

Review

## Genetic transformation of lettuce (*Lactuca sativa*): A review

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Lettuce (*Lactuca sativa* L.) is a globally important leafy vegetable that can be grown worldwide. Due to the rapid growth of population and the human desire to progress, there have been a lot of studies made by researchers, especially in genetic engineering. Improvements in regeneration system and transformation methodology have helped to increase the transformation efficiency and stable expression of transgenes in lettuce. Lettuce transgenic research carried out so far has mainly focused on using lettuce bioreactor to produce pharmaceutical protein and vaccines, improving nutritional and physiological value of lettuce. There are no comprehensive and detailed reviews available combining research developments with major regeneration system and basic genetic transformation in lettuce. This is an attempt to overview the progress in regeneration system, genetic transformation and biotechnological applications in the last decades as well as future implications.

**Key words:** Lettuce, regeneration system, genetic transformation, bioreactor.

### INTRODUCTION

Lettuce (*Lactuca sativa* L.) belongs to the Asteraceae family, one of the major crops grown worldwide. The plants often have a height of 15 to 30 cm, with colorful leaves running from bright green to red and yellow. Lettuce also have a wide range of shapes and textures,

from the dense heads of the iceberg type to the notched and scalloped (Martha, 2011). It is low in calories, good-tasted and nutritive, which is a good source of vitamin A, vitamin K and potassium to the human population. Its stems and leaves contain many active

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**Abbreviations:** 2,4-D, 2,4-Dichlorophenoxyacetic acid; 6-BA, 6-benzylaminopurine; hpt, hygromycin B phosphotransferase; HPLC-ELSD, high performance liquid chromatography-evaporation light-scattering detection; IAA, indole-3-acetic acid; NAA, 1-naphthyleneacetic acid; nptII, neomycin phosphor-transferase II gene; PPT, phosphinothricin; AI, avian Influenza; A.T., *Agrobacterium tumefaciens*-mediated transformation; ChIFN- $\alpha$ , chicken alpha interferon- $\alpha$ ; DAS-ELISA, double Antibody Sandwich-Enzyme Linked Immunosorbent Assay; ELISA, enzyme linked immunosorbent Assay; FMD, foot-and-mouth disease; GUS, glucuronidase; HBV, hepatitis B virus; HIV, human immunodeficiency virus; HPLC, high performance liquid chromatography; Human IFN, human interferon; Hyg, hygromycin; IL-2, interleukin-2; Km, kanamycin; Pb, projectile bombardment; PCR, polymerase chain reaction; PPT, phosphinothricin; RT-PCR, reverse transcription polymerase chain reaction; sCT, salmon calcitonin; SARS, severe acute respiratory syndrome; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Spec, spectinomycin.

ingredients such as mannitol (which take effects on diuretic and blood circulation promotion) and lactucerin (which play a role on hypnosis, analgesia, and adjuvant treatment of neurasthenia). Nowadays, extracts from *Lactuca sativa* L. have been used for curing sunburn and rough skin in creams and latexes (Odu and Okomuda, 2013).

However, plant diseases and insect pests are standing out in the cultivation and production of lettuce. Plant diseases seriously affect the quality and yield of lettuce. For example, *Lactuca sativa* *Sclerotinia* has occurred in the world of lettuce, which seriously harmed the basal part of stems and leaves (Waipara, 2006; Chitrampalam et al., 2010). So many experts committed themselves to studying transgenic lettuce. Various types of genes which were transferred into lettuces, being expressed and stably inherited in progenies, are summarized in Table 1.

Lettuce used as a foreign protein expression system has the following advantages (Guo, 2006):

- i) Exogenous gene expression product could be successfully post transcriptionally processed and modified with low cost and is relatively safe,
- ii) Lettuce is a well-loved vegetable that can be eaten directly. So lettuces with special functions could be used for the prevention or treatment of diseases, relieve the patients' spiritual and economic pressure as much as possible,
- iii) Lettuce resistance to cold ecological environment can be cultivated over a wider area, easy to scale and are grown almost all over the world,
- iv) The genetic transformation technology of lettuce is relatively mature, which provides favorable conditions for the use of lettuce to express foreign protein.
- v) As a plant bioreactor, lettuce's production cycle is shorter than a lot of plant, the production superiority of fast speed, simple condition and low cost will stand out if used in a large scale of producing some protein or vaccine.

## REGENERATION SYSTEM

An efficient and stable regeneration system is the basis of most genetic transformation technologies such as *Agro-bacterium* mediated and micro-projectile bombardment transformation. The tissue culture of lettuce starts early in lettuce and organogenesis has been the extensively used pathway compared to somatic embryogenesis for its wider adaptability among diverse genotypes. Protocols for obtaining stable regeneration in lettuce have been reported through organogenesis from callus (Gao, 2003), differentiated non meristematic tissues like leaf (Gao et al., 2011; Liu et al., 2011) and various seedling explants such as hypocotyls (Gao et al., 2002), cotyledons (Luo et al., 2010; Chen et al., 2012). A suitable transformation regeneration system should have

adequate source of explants, genetic stability of the regenerated plants, great regeneration ability, sensitive to *Agro-bacterium* or other conversion, and a modest antibiotic sensitivity. In the establishment of lettuce regeneration system, genotype, different hormone groupings, explant type, seedling age and other factors have been studied by researchers. Although some reports have shown a higher frequency of regeneration, materials or other reasons lead to poor reproducibility, this article reviews the relevant factors needed to accelerate and facilitate the application of this technique in lettuce transformation.

## GENOTYPE/CULTIVAR

It is well known that genotype has been the major factor which can significantly influence the regenerative capacity of explants. As in many other crops, shoot production in lettuce is also genotype-dependent. The frequency of shoot regeneration from cotyledon explants ranges from 84.6 to 100% of 4 lettuce species (Zhu et al., 2002). Explants of 5 cultivars were cultured under similar culture conditions, but only two lettuce cultivars (TN-96-39, TN-96-41) showed the best callus production, embryogenesis, regeneration and proliferation (Honari et al., 2008). The regeneration response in similar culture media showed big variability from cotyledon explants between the genotypes, differential frequency of regeneration ranging from 51 to 96% of 15 lettuce cultivars (Denise et al., 2002). Such variations have also been reported for many other genotypes. Genotype dependency for shoot regeneration has also been evidenced from leaf explants of *Lactuca sativa* L. cv. America Head Garden, America Violet Leaf and America Big Leaf Hydrangea (Liu et al., 2011). The effect genotype has on a species capacity to respond to shoot production has been recognized, so select an appropriate genotype is considerably important on the smoothness of studying genetic transformation.

## EXPLANT TISSUE

Differential sources of explants have been used for the induction of shoots in lettuce, including leaf, hypocotyl, root, stem, cotyledon and cotyledon petiole. By using same genotype; a regeneration frequency of 65, 85 and 95% was achieved through hypocotyl, cotyledon and cotyledon petiole, respectively, and the shoots in cotyledon and hypocotyl explants failed to respond greater than cotyledon (Li, 2007). Similar results were obtained with explants such as leaf, stem and root in Grand Rapids, meanwhile the rate of callus induction and bud generation from explants of leaves were significantly better than root and stem explants (Gao et al., 2011). However, an opposite conclusion appeared that the time and number of buds germination of cotyledon explants

**Table 1.** Genetic transformation of lettuce.

Genotype	Method	Detection	Selection	Plasmid vector	Function	Reference
Grand rapids	A.T.	PCR, PCR-Southern, RT-PCR	Hyg	pCAMBIA1301	FMD	Deng et al. (2007)
VitoriadeVerao	A.T.	PCR	Km	PG35SHBsAg	HBV	Jackson and Ekkehard (2008)
Glass lettuce	A.T.	PCR, RT-PCR	Km	pBI121-NA	AI	Fan et al. (2012)
Beijing lettuce	A.T.	PCR, PCR-Southern	Km	pEbislycBHyg, pBI121-gg	HIV	Jing et al. (2007)
Lactuca sativa	A.T.	PCR, Northern blot, Immunoblot, GM <sub>1</sub> -ELISA	Km	pMYV514	Cholera toxin	Huy et al. (2011)
Simpson elite	Pb	Southern blot, western blot, GM <sub>1</sub> BA, histochemistry, immunohistochemistry	Spec	pLS-LF-CTB-Pins	Cholera toxin	Tracey et al. (2007)
Snezhinka et al.	A.T.	PCR, RT-PCR	Km	pCB063, pCB064	Tuberculosis	Matvieieva et al. (2009)
Green Wave	A.T.	PCR, Southern blot, RT-PCR, ELISA, Western blot, Test animal trails	Km	pBIF-V	Plague	Sergio et al. (2010)
Lactuca sativa	A.T.	PCR, Northern blot, Western blot, Laser-Scanning Ionfocal Microscopy	Spec	pCV1, pCV2, pCV12	SARS	Li et al. (2006)
Lactuca sativa	A.T.	PCR, Northern blot, Immune blot, ELISA, GM <sub>1</sub> -ELISA	Km	pMYO51	Heat-labile enterotoxin	Kim et al. (2007)
Lactuca sativa	A.T.	PCR, Immune blot, ELISA, Western blot, FAS, Mouse feeding assay	Km	pBI121- <i>espA</i>	Enterohaemorrhagic <i>E. coli</i>	Luan et al. (2009)
Japanese soft glassy et al.	A.T.	GUS assay, PCR, SDS-PAGE, Western blot, Antiviral activity assay	Km	pBI121-IFN	Human IFN	Li et al. (2007)
America Grand rapids et al.	A.T.	PCR, RT-PCR, HPLC, GC-MS	Km	pBI-RS	ResveratrolSynthase	Zhu, (2008)
Zhouye	A.T.	PCR, Southern blot, RT-PCR	Km	p35S-2300-twinT-DNA::pil-msCT::noster	sCT	Cui et al. (2009)
Grand rapids	A.T.	PCR, ELISA, Differential spectroscopy	Km	pGBIVHbTα1hLf, pGBIRVHbTα1hLf	Human lactoferrin and Thymosin	Meng et al. (2005)
Lactuca sativa	A.T.	PCR, RT-PCR	Km	pBI-121-NK	nattokinase	Tian, (2007)
Lactuca sativa	A.T.	PCR, Southern blot, ELISA, Western blot	PPT, Km	-	IL-2	Yang et al. (2008)
Lactuca sativa	A.T.	GUS assay, RT-PCR, ELISA	Km	pSFIFN-α	ChIFN-α	Song et al. (2008)
Italian Yearly Late Bolt	A.T.	PCR, RT-PCR, HPLC-ELSD	Km	p2301-GMP-myc	Vitamin C	Wang et al. (2011)
<i>longifolia</i> Lam.	A.T.	PCR, RT-PCR, HPLC	Km	pCAMBIA2300-35S::LsHPT::NOS	Vitamin E	Ren et al. (2011)
Grand rapids	A.T.	PCR, Southern blot, RT-PCR, AA analysis	Km	pBI121-lrp	Lysine	Li et al. (2006)
Romaine	A.T.	RT-PCR, HPLC, <i>Lactobacillus casei</i> fermentation	Km	pFSndt5100- <i>Atp</i> sy- <i>foIE</i>	Carotenoids and Folic Acid	Fu et al. (2012)

Table 1. Contd.

Huaxuan No. 1	A.T.	PCR, Southern blot, RT-PCR, Nematode, Histological Analysis	Km	pBI121	Resistance to Root Knot Nematodes	Li et al. (2010)
Evola	A.T.	PCR, RT-PCR	Km	pBIPTA	Resistance to Aphids	Ahmed et al. 2007
Kaiser	A.T.	DAS-ELISA, Western blot, Southern blot, Northern blot	Km	pYK23	Resistance to <i>Mirafiori</i> virus	Yoichi et al. (2009)
Verônica	A.T.	PCR, RT-PCR	Hyg	pCambiaOxDc	Resistance to <i>Sclerotiniasclerotiorum</i>	Dias et al. (2006)
Chongchima	A.T.	Histochemical GUS staining, Southern blot, Northern blot, Drought and cold stress tests	Hyg	pCUMB	Resistance to drought and cold	Enkhchimeg et al.(2005)

were markedly better than leaf (Zhu et al., 2002).

These two opposite results demonstrated that genotype also influenced the induction of different explants tissue. Song et al. (2007) found a holographic phenomenon when using different part of cotyledon explants, the rate of bud induction in full cotyledon with or without petiole was higher than in the half and one-third of cotyledon. In addition, the rate of adventitious buds induction of integrated leaves were lower than the cotyledon petiole, which might be because the petiole had two incisions for absorbing more nutritional ingredients and hormone (Song et al., 2007). Although the explants diversification has been exploited and displayed a greater regeneration capacity, the leaf explants with callus-mediation had a greater potential in transformation through *Agrobacterium* as well as DNA bombardment (Gaurav et al., 2010).

### SEEDLING AGE

In the choice of seedling age, it is generally believed that cotyledon explants from seedlings 2-4 days after germination are in favor of adventitious bud regeneration. Shoots induction and callus regeneration is quite different in

different seedling ages and different types. The researchers also showed that 2-3d cotyledon explants just launched was the best in bud differentiation, even up to 100%. Similar results also appeared in the experiment of Chen et al., who compared the adventitious buds of Italy lettuce cotyledons differentiation rate of 3-4 days with 7-10; 3-4days lettuce cotyledons was significantly higher than the 7-10 days lettuce cotyledons (Chen et al., 2012). This may be because of the actively physiological metabolism of small seedling age cotyledons is vulnerable to the impact of external factors such as exogenous hormones. To the same organ and tissue, it is easier to be cultivated and regenerated for the juvenile state explants than adulthood plants.

However, to our surprise, for some species cotyledon age is not a barrier to efficient shoot regeneration. The cultivars Great Lakes, Greenway et al. were unaffected by cotyledon age, with no significant difference in the mean number of shoots produced for cotyledons excised 3-14 days after germination (Denise et al., 2002). In contrast, shoot production from explants of the other cultivars tested, showed a reduced ability to produce shoots as cotyledon age increased. To our knowledge this is the first report of a genotype dependent effect of explants age in lettuce. This might account for the considerable variation in the

percentage of explants forming shoots when previously published studies are compared (Denise et al., 2002).

### MEDIA COMPOSITION AND PLANT GROWTH REGULATORS FOR LETTUCE REGENERATION SYSTEM

MS and 1/2 MS medium have always been used for lettuce media composition. Plant growth regulators play a primary role in growth regulation rather than nutritional supplementation in plant regeneration and development (Slater et al., 2003). Plant growth regulators in different concentrations have obvious effect not only on callus explants tissues formation but also on shoot regeneration. Four growth regulators: NAA, IAA, 6-BA and 2, 4-D which are very familiar to us have been used in varied concentrations and combinations for shoot regeneration in tissue culture. Regeneration of seven shoots from cotyledon explants were obtained on MS medium enriched with 6-BA (0.1 mgL<sup>-1</sup>) and NAA (0.05mgL<sup>-1</sup>) (Liu et al., 1996). While at a high concentration of 6-BA (1.2 mgL<sup>-1</sup>), a similar number of shoots were obtained (Song et al., 2007). In contrast, MS medium supplemented with a high concentration of BA (0.44µM) did not increase the number of shoots produced per explants, but inhibited shoot development allowing

callus to proliferate (Denise et al., 2002). The combination of auxin like NAA and IAA has been extensively used in many composite crops resulting in advanced shoot initiation and producing a maximum number of shoots from explant of cotyledon. The result of Deng et al. demonstrated that  $0.2 \text{ mgL}^{-1}$  IAA in combination with  $1.5 \text{ mg L}^{-1}$  6-BA in the MS medium stimulated callus formation and improved the percentage of explants that regenerated (Deng et al., 2007). Successful rooting of regenerated shoots was not only achieved on 1/2 MS medium but also supplemented with NAA and IAA, and NAA was also found to be more effective than IAA in many reports (Zhu et al., 2002; Chen et al., 2012).

The species of Zhengyuan Italy lettuce were selected and the regeneration system was optimized in my work. Cotyledons as the explants were prepared by cutting of 5-day-old seedling and including 2-day-old cultured in the dark, and then transferred to the adventitious buds differentiation medium  $\text{MS}+0.5\text{mgL}^{-1}$  6-BA+ $0.3 \text{ mgL}^{-1}$  NAA with faster and better growing. The shoot regeneration rates were generally high, which was similar to the above research.

#### ADVANCES IN GENETIC TRANSFORMATION

Genetic transformation is an important tool in addressing increasing worldwide demands for lettuce with more academic and agronomic value. A variety of methods now exist for lettuce genetic transformation. The most frequently used methods are *Agro-bacterium* mediated transformation and particle bombardment, with the former having a much higher transformation frequency and efficiency, and the latter breaking the limitations of carrier method. Of the transgenic lettuce experiments described to date, the majority of them focus on three special areas critical to lettuce: pharmaceutical protein, vaccines, and nutritional value. Recent advances in the genetic transformation of lettuce have made it possible to transfer various chimeric genes of pharmaceutical and nutritional importance to the genome of recipient species. It is presumed that this technology may help to make up for some of the limitations of classical breeding associated with lettuce improvement (Fu et al., 2007).

#### TRANSGENIC LETTUCE TO PRODUCE VACCINE

The production of vaccine in transgenic plants is one of the hot spots of vaccine development nowadays. Oral vaccination, as a novel vaccine molecules expression system, could eliminate the economic burden and pain of injection and possess unparalleled advantages. These convenience factors could lead to better compliance for patients, both in developing and developed countries. In recent years, the ability of transgenic plants to induce an immune response via oral route has gradually been

confirmed, it is not only capable of expressing exogenous vaccine protein but also stimulate the effective protection of mucosal immune and system immune (Li and Xi, 2004). A lot of plant vaccines were successfully expressed in lettuce, including hepatitis B vaccine (Jackson et al. 2008), foot and mouth disease vaccine (Deng et al. 2007), avian influenza vaccine (Fan et al., 2012), et al, among which hepatitis B vaccine expressed in lettuce was frequently studied by researchers. It was confirmed that most of the transformation resistant lettuce plants detected by PCR, PCR-Southern and other molecular analysis grow very well, which laid the foundation of lettuce as a bioreactor to produce vaccine. In most cases, marker genes that confer antibiotic resistance such as neomycin phosphotransferase (*nptII*) and Hygromycin B-phosphotransferase (*hpt*) have been used in lettuce resistance selection.

Transgenic lettuce plant could exhibit higher protein accumulation, indicating that increased mRNA level in transgenic plants contributed to increased protein levels. It was important to decrease the feeding amount during immunization due to the high expression of vaccine antigen in transgenic plants. The low expression of antigen gene in transgenic plants was problematic to efficient induction of immune responses. To test the feasibility of oral vaccine, five-week-old mice were used in study to demonstrate the alleviation of symptomatic pancreatic and the preservation of insulin-producing  $\beta$ -cells. This was the first report of expression of therapeutic protein in transgenic chloroplasts of lettuce (Tracey et al., 2007). On the basis of the results obtained in previous study, human clinical trials would have been initiated, which will open up the possibility for the low-cost production and delivery of human vaccines, and a strategy for the treatment of various other autoimmune diseases (Tracey et al., 2007).

#### TRANSGENIC LETTUCE TO PRODUCE PHARMACEUTICAL PROTEIN

The production of pharmaceutical competent proteins and peptides in plants was another rapidly developing area in the application of transgenic plants in recent years. While the choice of an efficient expression system for production of a therapeutic protein or peptide was influenced by several factors like technical and economical. Numerous studies showed that human with little content of important clinical value of protein or polypeptide could be expressed in the plant system. Many proteins such as peptide hormones, insulin and interferon have been successfully expressed, in which peptide hormones have been studied more often, mainly calcitonin (Cui et al., 2009) and thymosin (Meng et al., 2005). The initial question to be solved was whether a given expression system could produce these proteins in an active form that could be administered to the patients. However, the low level of

expression was still the main problem facing in current production practice, so the expression in lettuce still needed to be substantially improved and its genetic stability to be further investigated.

In the present study, *Agrobacterium* mediated system has previously been examined to validate the expression of Human lactoferrin and Thymosin (Meng et al., 2005) or produce some nattokinase (Tian, 2007). ChIFN- $\alpha$  was correctly transcribed and expressed in lettuce plants, and the recombinant IFN obtained was active for conferring protection against VSV infection (Song et al., 2008). These findings could be valuable for prevention of many cardiovascular and cerebrovascular diseases and neoplastic diseases in old people.

### GOOD QUALITY RESEARCH OF TRANSGENIC LETTUCE

Lettuce is a type of leafy vegetable rich in vitamin. The nutritional value of its transgenic research was currently focused on the increase of content of vitamin A, vitamin C and vitamin E, and followed by the resistance of lettuce. Wang et al. transferred the gene-containing GDP-mannose pyrophosphorylase gene (GMP) plant expression vector p2301-GMP-myc into lettuce, while the content of vitamin C determined by HPLC-ELSD of most transgenic lettuce was higher than normal plants, even up to about 2.5 times, which have shown that over-expression of the GMP gene was an effective method to improve the vitamin C content of lettuce (Wang et al., 2011). Meanwhile, *Lactuca sativa* L. was transformed with *Atp5y* and synthetic *folE* gene, and the expression of lutein,  $\beta$ -carotene and total folate content in transgenic lettuce were measured by Realtime-PCR, HPLC and *Lactobacillus casei* fermentation. Compared with the wild type, the content of lutein,  $\beta$ -carotene and folic acid increased much in transgenic lettuce, to our surprise,  $\beta$ -carotene content was three times and folic acid was 1.85 times (Fu et al., 2012). These studies laid the foundation for modifying its metabolic pathways by genetic engineering in the meantime obtaining the new lettuce varieties rich in vitamins, carotenoids and folic acid.

### RESISTANCE RESEARCH OF TRANSGENIC LETTUCE

Although insect resistance, disease resistance and other excellent quality relative to economic traits were less studied, some breakthroughs have been made by researchers. For further work, resistance to Root Knot Nematodes and Aphids (*Lipaphiserysimi*) in transgenic lettuce (*Lactuca sativa*) made us better understand the mechanism of pest resistance (Zhang et al., 2010; Ahmed et al., 2007). Transgenic resistance to Mirafiori lettuce virus

in lettuce carrying inverted repeats of the viral coat protein gene provided a new way to resist disease, and the MiLV-resistant lettuce could be used as a resistant cultivar or as a breeding source (Yoichi et al., 2009). The transgenic lettuce over-expression of *Arabidopsis* ABF3 gene showed higher tolerances than wild-type plants against drought as well as cold stress, which could help to develop stress tolerance with an eventual improvement in crop yield (Enkhchimeg et al., 2005).

### THE PROSPECT OF APPLICATION

During the last 10 years, many advances in molecular biology and genetic engineering have been used to improve the agronomic and nutritional value of lettuce and introduce new attributes into existing cultivars. For genetic engineering efforts to be effective in delivering new cultivars, three interacting components are essential in tissue culture and genetic transformation. To start with, adventitious shoots induction of cotyledon was significantly influenced by genotype, so the research of explants types need to be strengthened; next, different types of hormone combination were mostly 6-BA and NAA, 6-BA and IAA, so other hormone combinations should be explored to improve regeneration frequency; last but not the least, the existing transgenic lettuce was focused on the genes transformation and expression, whereas stable inheritance of the genes and genetic traits was less studied.

Most of them have been successfully integrated in developing transgenic lettuce, but various other issues need to be addressed and resolved. Even the environmental risks and health hazards associated with transgenic crops are still sources of concern to all countries in the world.

A trans-genetic lettuce and cucumber system containing rabbit defensin gene NP-1 were constructed and optimized by *Agrobacterium*-mediated method in my research. To examine the disease resistance of transgenic plant, a series of molecular detection and inhibition zone test were performed. The results revealed that the protein possessed good resistance to *E. coil* and *Staphylococcus aureus* ( Song et al., 2013).

Although there are many problems that still need to be further explored, it can be said that the use of transgenic lettuce in production of pharmaceutical proteins and vaccines is a very effective one. Nowadays the transgenic soybeans have been approved by authorities, it is hoped that with proper assessment and field trials, many more transgenic crops will be applied in our everyday life in the future, including lettuce.

### Conflict of Interests

The author(s) have not declared any conflict of interests.

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