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Production of hairy root cultures of lettuce (*Lactuca sativa* L.)

Research Article

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Abstract: Hairy root cultures of lettuce (*Lactuca sativa L.*) were obtained by inoculation of cotyledonary leaves of *in vitro* lettuce seedlings (cvs. Nansen and Ljubljanska ledenka) with *Agrobacterium rhizogenes* A4M70GUS. Approximately in 96.7% cvs. Nansen and in 91.2% Ljubljanska ledenka inoculated explants produced hairy root when they were incubated on Murashige and Skoog (MS) half-strength medium without plant growth regulators. A total of 54% of all hairy root cultures expressed GUS activity. Every hairy root represented an independent transformation event. Line Ljubljanska ledenka 18 showed the highest biomass (5.5 times the biomass of control root). A PCR analysis of the genomic DNA confirmed the presence of marker and target genes in 15 hairy roots examined.

Keywords: *Genetic Transformation • Agrobacterium Rhizogenes • Lettuce*

Abbreviations

GUS – ß-glucuronidase;
MS – Murashige and Skoog medium;
X-Gluc – 5-bromo-4-chloro-3-indolyl-ß-D-glucuronide

1. Introduction

Lettuce (*Lactuca sativa* L.) is an annual plant, a leafy vegetable grown on nearly all continents. Lettuce (family *Asteraceae*) is believed to have originated from the Mediterranean. The evidence from Egyptian tomb paintings indicates that it was cultivated before 4500 BC. Lettuce classification is based on leaf shape and heading characteristics, though recently they have also been classified according to cropping seasons [1]. World lettuce production has been constantly increasing over the last years [2]. For instance, China produces almost half of the world's lettuce output, which amounts to 11 million Mt and is twice the size of the US output [3].

Genetic transformation is now a well-established technology. Like many other technologies, it has gone through different stages of evolution; scientific curiosity, research tool, novel applications and mass exploitation. The possibility using achievements in genetic engineering in scientific and applied medicine, veterinary and environmental science, seems very interesting. In this study genetic transformation with *Agrobacterium rhizogenes* was used for the introduction of exogenous genes into lettuce plants. *A. rhizogenes*-mediated infection of the plant tissue cultures received some attention 70 years ago as attempts were made to develop *in vitro* systems to study bacterial pathogenesis and hairy root [4]. Hairy root cultures have many advantages including biochemical and genetic

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stability, independence from seasonal and geographical conditions, rapid growth, and the ability to produce secondary metabolites at the levels comparable to those of the plants grown naturally. *A. rhizogenes* is used as genetic sources to create new genotypes and phenotypes with increased branching, wrinkled leaves, shorted internodes and modified flowering [5]. As a result, these cultures have been used to produce pharmaceuticals, cosmetics and food additives from many plant species [6–8]. Shih and Doran [9] demonstrated that hairy root is a feasible mean for *in vitro* propagation of plant virus, epitope vaccines and foreign proteins. Also, hairy root has a significant role in phytoremediation, a process that can remove or reduce the concentration of toxic organic and inorganic pollutants in soil, water and industrial effluents [7,10–16] (Figure 1).

Lettuce transformation is an effective tool for a lettuce breeding program [17]. Since Pillegi *et al*. [18], Ahmed *et al*. [19], Matvieieva *et al*. [20] and Lim *et al*. [17] reported *A. tumefaciens* transformation of lettuce we have developed the transformation with *A. rhizogenes* for the first time. The aim of this work is to exploit *in vitro* hairy roots of lettuce as a base for the development of phytoremediation systems – uptake phenols from water (mini bioreactor or hydroponics system for growing plants – after developing system for plant regeneration from hairy root).

2. Material and methods

2.1 Plant material and culture conditions

The seeds of *L. sativa* c.v. Nansen and Ljubljanska ledenka (Semenarna, Ljubljana) were sterilized in commercial bleach solution containing 5% (v/v) sodium hypochlorite for 15 min, followed by washing with sterile distilled water three times. The seeds were placed on half-strength basal MS medium (Murashige and Skoog, 1962) with 30 gl^{-1} sucrose, 100 mgl^{-1} myo-inositol, 7 gl^{-1} agar, pH 5.8 (25 ml/9 cm Petri dish; 20–30 seeds/dish). Seeds were germinated at 24 ± 2°C (under fluorescent light of 40 µmol $m^{-2}s^{-1}$ 16 h light / 8 h dark photoperiod). Cotyledonary leaves of 7-days old seedlings were used for the transformations.

2.2 Preparation of *A. rhizogenes* inoculum and induction of hairy roots

A. rhizogenes A4M70GUS carries the pRiA4 plasmid. This plasmid contains two T-DNA: T_L and T_R (Figure 2). The T_R-DNA consists of genes (*aux*1 and *aux*2) that control opine and auxin biosynthesis. The T_L-DNA consists of four loci (*rol*A, *rol*B, *rol*C and *rol*D). This was sufficient for producing the hairy root. The T_L-DNA has also *uid*A sequence (between *rol*C and *rol*D) driven by the 35S CaMV (*Cauliflower mosaic virus*) promoter followed by the nopaline synthase (*nos*) polyadenylation signal. *uid*A is reporter gene for enzyme ß-glucuronidase.

A. rhizogenes A4M70GUS were incubated on YEB medium (Yeast Extract Broth, [21]) pH 7.2, supplement with 100 mgl^{-1} neomycin. The bacterial strain was grown in darkness for 48 h at 24 ± 2°C and cultures were maintained at 4°C. Prior to co-cultivation with plant tissue, *A. rhizogenes* A4M70GUS were cultured in the same liquid media overnight at 28°C. Cotyledonary leaves were cut and incubated for 10 min in the bacterial suspension (OD_{600} of 0.6), dried with blotting paper, and cultivated on half-strength MS medium for five days in darkness at 24 ± 2°C. After co-cultivation, the explants were transsfered onto fresh same composition medium containing cefotaxime (300 mgl^{-1} Tolicar, Jugoremedia, Zrenjanin, Serbia) to remove bacteria. Formed roots were excised from cotyledonary leaves and transferred onto half-strength MS medium lacking plant growth regulators and sub-cultured on new medium every 30 days. The untransformed roots grown on the same medium were used as the controls.

2.3 Time-course of growth

A time course experiment was started with 0.15 g fresh weight (FW) of 15 days-old hairy root inoculated into 100 Erlenmayer flaks containing 40 ml half-strength liquid MS medium, on a gyratory shaker (110 r.p.m.), at 24 ± 2ºC, in the dark. Measurements were performed by harvesting roots every 5 days during 35 days of culture. The experiment was done in triplicate.

2.4 Statistical analysis

The data are presented as average values with their standard error. Means were compared by one-way analysis of variances (ANOVA).

2.5 GUS histochemical assay

GUS histochemical assays were performed using the method described by Jefferson *et al.* [22]. For assays, hairy roots were placed into individual wells of a 96-well microtitre plate, each well containing 200 µl of X-Gluc substrate at pH 7.0 and incubated for 24 h at 37ºC in the dark.

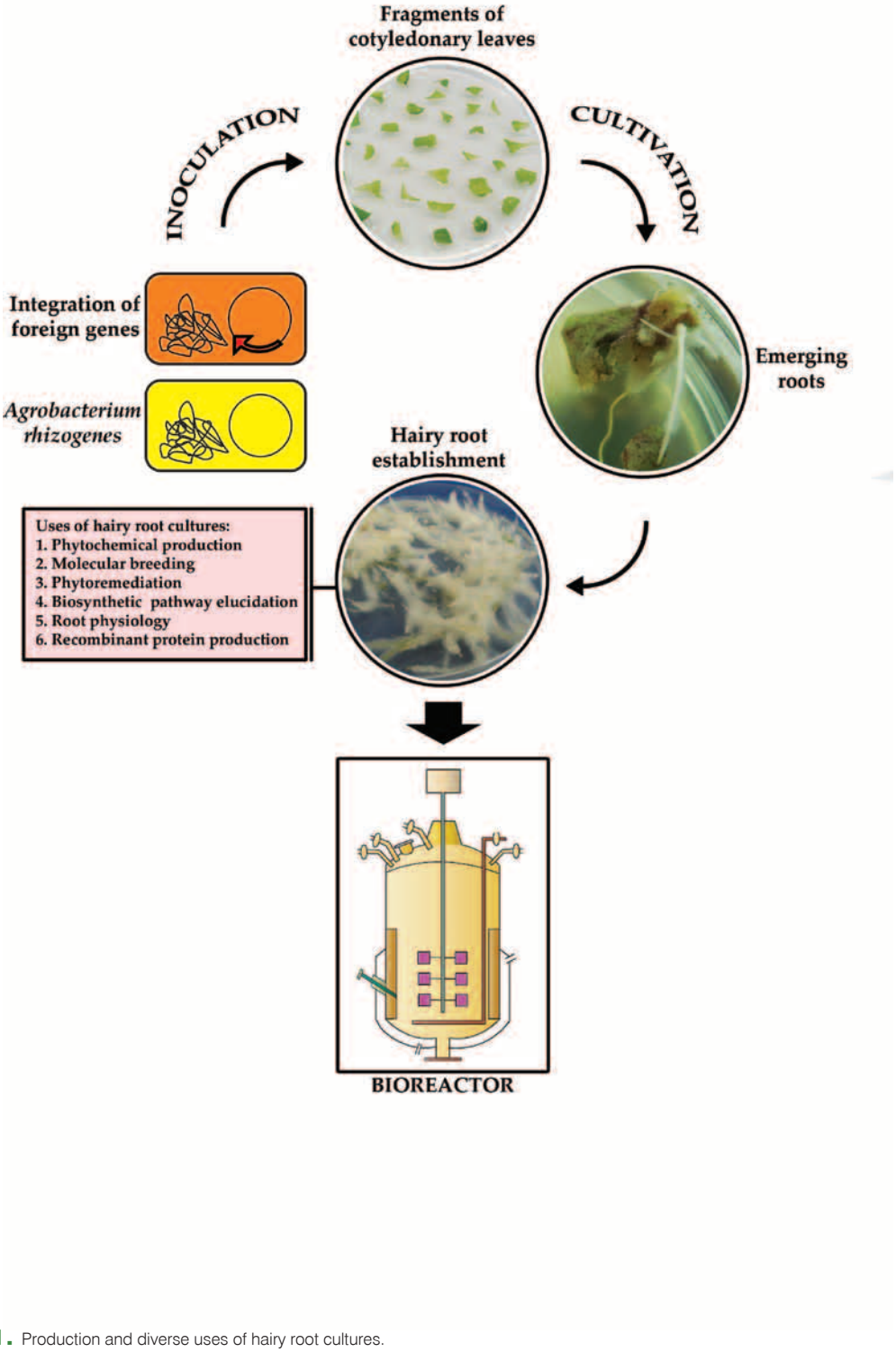

Figure 1. Production and diverse uses of hairy root cultures.

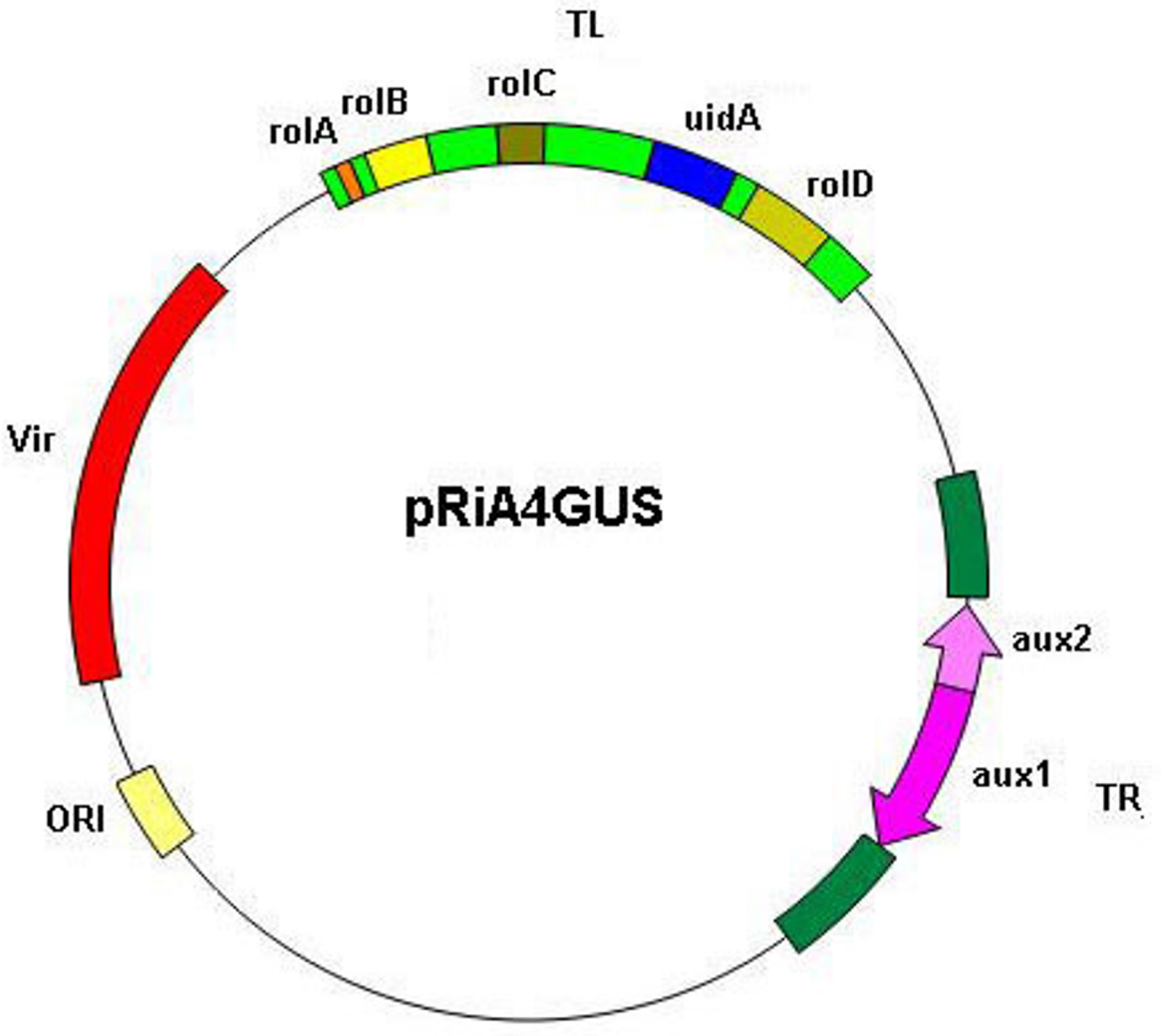

Figure 2. Diagram of pRiA4GUS with key genes.

2.6 PCR analysis

To confirm the transformation, genomic DNA was isolated from 200 mg of fresh hairy root using CTAB method described by Zhou *et al.* [23]. The following primers specific to the *uid*A, *rol*A, *rol*B and *rol*C genes (Table 1) were used for PCR. To confirm the absence of *Agrobacterium* from hairy root lines, PCR was carried out using primers specific for *vir*D1 gene (Table 1), located outside the T-DNA. PCR parameters were: initial denaturation temperature of 95ºC for 5 min followed by 40 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 2 min, with a final extension at 72ºC for 10 min using a thermal cycler (Techne, Genius). The amplified DNA products were visualized after electrophoresis on a 1.5% (w/v) agarose gel stained with ethidium bromide. Non-transformed roots, and *A. rhizogenes* A4M70GUS, were used as negative and positive control, respectively.

3. Results and discussion

3.1 Characterization of cultured hairy root lines

Roots appeared on cotyledon leaves of both cultivars lettuce 10 days after inoculation with *A. rhizogenes* A4M70GUS. The hairy roots were sub-cultured on a medium with cefotaxime 30 days after the inoculation. Every single root was considered as one line. After 30 days the percentage of explants which formed roots was very high (96.7% in Nansen and 91.2% in Ljubljanska ledenka). Similar transformation efficiency with *A. rhizogenes* A4M70GUS were reported earlier

Table 1. List of primers used for PCR analyses in this study

Gene	Primer sequences 5' -3'	Size of amplified fragment (bp)
uidA	CCC GGC AAT AAC ATA CGG CGT G	366
	CCT GTA GAA ACC CCA ACC CGT G	
virD1	ATG TCG CAA GGC AGT AAG	441
	CAA GGA GTC TTT CAG CAT G	
*rol*A	GTT AGG CGT GCA AAG GCC AAG	203
	TGC GTA TTA ATC CCG TAG GTC	
rolB	AAA GTC TGC TAT CAT CCT CCT ATG	348
	AAA GAA CGT GCA AGC TAC CTC TCT	
rolC	TAC GTA GAC TGC CCG ACG ATG ATG	342
	AAA CTT GCA CTC GCC ATG CCT CAC	

by Sretenović-Rajčić *et al*. [24] and Milošević *et al*. [25]. However, the effects of growth regulators on lettuce regeneration have not been fully evaluated, and no simple general procedure for the transformation of lettuce exists [17]. The efficiency of hairy roots formation depended on the strain of *A. rhizogenes* used and its interaction with plant species and tissue type [4,26,27]. Most plant tissues and organs, including the hypocotyl, leaf, stem, stalk, petiole, shoot tip, cotyledon, protoplast, storage root, and tuber, have shown capacity to be infected and genetically transformed by *A. rhizogenes*, with the resulting production of hairy roots [6,28–30]. However, the response varied depending on the *A. rhizogenes* strain and its interaction with the plant species and tissue type. In our experiments cotyledonary leaves of 7-days old seedlings were adequate explants. Momčilović *et al*. [31] used four species *Gentiana* (*G. acaulis*, *G. cruiciata*, *G. lutea* and *G. purpurea*) for the inoculation of shoots, leaves or decapitated stems with *A. rhizogenes*. Only shoots a few cm long responded by producing hairy roots, while others failed to respond.

The hairy root lines of lettuce grew on agar solidifid and liquid plant growth regulators-free medium, showing the typical features of hairy roots, plagiotropic growth, large number of root hair and lateral branches (Figure 3). The hairy root lines showed considerable phenoypic variations, particularly growth rate (Figure 4) and roots morphology. N2 line exhibited circularly hairy root growth on Petri dish in all subcultures. Some hairy roots were thin, with usual lateral roots and many root hairs (exsample line Ljubljanska ledenka 2). Non-transformed root cultures (Nc and Ljlc) had a very low growth rate. Morphological differences were observed not only between different cultivars of lettuce, but also between different lines (Figure 3). Similar observations were reported for *G. lutea*, *Impatiens hawkerii* [25,31]. In the early 1990s, it was demonstrated that a combination of *rol*A, *rol*B and *rol*C loci was sufficient to produce the hairy root phenotype, depending on plant species, tissue type and transformation conditions [27].

Injuries of plant tissue are a necessary precondition for the successful genetic transformation mediated by genus *Agrobacterium*. They present sites for

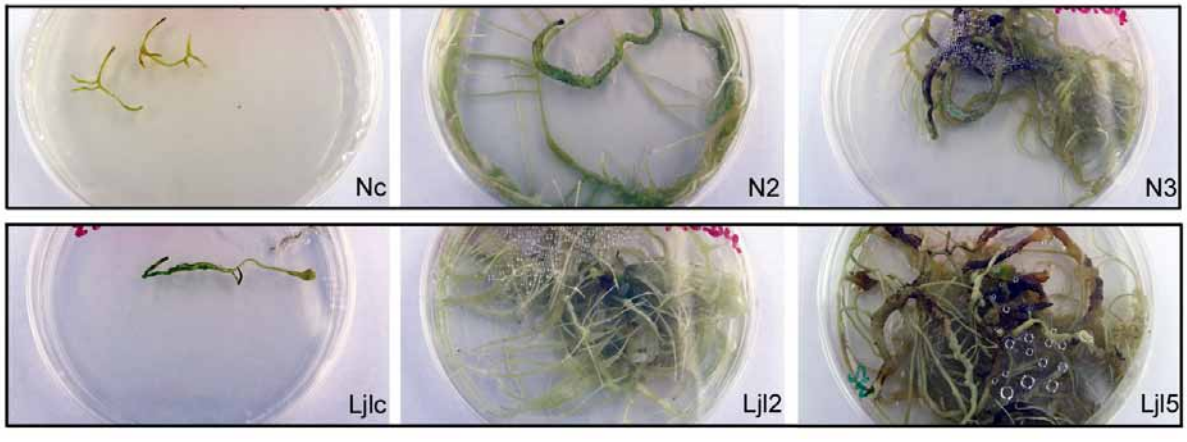

Figure 3. Growth of four weeks old hairy root culture of lettuce (Nc – Nasen control, N2, N3, Ljlc – Ljubljanska ledenka control, Ljl2, Ljl5).

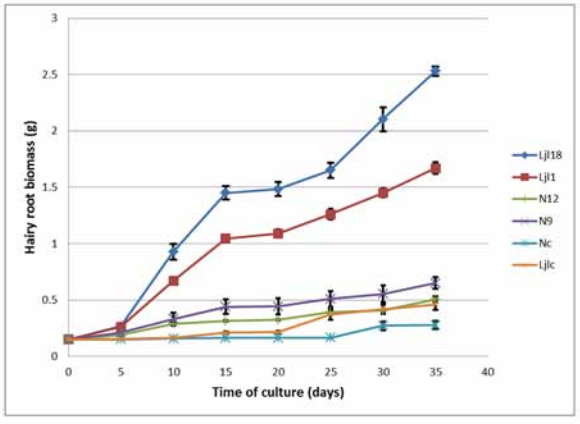

Figure 4. Time course of growth of *L. sativa* cv. Nansen and Ljubljanska ledenka fresh weight hairy root lines. Each point represents the mean of three replicates ± SD. Time of culture: 35 days.

the production of chemotactic signals which make bacterial transfer through the cell wall much easier, which is considered to represent a barrier [32]. Injuries of cotyledonary leaves caused by cutting probably contributed to high efficiency of induction of hairy root.

Substantial differences in the growth rate and biomass production among seven hairy root lines of each cultivar were observed after 35 days in culture. Index of the biomass increase varied considerably, ranging from 0.50 g per 40 ml half-strength liquid MS (Nansen 12 line) to 2.53 g (Ljubljanska ledenka 18 line). Line Ljubljanska ledenka 18 was found best for biomass accumulation, which was 5.5-fold higher than that of the control roots (Figure 4) and thus it was selected for further study of phytoremediation phenol from mini bioreactor systems. Tobacco hairy roots are able to remove phenol from solutions with its increasing concentrations (100-800 mg l^{-1}) [15]. However, *Brassica napus* hairy roots were able to remove phenol concentrations up to 500 mg l^{-1}, in the presence of H_2O_2, reaching high removal efficiency, within 1 h of the treatment and over a wide range of pH (4–9) [10]. Therefore, the variation in growth increment was disinguishable not only between the two examined lettuce cultivars, but also among different lines of the same cultivar. Among 14 lines (two the fastest growing lines from both cultivars and their controls are shown in Figure 4) extensively studied, in terms of biomass production line, Ljubljanska ledenka 18 grew more rapidly and branched more frequently than all others. Opposite to this line, growth analyses showed that line Nansen 12 had the lowest response. The variation in morphological and growth characteristics can also be attributed to the effect of *rol* genes. Line Nansen 12 has only *rol*C, while Ljubljanska ledenka 18 has three *rol* genes. Combination of *rol* genes is more efficient compared to their individual action, and induction of roots and their growth is more successful when *rol* genes act together, considering that they act synergistically [33]. *Rol* genes can take part in biosynthesis of plant growth regulators or they can increase plant sensitivity to those regulators [34].

All 14 lines demonstrated moderate production of biomass: the ratios of final fresh weights to their initial fresh weights were 3.33 to 16.87 for each subculture. Non-transformed root grew slowly and showed small growth rate (control of Nunsen 1.85 and Ljubljanska ledenka 3.07 times). Few studies have been performed on the analyses of hairy root growth [25,31,35–39]. Similar difference in growth rate of hairy roots lettuce was observed earlier [39]. The ratios of the final fresh weights to initial fresh weights of hairy roots *L. virosa* in Stojakowska *et al*. [39] investigation were 15.8. Hairy roots of *I. hawkerii* has biomass 26 times higher then non-transformed roots in liquid MS medium [25], biomass *G. lutea* was 6.9 to 18.8 times higher than control [31] but the highest ratio was found in *Scutellaria baicalensis* – 37 times [38]. In comparing of the two *L. sativa* cultivars, it seems that hairy root regeneration from cotiledonary leaves was better in Nansen, but growing of hairy roots in liqud medium was better in Ljubljanska ledenka cultivar.

3.2 GUS assay

GUS expression was used to evaluate the factors affecting transformation efficiency. All lines induced by *A. rhizogenes* A4M70GUS were subjected to GUS histochemical assay to determine the potential of lettuce cultivars to react with GUS stain. Positive X-gluc staining reaction was obtained for all lines, although the intensity of colour varied from lines to lines. GUS reaction was negative in the untransformed control roots (Figure 5). Positive GUS reaction is not reliable evidence of β-glukuronidase activity considering that endogenous enzyme in plant tissue can use same substrate and that most plant species express that enzyme at least in some tissues under certain conditions [40,41]. As a consequence this result is acceptable only as an indicator of the genetic transformation and further confirmed using other techniques or methods (*e.g.* by PCR analysis).

3.3 PCR analysis

All lines which failed to react with GUS stain were subjected to PCR analysis. All 15 lines, seven Nansen (Figure 6 A) and eight Ljubljanska ledenka (Figure 6 B) showed positive PCR amplification of *uid*A fragment of

366 bp (Figure 6). This result confirm the results of GUS assay. Presence of *rol*A gene was confirmed in five lines of c.v. Nansen and six of Ljubljanska ledenka (Figure 6).

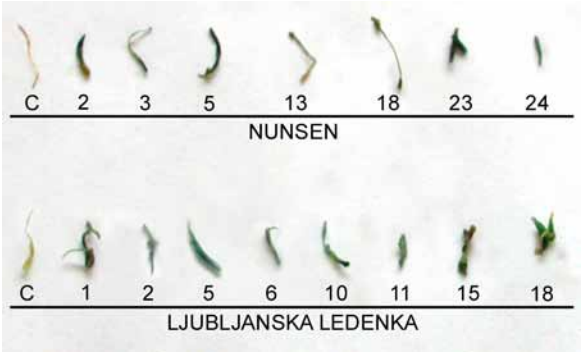

Figure 5. GUS histochemical assay of hairy root showing blue colouration in 15 lines lettuce and absence of GUS expression in control roots.

Whereas control roots did not shown any positive signal for 348bp fragment of *rol*B gene, two hairy root lines of Nansen and six of Ljubljanska ledenka were positive (Figure 6) indicating integration of this gene into the host cell genome. Amplicon of 342 bp corresponding to the *rol*C gene was obtained in the DNA of four Nansen and seven Ljubljanska ledinka lines (Figure 6). The presence of residual contaminating bacteria was checked by PCR analysis using *vir*D1 specific primer sequence. No amplification product was detected in any hairy root lines (Figure 6). However, positive control (Ar) showed amplificon of 441 bp *vir*D1 fragment.

Different characteristics among hairy root lines might be related to variation of the T-DNA insertion, *i.e.*, of the copy number and location of *Ri* genes integrated into the plant genom [42]. The perfect transformant would

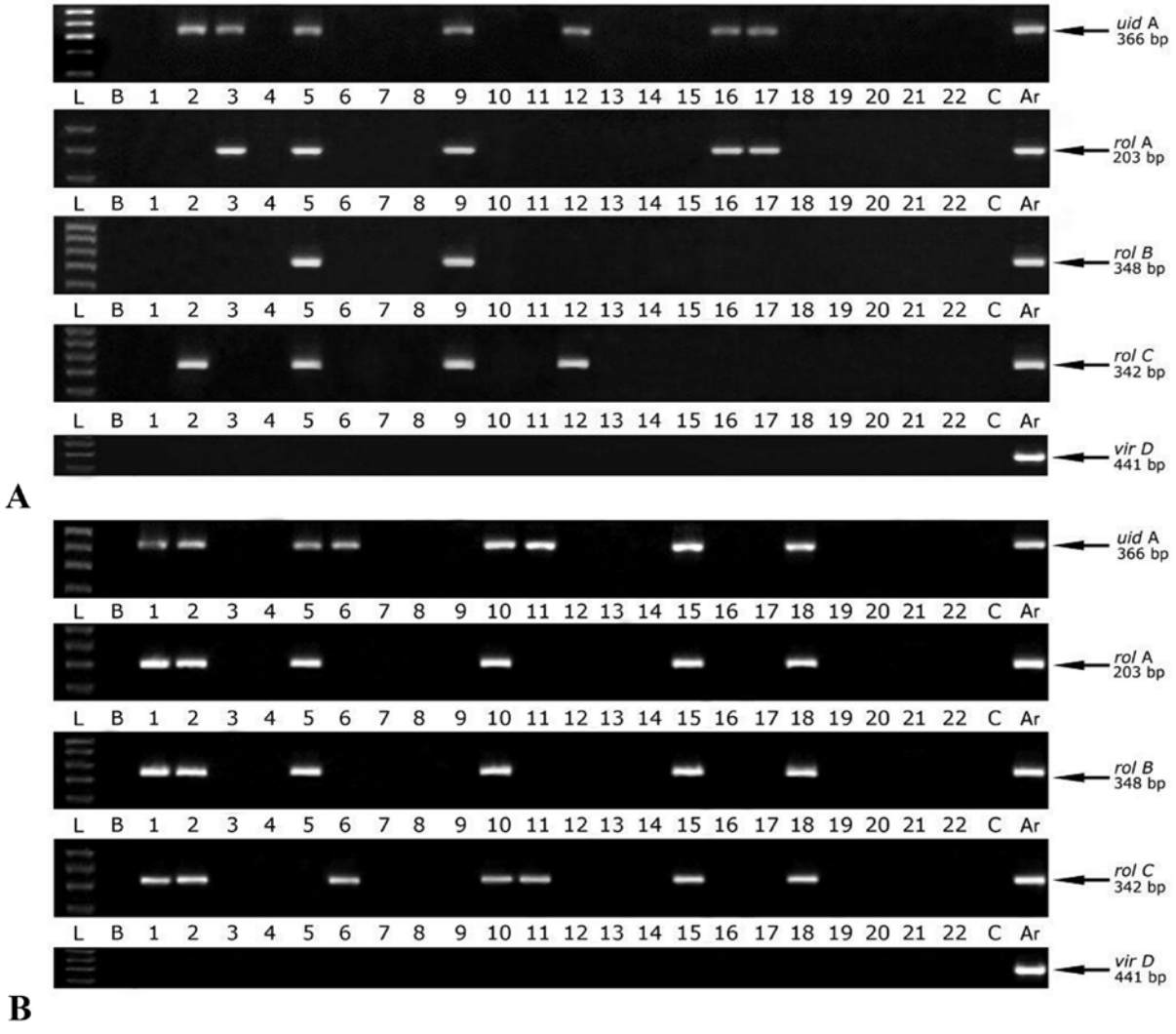

Figure 6. PCR analysis of genomic DNA isolated from hairy roots of lettuce cv. Nansen (A) and Ljubljanska ledenka (B). Lanes: L – ladder, B – blanc, 1-22 – hairy roots lines, C – untransformed control roots, Ar – positive control (pRiA4M70GUS).

contain a single copy of the transgene that would segregate as a mendelian trait, with uniform expression from one generation to the next. Ideal transformants can be found with difficulty, depending upon the plant material to be transformed and to same extent on the nature and the transgene complexity [43]. The different levels of their expression among hairy root lines could alter the endogenous auxins/cytokinins ratio or sensitivity to the previous plant growth regulator balance of each genotype [25, 44]. The estimation of a number of *rol* gene copies in genome of hairy root from lettuce, expression of *rol* and *aux* genes, and their influence on the morphology of obtained strains will be a subject of future studies. Plant regeneration from hairy roots on the plant growth regulators-free media often occurs spontaneously *G. cruciata* [27,31], *Blackstonia perfoliata* [48], *Brassica oleracea* [24], which is not the case in lettuce (similar to *G. lutea*, *G. acaulis* and *G. puntata* [31,45], *I. hawkerii* [25] and *Beta vulgaris* [46]. During the shoot regeneration process, plant growth regulators played a very important role. The ability of lettuce cultivars to regenerate may vary with the concentration and combination of growth regulators, particularly auxins and cytokinins [39]. Stimulatory effect of hormones on induction of organogenesis and shoot morphogenesis is goal of our future investigation.

Hairy root of various plant species can significantly detexify pesticides, antibiotics and other varius toxic industrial effluents [4,7]. Hairy root also have been used to compare the tolerance and removal of high levels of phenols [13,47]. Also, all regenerants from hairy root showed an increased ability to produce roots [49], which was very helpful in increasing root formation from the lettuce that can be using in phytoremediation process. In this context, first step of our investigation will be removal of phenol with hairy roots of lettuce from liquid medium for plant gowth with aim to developing model systems for bioreactors and hidroponic plant growth. This technology has been recognized as a cheap and eco-friendly alternative.

Acknowledgments

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