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Genetic improvement of Chinese cabbage for salt and drought tolerance by constitutive expression of a *B. napus* LEA gene

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Abstract

This is the first report on transformation of Chinese cabbage with late embryogenesis abundant (LEA) gene. Transgenic Chinese cabbage (*Brassica campestris* ssp. *pekinensis*) expressing a *B. napus* late embryogenesis abundant protein gene had been generated. Infection by *Agrobacterium* strain LBA4404 containing the binary vector pIG121–LEA, which carried LEA protein gene linking to CaMV promoter and terminator sequences, and the neomycin phosphotransferase II *(NP*TII) gene, was applied. Generated shoots were selected by their growth ability on revised MS medium containing kanamycin sulphate. Transgenic Chinese cabbage plants demonstrated enhanced growth ability under salt- and drought-stress conditions. The increased tolerance was reflected by delayed development of damage symptoms caused by stress. The increased tolerance also showed improved recovery upon the removal of stress condition. These results suggest that the genetic modification of Chinese cabbage by LEA protein gene holds considerable potentiality for crop improvement toward environment-stress tolerance.

Keywords: Drought tolerance; Kanamycin sensitive; Late embryogenesis abundant (LEA) gene; Salt-stress; Transgenic Chinese cabbage

1. Introduction

Late embryogenesis abundant (LEA) protein genes are highly expressed during late stages of seed development at normal growth condition, but many of the LEA class genes are also frequently expressed in vegetative tissues when plants are exposed to environmental stress [1]. Repeating units composed of short tracts of conserved amino acids are commonly found in LEA proteins and five groups of LEA proteins have been defined based on their amino acid sequence homologies [2]. Several groups of LEA protein genes have been demonstrated to confer water-deficit and salt-stress tolerance. LE25, a group 5 LEA protein from tomato (*Lycopersicum esculentum* L.) improved resistance to high salinity and freezing when expressed in *Saccharomyces cerevisiae* [3]; similarly, the hiC6 gene encoding a homologue of LEA enhances freezing tolerance of *S. cerevisiae* [4] and a wheat LEA protein Em, was demonstrated to function as an osmoprotective molecule in *S. cerevisiae* [5]. Expression of *HVA*1, a group 3 LEA protein from barley (*Hordeum vulgare* L.) conferred tolerance to soil water-deficit and salt-stress in transgenic rice plants [6–8], improved biomass productivity and water use efficiency under water-deficit conditions in transgenic wheat [9]. By far, group 3 LEA genes could be considered very important in agronomy.

Chinese cabbage (*Brassica campestris* ssp. *pekinensis*) is one of the most important vegetables consumed mainly during the winter season in Asia, especially, in China, Korea and Japan. The most recent techniques in plant genetic engineering have advanced and opened a new area for crop improvement. *Agrobacterium*-mediated transformation of *Brassica* crops has been reported to be successful with *Brassica napus* [10], *B. eracea* [11], *B. juncea* [12],

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B. carinata [13] and *B. campestris* [14]. However, transformation of Chinese cabbage (*B. campestris* p. *pekinensis*) has been difficult through the *Agrobacterium* approach and no reports on the production of transgenic Chinese cabbage with LEA protein genes have been published so far.

In this study, we have taken an improved approach of *Agrobacterium*-mediated transformation, to investigate transgenic efficiency and the function of a *B. napus* group 3 LEA protein gene in transgenic Chinese cabbage. Constitutive and high-level expression of the *B. napus* LEA protein gene in transgenic Chinese cabbage plants allowed us to test the role of the LEA protein gene in stress tolerance. We found that accumulation of the rape LEA protein in the vegetative tissues of transgenic Chinese cabbage plants conferred increased tolerance to water-deficit and salt-stress. Our results not only demonstrated a role of the *B. napus* LEA protein gene in stress protection but also suggested the potential possibility of LEA protein gene for genetic engineering of stress tolerance in Chinese cabbage.

2. Materials and methods

2.1. Agrobacterium strain and binary vector

A 1.0 kb *B. napus* LEA cDNA clone *ME-leaN4* was kindly supplied by Dr. Takahata (Faculty of Agriculture, Iwate University, Japan). *ME-leaN4* belongs to the group 3 LEA family, which was also characterized by predominant hydrophilic amino acids [15]. The site of β-glucuronidase (GUS) screenable marker gene in the binary vector pIG121 [16] was replaced by the 1.0 kb LEA fragment described above, under the control of the cauliflower mosaic virus (CaMV) 35 S constitutive promoter (Fig. 1). Manipulation of recombinant DNA was done according to standard procedures [17]. The binary vector was transferred into *Agrobacterium tumefactiens* strain LBA4404 by liquid nitrogen freeze–thaw method [18]. Glycerol stock of *A. tumefaciens* strain was thawed and then streaked onto solid YEP medium (pH 7.0) containing 100 mg/l streptomycin and 100 mg/l kanamycin. A single, isolated colony was picked to start a 50 ml liquid YEP medium culture with the same antibiotics described above. The culture was incubated overnight at 28 °C on a shaker (150 rpm) until the culture had grown to an OD_{600} of 0.5–0.6.

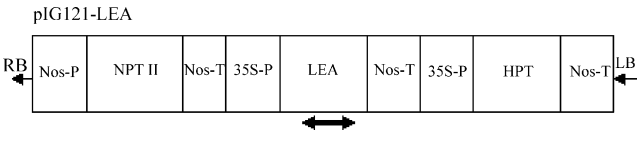

Fig. 1. Diagram of the T-DNA region of the binary vector pIG 121–LEA used for transformation. LB and RB are the left and right border sequences of the T-DNA region, respectively. The bold arrow indicates the PCR-amplified region, which was used to confirm the existence of LEA gene in the regenerated plants. *Nos-P*, promoter of the nopaline synthase gene; *NPTII*, neomycin phosphotransferaseII; *Nos-T*, terminator of the nopaline synthase gene; *35S-P*, CaMV 35S promoter; *LEA*, coding region of LEA gene; *HPT*, hygromycin phosphotransferase gene.

2.2. Plant transformation and selection

Chinese cabbage seeds were sterilized in 70% ethanol for 1 min and then in 1.6% sodium hypochlorite plus 0.1% Tween-20 for 20 min with vigorous shaking. After a thorough rinse in sterile distilled water, the seeds were placed on MS basal germination medium [19]. The hypocotyls with the cotyledons were used as explants for infection. The explants were pre-cultured for 2 days in MS medium with 2 mg/l BA, 1 mg/l NAA, 16 g/l Phytagar under the same photoperiod as stated above. The pre-cultured explants were infected with the overnight cultured *Agrobacterium* (OD_{600} = 0.5–0.6) by immersion and shaking at 100 rpm for 20 min. Explants were then co-cultivated for 3 days onto the same medium as in pre-culture.

After co-cultivation, explants were transferred to plates of selection medium (MS medium with 2 mg/l BA, 1 mg/l NAA, 4 mg/l $AgNO_3$, 300 mg/l Timentin [SmithKline Beecham], 10 mg/l kanamycin, 16 g/l Phytagar). Calli that formed on explants were sub-cultured to fresh-made selection medium every 4 weeks. Green shoots regenerated on selection medium were transferred to rooting medium (MS medium with 300 mg/l timentin, 10 mg/l kanamycin, 16 g/l phytagar) in plastic bottles. Then, rooted green shoots were transferred to vermiculite medium in 4.4 cm plastic pots and covered with a plastic bag to maintain a definite level of humidity. Finally, plants were transferred to larger pots and placed in the P1 greenhouse for further development and for recovery of seeds.

2.3. PCR, Southern and Northern analysis

The primers employed to detect the transgene was as follows: LEA forward sequence (F) 5′-GGCAAGGACAA-GACTTCCCA-3′, reverse sequence (R) 5′-CGGATCAG-TGCTCTGAGTAG-3′ to amplify a characteristic 550 bp fragment. The reaction consisted of 30 cycles with 45 s at 96 °C for denaturing, 45 s at 55 °C for annealing and 90 s at 72 °C for extension. For genomic Southern analysis, 10 μg total DNA from leaves was digested with *Hin*dIII (GIBCO, BRL Life Technologies) and separated by electrophoresis on a 0.8% agarose gel. Hybridization was performed with DIG-labelled DNA probe. The membrane was hybridized with a digoxigenine (Boehringer–Mannheim) labelled fragment, which contained the coding region of the *lea* protein gene generated by using the PCR DIG Probe Synthesis Kit (Boehringer–Mannheim). Southern hybridization procedure was as described by Tingay et al. [20] to confirm the integration of the LEA gene.

Extraction of total RNA and Northern blot analysis was as described by Sambrook et al. [17]. Briefly for Northern blotting, 20 μg of total RNA was separated on 1.5% (w/v)

denaturing agarose gel and transferred to positively charged nylon membranes (Boehringer–Mannheim). The membrane was hybridized with a digoxygenin (Boehringer–Mannheim) labelled probe to detect the gene-specific transcripts. The probe was the same as that used in Southern analysis, which contained the coding region of the *lea* protein gene prepared by PCR following the supplier's instructions and as described by Nishizawa et al. [21].

2.4. Seed germination on MS medium under salt-stress

To test the response of the seeds to salt-stress, 30 seeds of R_1 transgenic plants and 30 non-transgenic plant seeds were germinated on MS medium consisting of various concentrations of NaCl (0, 50, 100, 150 and 200 mM) and incubated at 25 °C in growth chambers under a 16-h light:8-h dark photoperiod, cool-white fluorescent light. After 2 weeks of incubation, percentage of germination was counted. Data were subjected to analysis of variance and the result of difference comparison by $LSD_{0.01}$ was shown by double asterisk in the figure. The seed germination experiment was replicated four times.

2.5. Salt tolerance in hydroponics and drought tolerance in soil

Seeds of R_1 transgenic plants were germinated on MS solid medium. Salt-stress in the hydroponics (pH 5.8) was produced by transplanting the seedlings into the hydroponic system containing 150 mM NaCl and cultured for 10 days. Hydroponic solution contained 25% full strength of macronutrients and micronutrients for regular liquid culture referring to Liu et al. [22] with slight modification. The salt concentration was maintained by exchanging the hydroponic solution every 3 days. Seeds of non-transgenic plants were used as control materials. Refined and sterilized seedling-nursing soil was used to grow Chinese cabbage in the greenhouse. Seeds from R_1 transgenic plants and non-transgenic plants were sown and germinated in the soil pots that were kept in flat-bottom trays containing water. Seedlings were grown for 2 weeks before they were exposed to drought stress. Drought stress experiment was conducted by withholding water till leaf-wilting symptom appeared.

3. Results

3.1. Production of transgenic Chinese cabbage plants

Chinese cabbage explants infected with *Agrobacterium tumefaciens* harboring the binary vector pIG121–LEA were cultured on hygromycin-containing medium for selection. Results of three transformation experiments were summarized in Table 1. Four hundred and five cotyledon explants with hypocotyls were infected in the transformation experiments. One hundred twenty-six kanamycin-resistant calli were selected and transferred onto regeneration medium. Nine independent lines of plants (80 plants) were generated and grown in the P1 greenhouse. The transformation efficiency of independent transgenic lines (transgenic plant lines/the number of cotyledon explants infected × 100) was 2.2%.

Table 1
Summary of transformation experiments

Transformation experiment no.	No. of cotyledon explants	No. of kanamycine-resistant calli	No. of lines (plants) regenerated
1	135	42	3 (28)
2	180	50	4 (35)
3	90	34	2 (17)
Total	405	126	9 (80)

3.2. Confirmation of transgenic plants by PCR, Southern and Northern analysis

The existence of the transferred LEA gene in the regenerated plants (R_0 generation) from kanamycin-resistant shoots was proven by PCR using specific primers. Out of 126 kanamycin-resistant calli, nine plant lines regenerated were confirmed to have the expected amplification product of 550 bp, indicating they were transformed. The control plants did not show any bands (Fig. 2).

*Hin*dIII-digestion of genomic DNA from each of the nine putative transgenic lines generated one to three copies of hybridization signals (Fig. 3), suggesting that perhaps one to three integrations of the LEA gene had occurred in the plant genome. There is only one digestion site by *Hin*dIII in the transformation vector, the fragment containing only the LEA coding region could not be released. The hybridization patterns of all transgenic plants were unique, indicating that these transgenic lines were derived from independent transformation events. No band was detected for control plant DNA as the LEA probe did not hybridize to genomic DNA from untransformed Chinese cabbage plants.

Expression of the LEA gene in the leaves of R_1 plants was analyzed by Northern blot analysis (Fig. 4). All of the nine

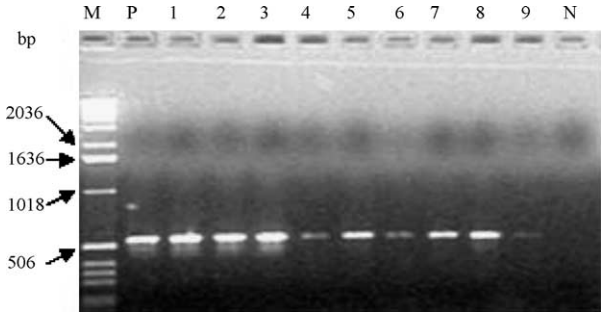

Fig. 2. Detection of the transgene from the Ro-regenerated Chinese cabbage plants by PCR with LEA-specific primers. M, 1 kb DNA ladder; P, positive control (plasmid vector of transformation); Lanes 1–9, independently transformed plant lines; N, non-transformed plants.

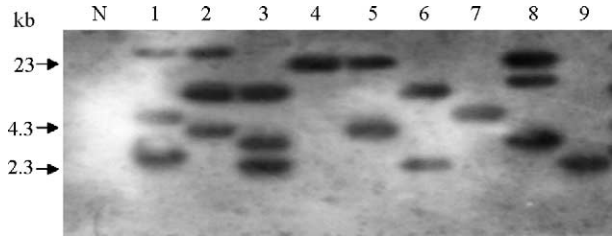

Fig. 3. Southern blot analysis of genomic DNA of R_1 transformants of Chinese cabbage digested with *Hind*III and probed with the LEA cDNA sequence. N, non-transformed Chinese cabbage plant (control); Lanes 1–9, independently transformed plant lines.

transgenic plant lines were confirmed to express the LEA protein gene, which showed a clear transcription band in the figure of Northern blot analysis. No band was detected from the non-transgenic plants. The results demonstrated the expression of LEA gene in transgenic plant leaves. Hybridization signals from transgenic line 1, 2, 3 and 8, which had three LEA gene copies inserted, appeared not significantly different from those of other transgenic lines with one or two copies of transgene.

3.3. Increased tolerance to salt-stress in MS medium and in hydroponics

After Northern analysis, it is interesting to learn whether the expression of LEA gene would have any beneficial effect on the growth performance of transgenic Chinese cabbage plants under salt-stress conditions. The effect of salt-stress on seed germination on MS medium was investigated. Germination experiments on MS with salt-stress were done for all the nine transgenic lines; the results were almost the same and are summarized in Fig. 5. Obvious difference was observed in germination rate between transgenic and non-transgenic plants when salt concentration was more than 90 mM (Fig. 5). As salt concentration increased to 150 mM, 30% of the transgenic seeds were germinated, whereas only 3% of the seeds of the non-transgenic plants germinated (Fig. 6(panels A and B)). At 180 mM of salt concentration, seeds of non-transgenic plants did not germinate at all, whereas the transgenic Chinese cabbage plants showed a 22% germination rate (Fig. 5). These results indicated that the seeds of transformed plants had enhanced ability to germinate and grow under salt-stress conditions.

Growth performance in hydroponics is shown in Fig. 6(panels C and D). After 10 days of growth in the hydroponics with 150 mM salt-stress, the seedlings of the

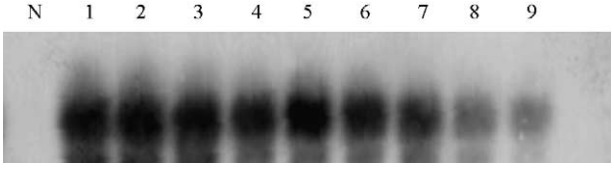

Fig. 4. Northern blot analysis with total RNA from the leaves of R1 transgenic Chinese cabbage. N, non-transformed Chinese cabbage plants; Lanes 1–9, independently transformed plant lines.

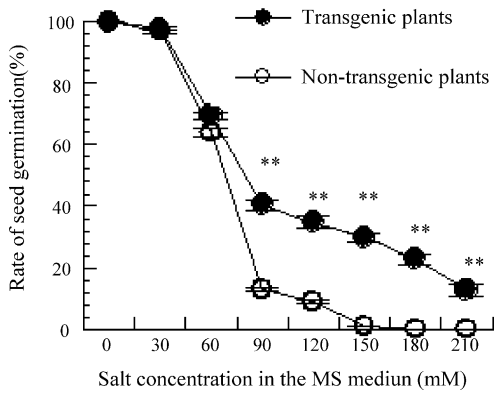

Fig. 5. Comparison on seed germination rate on MS medium between transgenic and non-transgenic Chinese cabbage plants. For transgenic plants, the data were the average of all the nine transgenic lines. Vertical bar means standard deviation; the asterisks (**) indicate significant difference at 0.001 probability level.

non-transgenic plants were inhibited and wilted, whereas those of the transgenic plants were in normal growth condition, indicating that transformation of LEA protein gene increased the salt tolerance of Chinese cabbage plants. No significant difference in growth status was observed among the nine transgenic lines that had different LEA gene insertion.

3.4. Enhanced drought tolerance of transgenic Chinese cabbage plants

Transgenic plants and non-transgenic plants were tested for drought tolerance by withholding water in the soil pot experiment. Transgenic and non-transgenic plants were grown for 3 weeks under a fully watered regimen at 25 °C and then transferred into a chamber of 25 °C and withholding water. After 2 weeks of water deprivation,

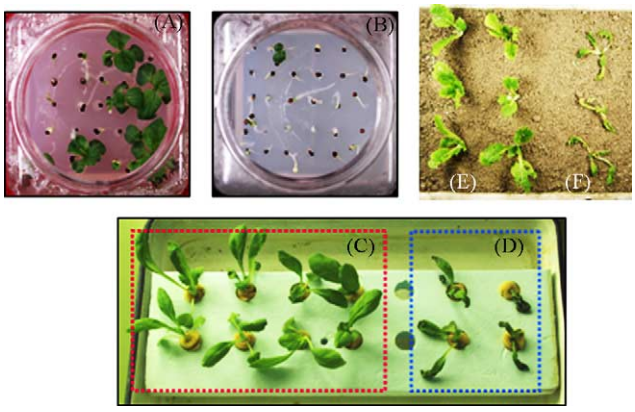

Fig. 6. Comparison on salt and drought tolerance between R_1 transgenic and non-transgenic Chinese cabbage plants. Effects of salt-stress by supplementing 150 mM NaCI in MS solid medium on germination and growth of transgenic plants line 2 (A) and non-transgenic plants (B); growth status of transgenic plants line 4 (C) and non-transgenic plants (D) in hydroponics with 150 mM NaCI stress and comparison on drought tolerance between transgenic line 5 (E) and non-transgenic Chinese cabbage plants (F) in soil tray.

non-transgenic plants wilted whereas transgenic plant did not (Fig. 6(panels E and F)). Resupplying water to the soil pot could not recover the growth of the non-transgenic plants that lost their leaves and died, while transgenic plants continued to grow. Experiment result demonstrated that deprivation of water was lethal to non-transgenic plants, but plants from transgenic plants survived and continued normal growth. Similar to the results of salt tolerance on MS medium and in hydroponics, no significant difference in drought tolerance was observed among the nine transgenic lines that had different LEA gene insertions.

4. Discussion

The low efficiency of *Agrobacterium*-mediated transformation of *B. campestris* had been pointed out to be related to its low susceptibility to *Agrobacterium* and the low regeneration frequency of infected tissues [14,23]. In most events of transformation of *Brassica* ssp., cotyledonary and hypocotyl explants have been often used. Compared with cotyledons, which had higher regeneration abilities but limited success of transformant production, hypocotyls that had lower regeneration frequency had a higher transformation rate [14,24]. Takasaki et al. [14] failed to obtain transgenic plants from the cotyledon explants, while Zhang [25] obtained transgenic plants with kanamycin- and hygromycin-resistant genes from cotyledons. Other factors, such as the duration of infection and co-cultivation also influenced the infection frequency. Our study aimed at developing an efficient transformation system for Chinese cabbage in order to introduce stress tolerant genes into this plant. The results showed that the use of seedling segment with both cotyledons and hypocotyls as explants, infection with *Agrobacterium tumefactiens* for 20 min and co-cultivation after inoculation for 3 days were effective to yield a relatively high transformation rate. The transformation system obtained in this study was the first of its kind that used both cotyledons and hypocotyls as explants. It may be applicable to other *Brassica* plants.

Regeneration of adventitious shoots from cotyledonary or hypocotyl explants was called direct regeneration and that from the callus formed in the explants had been called indirect generation [13,24]. Transgenic plants of Chinese cabbage (*B. campestris*) obtained in our study were generated by the indirect regeneration pattern, which was similar to the result reported by Babic et al. with *Brassica carinata* [13]. However, to the opposite, indirect regeneration has not been observed with the same *B. campestris* by Mukhopadhyay et al. [24]. It could be supposed that the use of cotyledon explants with hypocotyl was important to the success of transformation production with plants of *B. campestris*, which would be useful to modify agronomic traits by gene manipulation.

The late embryogenesis abundant genes were first identified as genes induced in seeds during maturation and desiccation [1]. They are frequently expressed in non-seed tissues in response to environmental stresses that include a water-deficit component. LEA proteins are highly hydrophilic, and thus, they are speculated to retain water molecules and prevent crystallization of cellular components under water-deficit, which results from drought, high salt and freezing stresses.

Over-expression of group 3 LEA protein gene in transgenic rice and wheat has shown to enhance resistance to specific water-deficit stresses or improve water efficiency and biomass productivity [6–9]. The results of this study also demonstrated that transgenic Chinese cabbage with group 3 LEA protein gene from *Brassica* spp. showed increased tolerance to water-deficit and salt-stress, which correlated well with the results of the former studies stated above. Transgenic R_1 plants maintained a higher germination rate than non-transgenic plants under salt-stress condition. Transgenic plants showed better performance in hydroponics with salt-stress. Appearance and development of wilting symptom caused by water-deficit was delayed. When water-stress condition was removed, transgenic plants resumed normal growth rapidly, while non-transgenic plants showed no recovery.

Imai et al. [3] and Honjoh et al. [4] had reported that LEA class genes conferred salt and freezing tolerance when expressed in yeast, but in our study, freezing tolerance in transgenic Chinese cabbage was not observed. Further more, although a correlation between the number of LEA gene copies inserted and growth performance of transgenic Chinese cabbage was not observed, as an individual example, our results from transgenic Chinese cabbage further support the role of LEA protein in promoting water-stress tolerance. The result suggests the potential ability of LEA protein genes to create new cultivars of Chinese cabbage with enhanced stress tolerance by genetic engineering.

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