Agrobacterium-mediated transformation of cotyledonary leaf of lettuce (*Lactuca sativa* L.) by the *GCHI* gene

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ABSTRACT

Lactuca sativa is widely used as a leafy vegetable belonging to the Asteraceae family. Cotyledonary leaves of *in vitro* lettuce seedlings were transformed by plasmids containing the *GCHI* gene. Also the effects of number of days of pre-cultivation and co-cultivation of explants were examined on the transformation frequency. After co-cultivation, the explants were transferred onto the regeneration medium containing 200 mg/l ceftriaxone and 20 mg/l kanamycin. The best callogenesis was obtained in the MS medium containing 0.05 mg/l NAA and 0.4 mg/l BAP. Shoot regeneration was achieved after transferring the calli onto the MS medium containing 0.05 mg/l NAA and 0.4 mg/l BAP. Finally, the shoots were rooted on the MS medium without growth regulators. A PCR analysis of the genomic DNA confirmed the presence of *GCHI* genes in both T_0 and T_1 plants. PCR analysis of T_0 plants revealed that 10 out of 52 plants were positive for the *GCHI* gene. The transformation frequency was 19%.

Keywords: *Agrobacterium*, Co-cultivation, Tissue culture, Transgenic lettuce.

INTRODUCTION

Guanosine triphosphate cyclohydrolase I, named also GTP cyclohydrolase I (GCHI), mediates the first and committing step of the pterin branch of the folate-synthesis pathway. This enzyme is the primary enzyme in the biosynthetic pathway leading to dihydrofolate and the pathway driving to tetrahydrobiopterin, two essential metabolic cofactors (Gibson *et al*., 2004; Aline *et al*., 2009). Folic acid is accounted as an essential vitamin and plays a key role in the human body. This vitamin is not synthesized by humans and animals and mostly plant food is the main source of the vitamin (Storozhenko *et al*., 2005). The use of genetic engineering methods allows us to obtain new genetically modified plants. Lettuce belongs to the Asteraceae family and is a widely used freshly consumed leafy vegetable. It is one of the objects of genetic engineering, first of all, because of quite simple cultivation under *in vitro* conditions and secondly, development of effective regeneration methods (Michelmore and Eash, 1988). Successful genetic transformation has been reported through *A. tumefacience* in many plant species, such as freezing resistance in lettuce (Pileggi *et al*., 2001), glucuronidase gene (Haddad *et al*., 2002) and oxidative protection during senescence in oilseed (Haddad *et al*., 2004a), aphid resistance transgenic tobacco expressing *pta* gene (Yao *et al*., 2003), brown planthopper and green leafhopper resistance in Indica rice (Nagadhara *et al*., 2003; Nguyen *et al*., 2013). Transgenic lettuce plants have been obtained using *A. tumefaciens* (Briza *et al*., 2010; Mccabe *et al*., 1999), electroporation (Chupeau *et al*., 1989), PEG-induced transformation (Lelivelt *et al*., 2005) and bombardment methods (Kanamoto *et al*., 2006). An efficient method for constructing transgenic

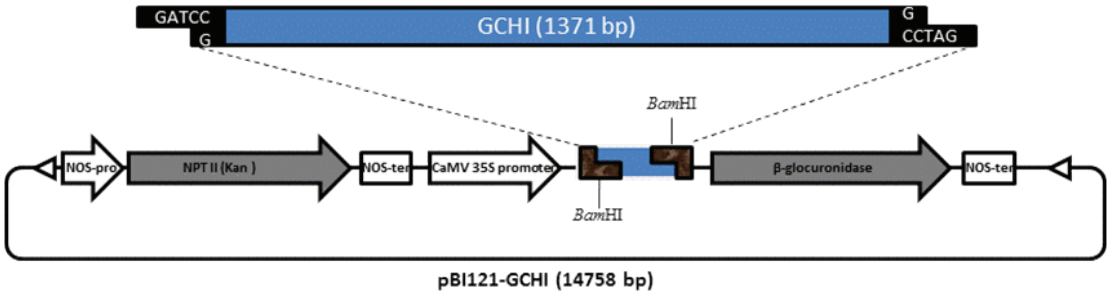

Figure 1. Recombinant pBI121 vector scheme carrying *GCHI* gene. The *GCHI* target gene had been cloned into the pBI121 vector plasmid in *Bam*HI restriction site downstream of the CaMV 35S promoter (Abofazeli *et al*., 2010).

lettuce cultivars using *A. tumefaciens* was described by Torres *et al*. (1993). Transgenic plants were developed to carry genes encoding the HBsAg antigen of hepatitis B virus in lettuce (Marcondes *et al*., 2008) and also the *GCHI* gene for increasing folic acid in developing wheat seeds (Shane *et al*., 2008). Lettuce plants carrying genes encoding the cholera toxin B subunit (Huy *et al*., 2011) and a tuberculosis antigen were also developed (Matvieieva *et al*., 2009; Matvieieva *et al*., 2012).

The aim of the present study was to introduce the *GCHI* gene into cotiledonary explants of lettuce by developing applied transformation procedures. Perior to the analysis of transformants, some *in vitro* factors i.e. medium composition, callogenesis, embryogenesis and shooting rate were evaluated to optimize plant regeneration.

MATERIALS AND METHODS

Agrobacterium strain and plasmid

The cDNA sequence of *GCHI* coding the first enzyme to synthesize folic acid was inserted into the pBI121 expression plasmid under the control of 35S promoter and NOS terminator, according to the method of Shane *et al*. (2008) (Figure 1). This recombinant vector pBI121-*GCHI* was transferred from *Escherichia coli* DH5α into *A. tumefaciens* strain pGV3101 (donated by Abofazeli, 2010).

Sensitivity test of explants to kanamycin and ceftriaxone

The sensitivity of lettuce cotyledon to kanamycin was assayed by culturing the cotyledons on the selection medium containing different concentrations of kanamycin (0, 10, 20, 30, 40 and 50 mg/l). Also, the sensitivity of *Agrobacterium* to ceftriaxone was assayed by culturing the *Agrobacterium* on the LB medium containing different concentrations of ceftriaxone (1, 5, 10, 15, 20, 50, 100, 150, 200, 300, 350 and 500 mg/l).

Plant material and tissue culture conditions

The seeds of lettuce (*L. sativa*) (Provided from Seed and Plant Improvement Institute of Karaj, Iran) were surface sterilized by 2.5% (w/v) clorox for 30 min, followed by washing with distilled sterile water 3 times. Then seeds were germinated on the blotting sterile paper and grown at 23 ± 2°C in a 16 h light and 8 h dark. The initial experiment was set up to optimize hormonal combinations in the regeneration medium from aseptically grown explants and cultured on the MS medium with different concentrations of NAA (0.05 and 0.1 mg/l) and BAP (0.1, 0.2 and 0.4 mg/l). All media were adjusted with 1 N NaOH to pH: 5.7, solidified with 7 g/l agar and autoclaved at 121°C for 20 min.

Transformation of lettuce cotyledonary explants

To carry out the transformation, transformed *Agrobacterium* cells were incubated on the LB medium supplemented with antibiotics (50 mg/l of each of kanamycin and ceftriaxone) for 16-24 h at 28°C. The optical density of bacteria inoculums was 0.8-1 at OD: 600 nm. Then, bacterial cells were centrifuged for 10 min at 4000 rpm. The pellet was resuspended in the same volume of liquid MS medium and left for 2 h, prior to the co-cultivation of explants.

Table 1. The MS medium composition for transgenic lettuce shoot regeneration.

Medium	Composition
MS1	30 g/l sucrose, 0.2 mg/l NAA, 0.2 mg/l BAP and 7 g/l agar
MS2	30 g/l sucrose, 0.05 mg/l NAA, 0.2 mg/l BAP and 7 g/l agar
MS3	30 g/l sucrose, 0.05 mg/l NAA, 0.2 mg/l BAP, 20 mg/l kanamycin, 200 mg/l ceftriaxon and 7 g/l agar
MS4	30 g/l sucrose, 0.05 mg/l NAA, 0.4 mg/l BAP, 20 mg/l kanamycin, 200 mg/l ceftriaxon and 7 g/l agar
MS5	30 g/l sucrose, 20 mg/l kanamycin, 200 mg/l ceftriaxon and 7 g/l agar

Table 2. Effect of the medium composition on transgenic lettuce root regeneration.

Medium	Composition	Growth rate (%)
MSR1	MS medium, 30 g/l sucrose, 20 mg/l kanamycin, 200 mg/l ceftriaxon	85
MSR2	½MS medium, 30 g/l sucrose, 20 mg/l kanamycin, 200 mg/l ceftriaxon	0
MSR3	MS medium, 30 g/l sucrose, 0.2 mg/l NAA, 20 mg/l kanamycin, 200 mg/l ceftriaxon	10
MSR4	½MS medium, 30 g/l sucrose, 0.2 mg/l NAA, 20 mg/l kanamycin, 200 mg/l ceftriaxon	0
MSR5	MS medium, 30 g/l sucrose, 0.5 mg/l NAA, 20 mg/l kanamycin, 200 mg/l ceftriaxon	0
MSR6	½MS medium, 30 g/l sucrose, 0.5 mg/l NAA, 20 mg/l kanamycin, 200 mg/l ceftriaxon	0

Table 3. Primers used to amplify the *GCHI* gene.

Primers	Primers sequnce (5'→3')	Size of amplified fragment (bp)
1F-GCHI	AACGGATCCATGGGCGCATTAGATGAAGG	1389
1R-GCHI	AGCGGATCCTCATCTTCCTGCAGAACCAG	

Cotyledonary leaves from 3 day-old seedlings were cut into 0.5 × 0.5 cm diameter and explants were left on the pre-cultivation MS1 medium (Table 1) for 0, 2, 4 and 6 days prior to the co-cultivation with the inoculum. Then, explants were inoculated with the *Agrobacterium* cells harboring strain pGV3101 having *GCHI* gene in the liquid MS medium for 2 min, dried with blotting paper and cultivated on the co-cultivation MS2 medium (Table 1) for 1, 2 and 4 days. These experiments were set up to determine the effect of time of the recultivation and co-cultivation of explants with *Agrobacterium* on callogenesis, embryogenesis and shoot regeneration.

Subsequently, explants were transferred onto the selection and regeneration MS3 medium (Table 1) and incubated under a 16/8 h light / dark photoperiod in the growth room. After 4 weeks, calli were transferred into the MS4 medium for embryogenesis and shoot regeneration. Then, kanamycin resistant shootlets were separated and transferred onto the MS5 medium for rooting. Since roots did not develop well, 6 different combinations of medium were examined for the rooting of the transformed shootlets (Table 2).

Molecular analysis

The presence of transgene was analyzed by the polymerase chain reaction (PCR) using specific primers in transformed and control (non-transformed) plants (Table 3). Genomic DNA was extracted from the green leaves of sterile lettuce plants using a CTAB method

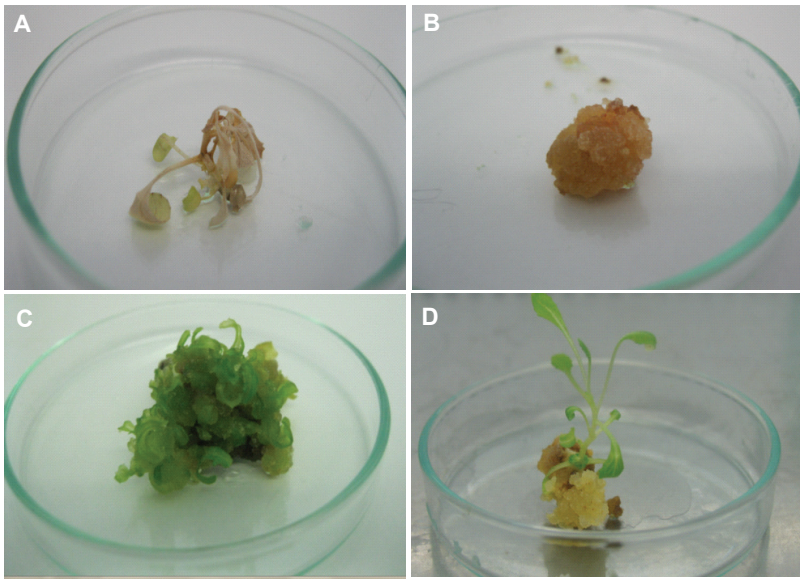

Figure 2. Selection and plant regeneration of transformed lettuce with *GCHI* gene. **A:** Cotyledonary explant on the callogenesis medium contaning 20 mg/l kanamycin and 200 mg/l ceftriaxone; **B:** Callogenesis in cotyledonary explant on the callogenesis medium (6 weeks after culture); **C:** Embryogenesis of a callus on the embryogenesis medium; **D:** Transgenic T_0 plantlet regenerated from transformed cotyledons.

(Matvieieva *et al*., 2009). Thermal cycling included 1st denaturation at 94°C for 5 min, followed by 35 cycles of 1 min at 94°C for denaturation, 1 min at 55°C for annealing, 2 min at 72°C for extension, and finally incubated at 72°C for 10 min.

Experiments were carried out as factorial based on the complete random design with three replications and data analysis was performed by SPSS software. Twenty petri dishes carrying 5 explants each were prepared for both transformation and regeneration.

RESULTS AND DISCUSSION

Effect of kanamycin and ceftriaxone concentration

Kanamycin (20 mg/l) completely blocked regeneration from untransformed explants and therefore, could be used to select transformed cells (Figure 2A and B). In some other similar reports, researchers used 25 mg/l (Matvieieva *et al*., 2009) and also 50 mg/l (Ahmed *et al*., 2007) kanamycin for selecting transformed cells. In the present study, preliminary transformation experiments indicated that ceftriaxone (200 mg/l) severely inhibited *Agrobacterium* regrowth on the cotyledonary explants and 200 mg/l ceftriaxone was found to be optimum. It seems that such a concentration of this antibiotic is sufficient for the inhibition of *Agrobacterium* growth after co-cultivation with the plant materials. In other similar studies, researchers have used 50 mg/l ceftriaxone to inhibit *Agrobacterium* growth after co-cultivation with plant material (Ahmed *et al*., 2007). Ceftriaxone could be recommended as an alternative to cefotaxime for its less cost and almost the same effects (at least in our study) to inhibit *Agrobacterium* re-growth.

Standardization of plant selection and regeneration from lettuce cotyledonary explants

As Table 4 shows, the highest percentage (100%) of explants showing callogenesis were obtained on the MS medium containing 0.05 mg/l NAA and 0.2 mg/l BAP (Figure 2B). Also, the highest percentage (85%) of calli showed embryogenesis and 60% of the embryos exhibited shooting on the MS medium containing 0.05 mg/l NAA and 0.4 mg/l BAP (Figure 2C, D). As shown in Table 5, the ANOVA analysis illustrated a significant difference at 0.01 probability for both NAA and BAP. However, NAA × BAP interaction was not significant. Similar results have been reported by other researchers (Lelivelt *et al*., 2005). The highest percentage (85%) of explants that exhibited rooting was observed on the hormone free MS medium containing 30 g/l sucrose, 20 mg/l kanamycin and 200 mg/l ceftriaxone. This result

Table 4. Effect of medium composition on the percentage of transgenic lettuce plants.

Medium	Concentration of growth regulators (mg/l)		Characters		
	NAA	BAP	Callogenesis (%)	Embryogenesis (%)	Shooting (%)
M1	0.1	0.1	51	43	31
M2	0.1	0.2	83	59	44
M3	0.1	0.4	90	65	39
M4	0.05	0.1	89	61	48
M5	0.05	0.2	100	74	45
M6	0.05	0.4	79	85	60

Table 5. Analysis of variance on callogenesis, embryogenesis and shooting in cotyledonary explants.

Source of variation	DF	Mean of square		
		Callogenesis	Embryogenesis	Shooting
NAA	1	800.00**	1184.222**	813.389**
BAP	2	751.167**	681.556**	162.889**
NAA × BAP	2	1037.167**	13.556^{ns}	141.556**
Error	10	8.4	4.389	3.056
C.V. (%)		3.59	3.28	4.04

** Significant at 0.01 probability level, ^{ns} non significant.

Table 6. Effects of Co-cultivation period on survival percentage of cotyledonary explants lettuce. The regeneration medium contained 20 mg/l kanamycin and 200 mg/l ceftriaxone.

Explant type	Co-cultivation period (days)	Surviving explants (%)
Leaf cotyledon	0	0
	2	71
	4	63
	6	61

agrees with Matvieiva *et al*. (2012) findings. The shoots regenerated from kanamycin resistant calli were continuously proliferated by stem cutting on the MS medium. However, it was rather difficult to induce enough roots in such shoots, and thus their acclimatization and transplantation into soil was tedious. After acclimatization, they were grown to the flowering stage in a growth chamber by changing the photoperiod from short (8 h) to long day (16 h) conditions. They successfully produced T_1 seeds, 65% of which germinated on the MS medium containing 20 mg/l kanamycin. The grown plants appeared normal in morphology.

Genetic transformation of cotyledonary explants

Cutting explants into 2 pieces improved the chance of infection by *Agrobacterium* and transformation efficiency (Matvieieva *et al*., 2012). The best time for precultivation of explants was 2 days, to improve transformation by the contact between plant and bacteria (Table 6). Cotyledons were inoculated for 2 min with the *A. tumefaciens* cells harboring strain pGV3101. The transformation frequency was 19%. In some reports, explants were inoculated with *A. tumefaciens* strain C58 pGV3101 in *Brassica napus* and *Arabidopsis thaliana* (Haddad *et al*., 2004b), EHA105 (Hua Boa *et al*., 2009) and LBA4404 in spinach (Naderi *et al*., 2012) at various incubation periods to obtain healthy transformants. In a research, the post infection process, an incubation period of 3 min was selected in the subsequent transformation experiments (Ahmed *et al*., 2007). Moreover, the optical density of *Agrobacterium* inoculums was

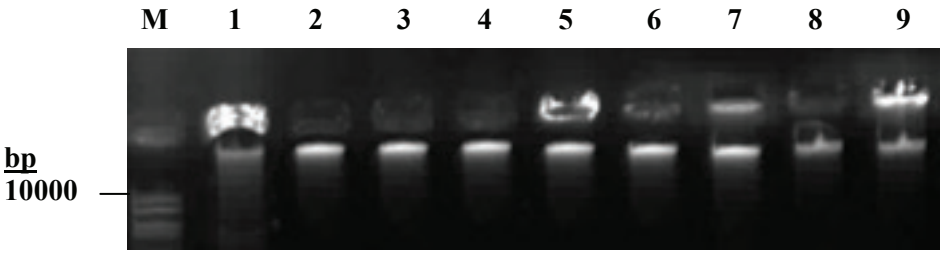

Figure 3. Genomic DNA was extracted from T plants growing in MS medium containing 20 mg/l kanamycin and 200 mg/l ceftriaxone for molecular analysis of transformants.

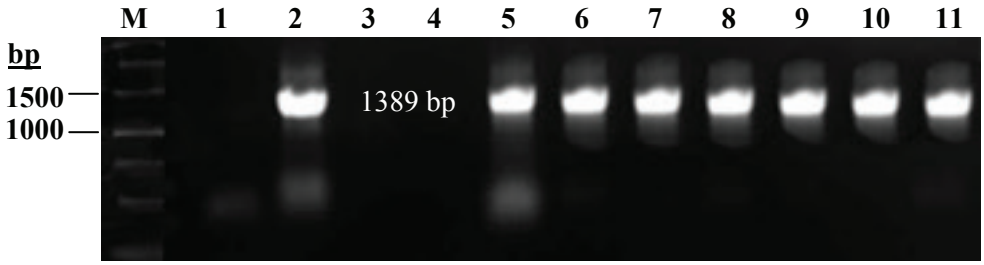

Figure 4. PCR analysis of genomic DNA extracted from T_0 lettuce plants to detect the presence of *GCH1* gene. The PCR products were separated on a 1.2% agarose gel. M:1 kbp size marker; 1: nonrecombinant plant; 2: positive control; 3 and 4: negetive controls; 5-11: T_0 trangenic plants containing the *GCH1* gene.

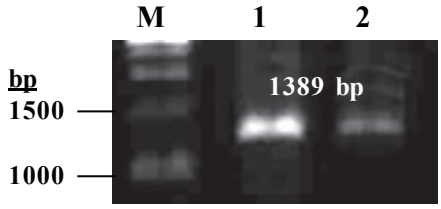

Figure 5. PCR analysis of genomic DNA of T_1 plants (Lane 1 and 2) showing the presence of *GCH1* gene. (M: 1 kbp size marker).

0.8-1 at 600 nm. In another study the optical density of *Agrobacterium* inoculums was reported 1.5 at 600 nm (Matvieieva *et al*., 2012). No acetosyringone or syringaldehyde was used to stimulate bacterial transformation, since these compounds do not increase the transformation rate in lettuce (Torres *et al*., 1993). As lettuce itself releases high amounts of different phenolics it does not need such compounds. Similar to the reference explants, 96 h of co-cultivation period was optimized for lettuce explants inoculation in the present study. The cotyledonary explants were washed with MS medium containing 20 mg/l kanamycin and 200 mg/l ceftriaxone and transferred onto the selection medium. Similar results were reported in raspberry (Cao *et al*., 1998), and in peanut (Briza *et al*., 2010). Pre-cultivation also showed a positive effect on transformation. In a similar way, other researchers reported that when seedlings were precultivated for 4-6 days, the level of GUS transient expression was significantly greater than that of the control (Moralejo *et al*., 1998). For developing a highly efficient method of transforming embryogenic callus, shoot explants have been co-cultivated for 48 h in cotton (Shuangxia *et al*., 2005), 72 h in *Odontoglossum ringspot* (Zhang, 2010), 96 h in spinach (Nagadhara *et al*., 2003) and 48 h in tobacco (Matvieieva *et al*., 2010; Shuangxia *et al*., 2005. Matvieieva *et al*. (2010). Shuangxia *et al*. (2005) further reported that the improvement of DNA uptake could be due to the stimulation of cell division by the hormones in the precultivation medium, since mitotic cells would be more susceptible to *Agrobacterium* and would have a higher level of transcription. Pre-cultivation of the explants on the regeneration medium prior to the inoculation of *Agrobacterium* increased the frequency of transformation in pigeon pea to 62% from cotyledonary nodes and 27% from shoot tips (Geeta *et al*., 1999), 28.8% from callus and 1.2% from regenerated shoots (Lawrence and

Table 7. Effects of recultivation and Co-cultivation period on callogenesis, embryogenesis and shooting percentage of cotyledonary explants. The regeneration medium contained 20 mg/l kanamycin and 200 mg/l ceftriaxone.

Recultivation period (day)	Co-cultivation period (day)	Callugenesis (%)	Embryogenesis (%)	Shooting (%)
0	1	0	0	0
	2	0	0	0
	4	0	0	0
2	1	11	0	0
	2	91	39	19
	4	98	53	53
4	1	89	31	9
	2	88	59	10
	4	96	44	11
6	1	89	22	0
	2	95	32	29
	4	91	31	33

Table 8. Analysis of variance on precultivation and Co-cultivation in callugenesis, embryogenesis and shooting in cotyledonary explants.

Source of variation	DF	Mean of square		
		Callugenesis	Embryogenesis	Shooting
Precultivation	3	4766.582**	1876.852**	728.704**
Co-cultivation	2	2152.778**	625.00*	936.111**
Precultivation × Co-cultivation	6	2326.852**	565.741**	306.481**
Error	22	78.535	120.455	23.990
C.V. (%)		13.57	52.68	47.69

** Significant at 0.01 probability level, * Significant at 0.05 probability level.

Koundal, 2001). In our study, explants changed color to yellow and died after several days, when they were immersed in the inoculation liquid immediately after being removed from the plantlet. The lowest percentage of transgenic plants was obtained when explants were placed in the pre-cultivation medium for 6 days and in the co-cultivation medium for 1 day, respectively. Simple and interaction effects of pre-cultivation, co-cultivation, considering callogenesis, embryogenesis, and shooting had significant effects at 0.01 probability level (Tables 7 and 8). However, co-cultivation × embryogenesis had a significant effect at 0.05 probability.

Molecular analysis of transgenic plants

To confirm transgenic plants, DNA was extracted from the regenerated plants selected in the presence of Kanamycin (Figure 3). PCR analysis of transformants revealed that 19% of the regenerated plants were positive for the *GCHI* gene (Figure 4). Transgenic lettuce seeds were cultivated in the ½MS medium containing 20 mg/l kanamycin and the DNA was extracted from leaves. T_1 plants were obtained from *in vitro* T_0 selected transformed plants under constant selective pressure of kanamycin. However, previous papers reported different results (Pileggi *et al*., 2001; Ahmed *et al*., 2007; Aline *et al*., 2009). PCR analysis heridity revealed that T_1 progeny were transformed for the *GCHI* gene successfully (Figure 5). Therefore, we optimized the heridity protocol of the indirect regeneration of plants from the cotyledonary leaves of lettuce.

Based on results, 0.05 mg/l NAA and 0.4 mg/l BAP caused an increase in regeneration. The pre-cultivation for 2 days and 4 days of co-cultivation brought about the best result of transformation. PCR analysis of the genomic DNA revealed the presence of the target gene

in the 19% of the examined plants.

In summary, *A. tumefaciens* can be used for transferring the target gene into lettuce plant genomes. The co-cultivation period illustrated a significant positive effect on the efficiency of transformation in lettuce. By increasing co-cultivation time, the rate of transformed plants increased. We optimized the protocol of the indirect regeneration of plants from the cotyledonary leaves of lettuce. We used a local lettuce cultivar which has the high regeneration potential, as the plant material, with the availability of a rapid and efficient transformation-regeneration system from cotyledons. It is now feasible to introduce genes for desirable traits.

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