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Agrobacterium-mediated transformation of *Cichorium intybus* L. with interferon- 2b gene

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An efficient method for the plant regeneration and Agrobacterium-mediated transformation with interferon- 2b gene has been developed for chicory C. intybus L. cv. Pala rossa. The regeneration with efficiency about 100% was induced on the MS medium supplemented with 0.5-2.5 mg/l kinetin and 0.5 mg/l NAA. The transformed plantlets were recovered with 26.9% regeneration frequency on basal medium with 25 mg/l kanamycin. According to PCR-analysis the npt II and ifn- 2b genes were integrated into the genome of the transformed plants.

Keywords: transformation, Agrobacterium, Cichorium intybus, interferon

Introduction. *C. intybus* L is a biennial representative of *Asteraceae* family. Chicory is grown in many geographic regions – Europe (Belgium, Germany, France), the USA, South America, and India. The attention to this plant is explained by the fact that it is used in both cooking (salad sorts, in particular) and production of coffee substitute. Its medicinal features are attributed to the presence of inulin, coumarins, flavonoids, and vitamins [1]. Chicory is anti-hepatotoxic, anti-ulcer, anti-inflammatory, cardiotonic, and diuretic means, used for treatment of diabetes, AIDS, tumours, tachycardia, etc. [2-4]. Chicory is also used in production of a series of medical preparations, such as LIV52. Thus, it is an interesting object of cell engineering which may be used for creating plants with immunomodulating features.

One of the conditions of efficient transformation of plants is the availability of regeneration methods. This allows obtaining maximal number of shoots from such plant explants as roots, leaves, and stalk buds [1, 5, 6] with almost 100% regeneration frequency [7].

The quantity of investigations in genetic transformation of chicory is rather limited. The main goal of experiments was to develop plants with changed phenotype, with induction of flowering [8], resistance to herbicides [9], synthesis of fructan [10], in particular. Since chicory is used in cooking without any prelimi-

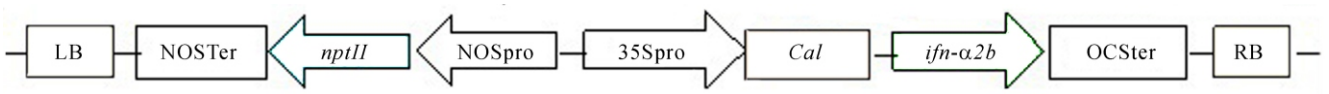

Fig.1 Scheme of T-DNA vector *pCB124* for Agrobacterium-mediated transformation of *Cichorium intybus:* LB and RB - left and right boundaries of T-DNA; *nptII* - gene of neomycin phosphortransferase II; *ifn- 2b* - gene of interferon- 2b; NOSpro and NOSter - promotor and terminator of nopalinsynthase gene, respectively; 35Spro - promotor of 35S-protein gene from the genome of cauliflower mosaic virus; OCSter - terminator of octopinsynthase gene; *cal* - calreticulin

nary thermal treatment, this plant may serve as an object of genetic transformation, e.g. in creating plants with immunomodulating features. There are current methods, allowing initiation of synthesis of biologically active substances in plants [11]. The pharmaceutical recombinant biologically active proteins of plant origin have a series of advantages compared to the preparations of microbial origin. The former are not cost-consuming in production and storage, they are also applicable for mass production [12].

-interferon is a regulator of the mammalian immune system. It enhances the function of cytotoxic T-lymphocytes and phagocytic activity of macrophages, promotes lysis of infected cells, and slows down the development of viral infections. Therefore, the development of new methods of obtaining clean interferon preparation is an urgent and promising task of biotechnologies. There are some pharmaceutical preparations of recombinant interferon, developed on the basis of bacteria using the methods of genetic engineering.

Genetically modified plants with interferon gene were obtained over 20 years ago [13]. Those works were aimed at using anti-viral activity of interferon in order to protect plants from diseases [14, 15]. The creation of transgenic plants with interferon gene is a new and actively developing direction of current investigations. Though currently modified plants have not found their practical application yet, the possibility of expression of interferon gene in the plants of potato [16], rice [17], and salad [18] was vividly demonstrated. The recombinant interferon was shown to have immunogenic activity [19], thus, it may be used with therapeutic purposes.

Materials and Methods. The seeds of chicory *C. intybus* L. cv *Pala rossa* were used as primary material. The seeds were sterilized in 70% ethanol (1 min), 25% solution of commercial preparation *Belizna* (10 min), then washed in distilled water (60 min). Later the seeds were grown in agarose medium MS [20] at 16-hour light photoperiod, 24°C.

Cotyledonous and leaves of 10-12-day-old seedlings were used for regeneration of shoots and transformation. They were transected and cultivated at 16-hour light photoperiod, 24°C.

The frequency of plant regeneration from cotyledonous and leaf explants was defined using cultivation on media, different in the content of salts and phytohormones (Table 1).

The transformation was performed using *Agrobacterium tumefaciens* (strain GV3101) with vector construction *pCB124* (Fig. 1). T-DNA of *pCB124* vector contained a selective gene *nptII*, target gene *ifn- 2b*, and calreticulin – a leader sequence, allowing accumulation of a target protein in endoplasmic reticulum.

Bacteria were grown at LB medium [23] with antibiotics (100 mg/l carbenicillin, 50 mg/l rifampicin, 25 mg/l gentamicin) for 48 hours at 27°C. Bacterial cells were precipitated by centrifugation (300 g, 10 min); the precipitate was pre-suspended in the solution of 10 mM $MgSO_4$. Transected leaves were incubated in bacterial suspension for 30 min, dried with filtration paper, and cultivated on agarose MS medium for two days. Later the explants were sequentially applied on medium No.1 (for one week) and No.2 (Table 1) with the addition of antibiotics – kanamycin (25 mg/l) and cefotaxime (600 mg/l). The shoots were rooted on the MS medium without hormones, but with antibiotics in the same concentrations.

Genome DNA was extracted from green leaves of sterile plants according to [23]. PCR of genome DNA was performed on Mastercycler personal 5332 amplifier (Eppendorf) with thermostatic lid in vials with ultra-thin walls. The reaction mixture contained single-mission PCR-buffer, containing ammonium sulphate, 0.2 μmol of corresponding primers (Table 2), 200 μmol of each desoxinucleotidetriphosphate, 0.5

Table 1
Content of nutrient media for investigation on plant regeneration from cotyledonous and leaf explants of chicory

Medium constituents	Content in the medium, mg/l							
	№ 1	№ 2	№ 3	№ 4	№ 5	№ 6	№ 7	№ 8
Macroelements	MS*	MS	MS	MS	B_5**	B_5	B_5	B_5
Microelements	MS	MS	MS	MS	MS	MS	MS	MS
Thiamine	1	1	1	1	1	1	1	1
Pyridoxine	1	1	1	1	1	1	1	1
Nicotinic acid	1	1	1	1	1	1	1	1
Biotin	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01
Ca-pantothenate	1	1	1	1	1	1	1	1
Inositol	100	100	100	100	100	100	100	100
Kinetin	2,5	0,5	–	–	2,5	0,5	–	–
Benzylaminopurine	–	–	2,5	0,5	–	–	2,5	0,5
-NAA	0,5	0,05	0,5	0,05	0,5	0,05	0,5	0,05
Morpholinoethansulphonic acid	1000	1000	1000	1000	1000	1000	1000	1000
Casein hydrolyzate	300	300	300	300	300	300	300	300
Sucrose	30000	30000	30000	30000	30000	30000	30000	30000
Agar	6000	6000	6000	6000	6000	6000	6000	6000

Note: *Macro- and microelements for MS [20]; **macroelements for B_5 [21]

Table 2
Primers, used to confirm the presence of nptII and ifn- 2b

Gene	Primer	Size of amplified fragment, b.p.
nptII	5'- cctgaatgaactccaggacgaggca-3' 5'- gctctagatccagagtcccgctcagaag-3'	622
ifn- 2b	5'-ctcctgcttgaaggacag-3' 5'-ggagtcctccttcatcag-3'	264

units of Taq-polymerase, and 10-50 ng of DNA-sample. The total amount of reaction mixture equalled 20 µl.

The amplification conditions were as follows: primary denaturation – 90°C, 3 min; 30 cycles of amplification (94°C, 30 sec – 60°C, 30 sec – 72°C, 30 sec); final polymerization – 72°C, 5 min.

Results and Discussion. The efficiency of obtaining genetically modified plants via *Agrobacterium*-mediated transformation depends on regeneration capability of explants used. Therefore, we studied the specificities of regeneration of chicory plants on nutrient media, different in the content of macroelements and phytohormones (media No.1-8, Table 1).

The experiments revealed the absence of significant differences between the frequencies of shoot regeneration of two types of explants – cotyledonous and leaves – on all the media, except for medium No.7. The formation of shoots was observed after 10-15 days.

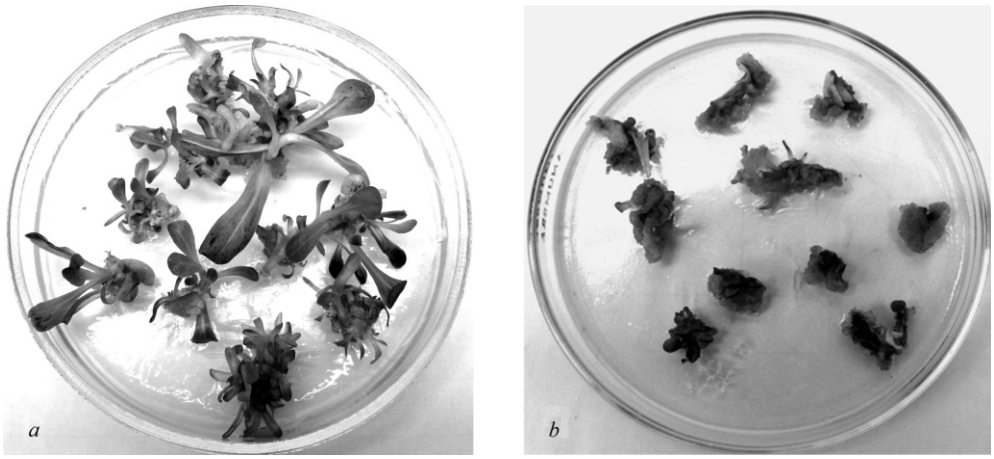

Fig.2 Influence of mineral components of medium on regeneration of chicory plants: *a* – MS macroelements, 0.5 mg/l kinetin, 0.5 NAA; *b* – macroelements B_5, 0.5 mg/l kinetin, 0.5 mg/l NAA

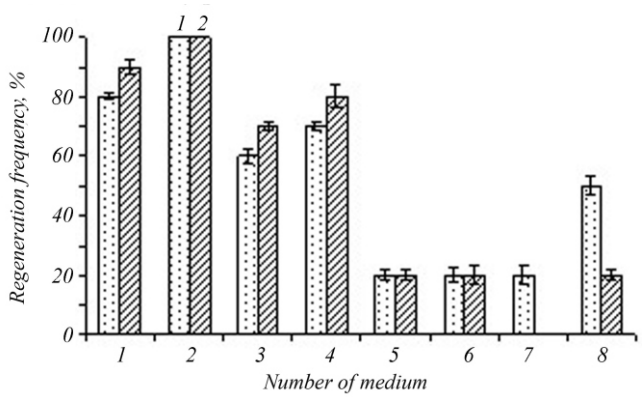

Fig.3 Frequency of regeneration of shoots from cotyledonous (*1*) and leaves (*2*) of chicory on different media

The comparison of the regeneration frequency on media with different content of macroelements demonstrated that the availability of MS macrosalts is the optimal condition. Thus, after 3 weeks the frequency of regeneration of shoots on these media equalled 60-100%, whereas the same index was 0-50% in the presence of B_5 macroelements (Fig. 2, 3). MS and B_5 media are different in the amount of nitrogen (in the form of cations NH_4^+ and anions NO_3 -), the concentration of which in MS medium is much higher. The increase in nitrogen concentration is a possible stimulus for regeneration process of chicory plants.

The comparison of the impact of phytohormones on the process of shoot regeneration revealed that the use of medium with kinetin increases the regeneration frequency compared to the medium with benzylaminopurine (BAP). For instance, the regeneration frequency on medium No.2 (MS, 0.5 mg/l kinetin) was higher than that for medium No.4 (MS, 0.5 mg/l BAP) – 100 and 70%, respectively. Besides, the time period, necessary for regeneration initiation on media No.1 and No.2 with kinetin, was much shorter (7-10 days) than that for media No.3 and No.4 with BAP (14-20 days). The increase in cytokinins (both kinetin and BAP), 0.5-2.5 mg/l, did not result in the increase in regeneration frequency.

Therefore, it was demonstrated that the use of nutrient medium with MS macroelements and kinetin allows obtaining regeneration of chicory plants with the maximal frequency (up to 100%). Since high efficiency of shoot regeneration is one of the reasons of successful transformation, this medium was used in experiments of creating genetically modified plants.

The transformation was performed using *A. tumefaciens* with vector construction *pCB124*, T-DNA of which contained selective gene *nptII* and target gene *ifn- 2b*. The expression of *npt-II* gene gives more advantages to transformed cells, proliferation of which takes place in the presence of selective concentration of kanamycin. To determine the latter, we tested five concentrations of antibiotic – 10, 25, 50, 100, and 150 mg/l. At the kanamycin concentration of 25 mg/l and higher there were no green shoots, regenerating from cotyledonous leaves, and the explants perished. Thus, the concentration of 25 mg/l was defined as the selective one.

In case of cultivating explants on media No. 1 and 2 with the addition of kanamycin (25 mg/l) and

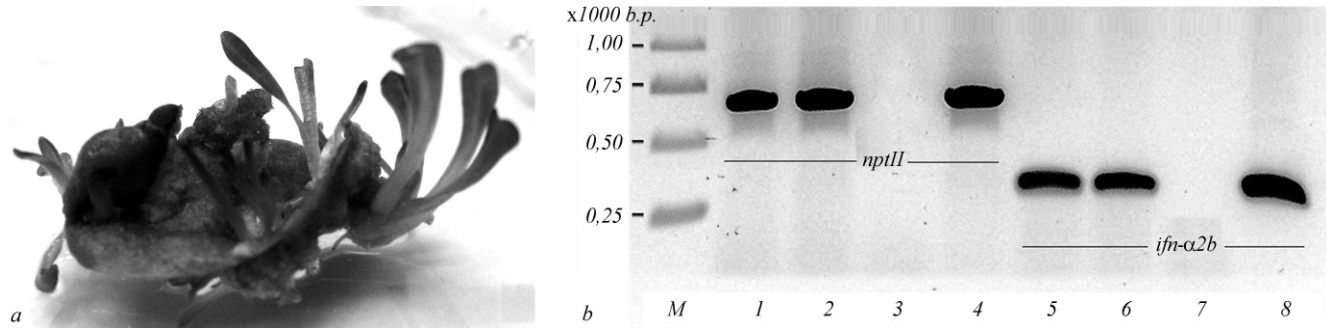

Fig.4 Regeneration of canamycin-resistant plants of chicory cv *Pala rossa* (*a*) and PCR-analysis of total DNA from plants, transformed with *pCB124* construction, for the presence of *nptII* and *ifn-* genes (*b*: *1*, *2*, *5*, *6* – transformed plants; *3*, *7* – control plants; *4*, *8* – DNA of *pCB124* plasmid; *M* – marker)

cefotaxime (600 mg/l) for bacteria elimination, the shoot formation was observed after 7-14 days (Fig. 4, *a*).

The transformation frequency (i.e. regeneration frequency of green plants in the presence of selective concentration of canamycin) was determined according to the ratio of the number of explants with the shoots, green on selective medium, to the total number of explants in per cent. It equalled 26.9%, which must be connected with high regeneration capability of these plants (up to 100%). There were no white plants at the selection of explants on the medium with antibiotics.

PCR-analysis of total DNA of eight plants, regenerated on selective medium, revealed the presence of both selective *nptII* gene and target *ifn- 2b* gene (Fig.4, *b*).

Consequently, the highest frequency of plant regeneration (up to 100%) was observed at cultivation of cotyledonous and leaves of chicory on the medium, containing MS macroelements, kinetin (0.5-2.5 mg/l) and -NAA (0.5 mg/l). *Agrobacterium*-mediated transformation with *pCB124* construction allowed obtaining transformed plants with the frequency of 26.9%. PCR-analysis demonstrated that DNA of all plants under study had both selective *nptII* and target *ifn- 2b* gene. The use of *nptII* gene in *pCB124* construction is efficient, since it allows selecting exactly transformed plants. Therefore, the method of *Agrobacterium*-mediated transformation may be used with the purpose of obtaining transgenic chicory plants with interferon- 2b gene.

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Перенесення гена біосинтезу інтерферону- 2b в рослини цикорію (*Cichorium intybus* L.) методом агробактеріальної трансформації

Резюме

Генетична трансформація цикорію C. intybus L. становить інтерес з огляду на можливість створення рослин-імуномодуляторів, які містять ген інтерферону- 2b. Для отримання таких рослин оптимізовано умови регенерації цикорію сорту Пала росса та здійснено трансформацію за допомогою Agrobacterium tumefaciens. Показано, що частота регенерації на середовищі з макроелементами MS, кінетином (0,5–2,5 мг/л) та -нафтилоцтовою кислотою (0,5 мг/л) сягає 100 %. З використанням полімеразно-ланцюгової реакції встановлено, що ДНК усіх проаналізованих рослин містить як селективний ген nptII, так і цільовий ifn- 2b-ген. Таким чином, методика агробактеріальної трансформації може бути застосована для отримання генетично модифікованих рослин цикорію з геном, що зумовлює синтез інтерферону- 2b.

Ключові слова: трансформація, Agrobacterium, Cichorium intybus, інтерферон.

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Перенос гена биосинтеза интерферона- *2b* в растения цикория (*Cichorium intybus* L.) методом агробактериальной трансформации

Резюме

Генетическая трансформация цикория C. intybus L. представляет интерес с точки зрения возможности создания растений-иммуномодуляторов, содержащих ген биосинтеза интерферона- 2b. Для получения таких растений оптимизированы условия регенерации растений сорта Пала росса и проведена трансформация с помощью Agrobacterium tumefaciens. Показано, что частота регенерации на среде с макроэлементами MS, кинетином (0,5–2,5 мг/л) и -нафтилуксусной кислотой (0,5 мг/л) достигает 100 %. С использованием полимеразной

цепной реакции установлено, что ДНК всех проанализированных растений включает как селективный ген nptII, так и целевой ifn- 2b-ген. Таким образом, методика агробактериальной трансформации может быть использована для получения генетически модифицированных растений цикория с геном, обусловливающим синтез интерферона- 2b.

Ключевые слова: трансформация, Agrobacterium, Cichorium intybus, интерферон.

REFERENCES

1. *Rehman R. U., Israr M., Srivastava P. S., Bansal K. C., Abdin M. Z. In vitro* regeneration of witloof chicory (*Cichorium intybus* L.) from leaf explants and accumulation of esculin // In vitro Cell. Develop. Biol.–2003.–**39,** N 2.–P. 142–146.
2. *Gadgoli C., Mishra S. H*. Antihepatotoxic activity of *Cichorium intybus* // Ethanopharmacology.–1997.–**58,** N 2.–P. 131–134.
3. *Hughes R., Rowland I. R*. Stimulation of apoptosis by two prebiotic *Chicory fructans* in the rat colon // Carcinogenesis.–2001.–**22,** N 1.–P. 43–47.
4. *Ahmad K. D., Gilani S. N., Akhtar A. H., Khan L*. Antiulcerogenic evaluation of aqueous extracts of *Cichorium intybus* and *Phyllanthus emblica* in normal and aspirin-treated rats // Pakistan J. Sci. and Indust. Res.–1998.–**41,** N 2.–P. 92–96.
5. *Profumo P., Gastaldo P., Caffaro L., Dameri R. M., Roti Michelozzi G., Bennici A.* Callus induction and plantlet regeneration in *Cichorium intybus* L.: II. Effect of different hormonal treatments // Protoplasma.–1985.–**126**, N 3.–P. 215–220.
6. *Yucesan B., Turker A. U., Gurel E*. TDZ-induced high frequency plant regeneration through multiple shoot formation in witloof chicory (*Cichorium intybus* L.) // Plant Cell, Tissue and Organ Culture.–2007.–**91**, N 3.–P. 243–250.
7. *Velayuthan P., Ranjithakumari B. D., Baskaran P.* An efficient *in vitro* plant regeneration system for *Cichorium intybus* L. – an important medicinal plant // J. Agricult. Technol.–2006.–**2**, N 2.–P. 287–298.
8. *Bais H. P., Venkatesh R. T., Chandrashekar A., Ravishankar G. A. Agrobacterium rhizogenes*-mediated transformation of Witloof chicory – *in vitro* shoot regeneration and induction of flowering // Curr. Sci.–2001.–**80**, N 1.–P. 83–87.
9. *Vermeulen A., Vaucheret H., Pautot V., Chupeau Y*. Agrobacterium mediated transfer of a mutant *Arabidopsis* acetolactate synthase gene confers resistance to chlorsulfuron in chicory (*Cichorium intybus* L.) // Plant Cell Rep.–1992.–**11**, N 5–6.–P. 243–247.
10. *Vijn I., van Dijken A., Sprenger N., van Dun K., Weisbeek P., Wiemken A., Smeekens S*. Fructan of the inulin neoseries is synthesized in transgenic chicory plants (*Cichorium intybus* L.) harbouring onion (*Allium cepa* L.) fructan:fructan 6G-fructosyltransferase // The Plant J.–1997.–**11**, N 3.–P. 387–398.
11. *Rice J., Ainley W. M., Shewen P.* Plant-made vaccines: biotechnology and immunology in animal health // Anim. Res. Rev.–2005.–**6,** N 2.–P. 199–209.
12. *Daniell H., Streatfield S. J., Wycoff K.* Medical molecular farming: production of antibodies, biopharmaceuticals and edible vaccines in plants // Trends Plant Sci.–2001.–**6,** N 5.–P. 219–226.
13. *Orchansky P., Rubinstein M., Sela I.* Human interferons protect plants from virus infection // Proc. Nat. Acad. Sci. USA.–1982.–**79**, N 7.–P. 2278–2280.
14. *Antoniw J. F., White R. F., Carr J. P.* An examination of the effect of human -interferons on the infection and multiplication of tobacco mosaic virus in tobacco // Phytopath. Z.–1984.–**109**, N 4.–P. 367–371.
15. *Rudas V. A., Piven' N. M., Rivkin M. I ., Vershinin A. V., Infante D. C.* The genetic transformation of *Lycopersicon peruvianum* var. *dentatum* by using *Agrobacterium tumefaciens* carrying a plasmid with the human beta-interferon gene // Tsitol. Genet.–1997.–**31**, N 2.–P. 17–22.
16. *Ohya K., Matsumura T., Ohashi K., Onuma M., Sugimoto C*. Expression of two subtypes of human IFN- in transgenic potato plants // J. Int. and Cytokine Res.–2001.–**21**, N 8.–P. 595–602.
17. *Masumura T., Morita S., Miki Y., Kurita A., Morita S., Shirono H., Koga J., Tanaka K.* Production of biologically active human interferon- in transgenic rice // Plant Biotechnol.–2006.–**23**, N.1.–P. 91–97.
18. *Li J., Chen M., Liu X.-W., Zhang H.-Ch., Shen F. F., Peng G. W.* Transient expression of an active human interferon-beta in lettuce // Scientia Horticult.–2007.–**112**, N 3.–P. 258–265.
19. *Chen T.-L., Lin Y.-L., Lee Y.-L., Yang N.-S., Chan M.-T.* Expression of bioactive human interferon-gamma in transgenic rice cell suspension cultures // Transgen. Res.–2004.–**13**, N 5.–P. 499–510.
20. *Murashige T., Skoog, F*. A revised medium for rapid growth and bio-assays with tobacco tissue cultures // Physiol. Plant.–1962.–**15**, № 3.–P. 473–497.
21. *Gamborg O. L., Miller R. A., Ojim, K.* Nutrient requirements of suspension cultures of soybean root cells // Exp. Cell Res.–1968.–**50**, № 1.–P. 148–151.
22. *Maniatis T., Sambrook J., Fritsch E. F*. Molecular cloning: A laboratory manual.–New York: Cold Spring Harbor Lab. publ., 1982.–545 p.
23. *Draper J., Scott R*. The isolation of plant nucleic acids // Plant Genetic Transformation and Gene Expression: A Laboratory Manual / Eds J. Draper et al.–Oxford etc.: Blackwell Sci. Publ., 1988.–355 p.

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