
cv. Areco	Control	0	254	250	(98.4)	601	2.37	2.40
	Tim.+Hyg.	500 + 20	122	31	(25.4)	*84	0.69	2.71
	Tim.+Hyg.	500 + 25	104	44	(42.3)	*135	1.30	3.06
	Tim.+Hyg.	500 + 30	126	35	(27.8)	**78	0.62	2.23

In parallel, molecular analysis was performed to detect transgenes. Fifty four clones were evaluated for the presence of *hpt* sequence in their genome by nested PCR. In 7 clones the presence of the transgene was detected, all these samples were simultaneously negative (according to *picA*-specific nested PCR) for the presence of bacterial *picA* gene, *i.e. Agrobacterium* contamination of tissues was firmly excluded.

Discussion

Regeneration response to shoot inducing medium was different in flax cultivars (genotypes) studied. Such difference is common also in many other plant species (*e.g.* Brown and Atanassov 1985, McCormick *et al.* 1986). In flax successful systems of plant regeneration were established both for organogenesis (*e.g.* Link and Eggers 1946, Gamborg and Shyluk 1976) and somatic embryogenesis (Gomes da Cunha and Ferreira 1996), but organogenesis is still much more common and efficient. Genotype-specific *in vitro* regeneration response was even more pronounced after addition of hygromycin B (Tables 1, 2). This fact may negatively influence attempts to transform specific genotype of interest (*e.g.* in cv. Jitka limited number of hypothetically transformed plants was obtained). Hygromycin B at concentration of 20 mg dm^{-3} (in the presence of ticarcillin) efficiently suppressed the regeneration of

untransformed control plants. Only limited number of dark green shoots was formed under selective conditions and they did not elongate over 20 mm. Most of other shoots were yellowish and underwent necrosis. Elevated concentrations of hygromycin B (to 30 mg dm^{-3}) strongly influenced further growth (elongation) of shoots formed and proportion of yellowish and necrotic shoots induced. Of two cultivars tested, cv. Jitka was the most sensitive to the effect of hygromycin B. Due to the intensive necrosis even at 20 mg dm^{-3} hygromycin there is no chance to use higher hygromycin doses for selection of transgenic plants. Contrary to cv. Jitka, 30 mg dm^{-3} hygromycin did not prevent bud formation (number of buds formed on explant) in cv. Areco, but only their elongation was strongly suppressed (data not shown here). On the other hand, doses over 20 mg dm^{-3} hygromycin stimulated intensive callogenesis what may influence somaclonal variability of regenerants. During our experiments a new publication of Bretagne-Sagnard and Chupeau (1996) has appeared where another antibiotic spectinomycin was successfully applied in selection of transgenic flax.

With regard to high sensitivity of nested PCR performed on limited number of regenerants from transformation experiments we assume that regenerated plant could be chimerical. About fifty another regenerants were detected negatively by simple *hpt*-specific PCR which is of much lower detection sensitivity. Of course, the possibility of escapes could not be excluded, too. Due to the fact, that the vector construct used in our experiments did not contain any marker gene (*e.g.* UidA coding for bacterial β-glucuronidase; GUS), it was not possible to trace patterns of transformation intensity on flax hypocotyls. Dong and McHughen (1991) using model marker GUS gene have revealed strong influence of both length of explant preculture and time of cocultivation on patterns of explant transformation. Based on their results the authors expect the possible improvement of efficiency of their own transformation procedure. It should be noted that in our experiments both vector construct and flax cultivars differed from that used by Dong and McHughen (1991) which may cause difference in optimizing the transformation procedure. More data relating to problems of chimerism and "escapes" will be obtained following tests of seed progenies of selfed plants and additional studies including molecular diagnostics of segregants of T_2 plants grown in field conditions in year 1999.

Transgenic calli formed from bacterium-treated tissues (hypocotyls and cotyledons) were shown to be a complex mixture of independently transformed and non-transformed cells from which chimeric shoots were often regenerated (McHughen and Jordan 1989).

References

Aragao, F.J.L., Barros, L.M.G., Brasileiro, A.C.M., Ribeiro, S.G., Smith, F.D., Sanford, J.C., Faria, J.C., Rech, E.L.: Inheritance of foreign genes in transgenic bean *Phaseolus vulgaris* (L.) co-transformed via particle bombardment. - Theor. appl. Genet. **93**: 142-150, 1996.

Basiran, N., Armitage, P., Scott, R.J., Draper, J.: Genetic transformation of flax (*Linum usitatissimum*) by *Agrobacterium tumefaciens*. Regeneration of transformed shoots *via* a callus phase. - Plant Cell Rep. **6**: 396-399, 1987.

Bretagne-Sagnard, B., Chupeau, Y.: Selection of transgenic flax plants is facilitated by spectinomycin. - Transgenic Res. **5**: 131-137, 1996.

Brown, D.C.W., Atanassov, A.I.: Role of genetic background in somatic embryogenesis in *Medicago*. - Plant Cell Tissue Organ Cult. **4**: 111-122, 1985.

Dong, J.-Z., McHughen, A.: An improved procedure for production of transgenic flax plants using *Agrobacterium tumefaciens*. - Plant Sci. **88**: 61-71, 1993.

Gamborg, O.L., Shyluk, J.P.: Tissue culture, protoplasts, and morphogenesis in flax. - Bot. Gaz. **137**: 301-306, 1976.

Hepburn, A.G., Clarke, L.E., Blumdy, K.S., White, J.: Nopaline Ti-plasmid, pTiT37, T-DNA insertions into flax genome. - Mol. appl. Genet. **2**: 211-224, 1983.

Jordan, M.C., McHughen, A.: Glyphosate tolerant flax plants from *Agrobacterium* mediated gene transfer. - Plant Cell Rep. **7**: 281-284, 1988a.

Jordan, M.C., McHughen, A.: Transformed callus does not necessarily regenerate transformed shoots. - Plant Cell Rep. **7**: 285-287, 1988b.

Koncz, C., Martini, N., Szabados, L., Hrouda, M., Bachmair, A., Schell, J.: Specialized vectors for gene tagging and expression studies. - In: Gelvin, S.B. (ed.): Plant Molecular Biology Manual B2. Pp. 1-22. Kluwer Academic Publishers, Dordrecht-Boston-London 1994.

Kononov, M.E., Bassuner, B., Gelvin, S.B.: Integration of T-DNA binary vector "backbone" sequences into the tobacco genome evidence for multiple complex patterns of integration. - Plant J. **11**: 945-957, 1997.

Langley, R.A., Kado, C.I.: Studies on *Agrobacterium tumefaciens*: conditions for mutagenesis by N methyl-N-nitrosoquanidine and relationship of *A. tumefaciens* mutants to crown gall induction. - Mut. Res. **14**: 277-286, 1972.

Ling, H.Q., Binding, H.: Transformation in protoplast cultures of *Linum usitatissimum* and *L. suffruticosum* mediated with PEG and with *Agrobacterium tumefaciens*. - J. Plant Physiol. **151**: 479-488, 1997.

Link, G.K.K., Eggers, V.: Mode, site, and time of initiation of hypocotyledonary bud primordia in *Linum usitatissimum* L. - Bot. Gaz. **107**: 441-454, 1946.

McCormick, S., Niedermeyer, J., Fry, J., Barnason, A., Horsch, R., Fraley, R.: Leaf disc transformation of cultivated tomato (*L. esculentum*) using *Agrobacterium tumefaciens*. - Plant Cell Rep. **5**: 81-84, 1986.

McHughen, A.: *Agrobacterium* mediated transfer of chlorsulfuron resistance to commercial flax cultivars. - Plant Cell Rep. **8**: 445-449, 1989.

McHughen, A., Holm, F.A.: Transgenic flax with environmentally and agronomically sustainable attributes. - Transgenic Res. **4**: 3-11, 1995.

McHughen, A., Jordan, M.: Recovery of transgenic plants from "escape" shoots. - Plant Cell Rep. **7**: 611-614, 1989.

McHughen, A., Jordan, M., Feist, G.: A preculture period prior to *Agrobacterium* inoculation increases production of transgenic plants. - J. Plant Physiol. **135**: 245-248, 1989.

McHughen, A., Rowland, G.G., Holm, F.A., Bhatty, R.S., Kenaschuk, E.O.: CDC Triffid transgenic flax. - Can. J. Plant Sci. **77**: 641-643, 1997.

Mlynárová, L., Bauer, M., Nap, J.-P., Pret'ová, A.: High efficiency *Agrobacterium*-mediated gene transfer to flax. - Plant Cell Rep. **13**: 282-285, 1994.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue cultures. - Physiol. Plant. **15**: 473-497, 1962.

Scheid, O.M., Afsar, K., Paszkowski, J.: Gene inactivation in *Arabidopsis thaliana* is not accompanied by an accumulation of repeat-induced point mutations. - Mol. gen. Genet. **244**: 325-330, 1994.

Tejklová, E.: [Long-term *in vitro* shoot-tip culture and plant regeneration in flax]. - Rost. Výroba (Praha) **28**: 1009-1022, 1992. [In Czech.]

Zhan, X.C., Jones, D.A., Kerr, A.: Regeneration of flax plants transformed by *Agrobacterium rhizogenes*. - Plant mol. Biol. **11**: 551-559, 1988.