Original Paper Plant Biotechnology, **17** (1), 21-25 (2000)

The Use of Callus Cultures for Searching Proteins Associated with Increased Freezing Tolerance During Cold Acclimation in *Lonicera caerulea*

Hiroyuki IMANISHI*, Kumiko TAKADA, Kiyoshi MASUDA, Takashi SUZUKI and Takashi HARADA

Horticultural Sciences, Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan
**Corresponding author* E-mail address :hima@agri.akita-pu.ac.jp

Received 7 July 1999; accepted 26 October 1999

Abstract

To elucidate the molecular events underlying cold acclimation in woody plants, we established an *in vitro* system that responds to cold treatment, using calli of blue-berried honeysuckle (*Lonicera caerulea* L. var. *emphyllocaryx* Nakai). The calli that had been exposed to low temperature at 5 ℃ increased their cold tolerance after 8 days of the treatment, reaching a plateau on day 12. The cold-treated calli had an approximately 60% survival rate at -7.5 ℃, while the survival rate of non-acclimated calli at the same temperature was below 10%. The cold-treated calli lost their freezing tolerance within 2 days of incubation at 25 ℃. The proteins extracted from these calli were analyzed by 2-dimensional polyacrylamide gel electrophoresis. As a result, a 42 kDa (*pI* 5.4) protein was shown to accumulate in abundance during cold acclimation. The protein decreased by culture at 25 ℃. These results suggest that the callus of *L. caerulea* did cold-acclimate in response to low temperature conditions, and that it could be used for the investigation of proteins involved in the incremental development of freezing tolerance.

1. Introduction

In many plant species exposure to low-nonfreezing temperatures induces a greater tolerance to freezing (Levitt, 1980). This process, whereby plants develop low temperature tolerance, is termed cold acclimation (Levitt, 1980). Many investigations have tried to elucidate the physiological and molecular events that take place during cold-acclimation (Levitt, 1980; Sakai and Larcher, 1987), and one approach has been to seek out proteins involved in increasing the freezing tolerance of the cells. It has been reported that low, nonfreezing temperatures induce the exclusive accumulation of some proteins in leaves and stems in plants such as *Arabidopsis thaliana* (Kurkela and Franck, 1990; Nordin *et al.*, 1991; Gilmour *et al.*, 1992), alfalfa (Mohapatra *et al.*, 1989), spinach (Guy and Haskell, 1987), *Brassica* (Meza-Basso *et al.*, 1986; Weretilnyk *et al.*, 1993) and wheat (Perras and Sarhan, 1989). In woody plants grown in fields, the proteins that showed a specific increase in response to chilling treatment were found in the floral buds of blueberry (Muthalif and Rowland, 1994) and in the bark and xylem tissues of peach (Arora *et al.*, 1992).

Compared with intact plants, studies on cold acclimation in calli and cultured cells are largely limited (Guy *et al.*, 1987). If a system that increased freezing tolerance in response to low temperature could be established then the tissues grown *in vitro* should provide novel materials. Especially, in woody plants, the situation may be so, since the cold treatments of woody plants grown in fields and the extraction of molecules such as nucleic acids and proteins from them are largely laborious.

Lonicera caerulea L. var. *emphyllocaryx* Nakai is a deciduous shrub, which is native to northern areas of Asia and Eastern Russia. This plant has been known to show extensive frost hardiness in winter and in experimental conditions of exposure to a period of low, nonfreezing temperatures (Imanishi *et al.*, 1998; Imanishi *et al.*, 1999). Thus, these plants appear to be very suitable for establishing an *in vitro* system for cold acclimation. In this study, we demonstrate both the development of freezing tolerance in *L. caerulea* calli by treatment with a low temperature and the loss of freezing tolerance upon returning the plants to regular culture conditions. In addition, we report the accumulation of a 42 kDa protein in *L. caerulea* calli, induced by incubation at a low temperature.

2. Materials and Methods

2.1 Plant material

The calli of *Lonicera caerulea* L. var. *emphyllocaryx* Nakai derived from shoot apices were maintained in 100 m*l* Erlenmeyer flasks on a 25 m*l* semi-solid medium containing half-strength Murashige and Skoog's (MS) inorganic salts (Murashige and Skoog, 1962), MS organic constituents, 10 μM 1-naphtahleneacetic acid and 10 μM BA (pH 5.7) (callus culture medium). The calli were grown under a photoperiod of 16 h light (60 μmol · m^{-2} · sec^{-1}, fluorescent tubes) and 8 h darkness. One-month-old calli were used in the experiments reported here.

2.2 Cold acclimatization and de-acclimatization

Calli grown in Erlenmeyer flasks on the callus culture medium at 25 ℃ with a 16 h photoperiod were transferred to cold-acclimatized conditions consisting of a constant 5 ℃ and an 8 h or 16 h photoperiod provided by cool-white fluorescent lamps (60 μmol · m^{-2} · sec^{-1}). The calli kept at 5 ℃ for 14 d were de-acclimatized by transferring them from 5 ℃ to 25 ℃ under a 16 h photoperiod.

2.3 Determination of freezing tolerance

To determine the freezing tolerance, callus segments, approximately 2 mm cubes, were cut from the calli and immersed in 1 m*l* plastic tubes which were 3.5 mm in diameter and filled with water. They were cooled stepwise in a programmed freezer to 5, 2.5, 0, -5, -7.5, -10, -12.5, -15, -17.5, and -20 ℃, with ice-seeding at -3 ℃. The chamber was cooled at a constant rate of -0.42 ℃ /min between each indicated temperature and then held for 30 min at each temperature. The frozen tissues were then thawed in a water bath at 37 ℃ until the ice crystals disappeared. The thawed callus segments were taken off the tubes and cultured in 100 m*l* flasks on a 25 m*l* callus culture medium.

The fresh weight of the calli was measured after six weeks of culture to estimate the growth of the callus. The viability of explants was determined as follows: viability (%)=[(final fresh weight of tissues grown from frozen explants-initial fresh weight of the explants)/(final fresh weight of tissues derived from unfrozen explants-initial fresh weight of the explants)] x 100.

2.4 Electrophoresis of proteins

The calli were pulverized in liquid nitrogen with a mortar and pestle and homogenized in nine volumes of 6 M urea, 20 mM tris-HCl, pH 7.6, 2 mM EDTA, 1% Triton X-100 supplemented with Complete (protease inhibitor mixture, Boehringer, Mannheim, Germany). After removing cell debris by centrifugation at 10,000 x g for 10 min at 4 ℃, the proteins in supernatants were precipitated by adding 4 volumes of cold acetone. The precipitates were pelleted by centrifugation, washed with 80% acetone, and subsequently with 100% acetone, and dried in air. For electrophoresis, the proteins were solubilized in a sample solution containing 9 M urea, 1 mM EDTA, 5% 2-mercaptoethanol, 0.4% NP-40 (Sigma, Mo.) and 2% Servalyt 3/10 (Serva, Heidelberg) and then separated by two-dimensional polyacrylamide gel electrophoresis. The first-dimension separation was by non-equilibrium pH gradient electrophoresis (NEpHGE) as described by O'Farrell *et al.* (1977), using 2% Servalyt 3/7 as a carrier ampholyte. The second-dimension was by SDS-PAGE with a 5-15% polyacrylamide gradient using a buffer system according to Laemmli (1970). The proteins in the gels were stained with CBB R-250 and de-stained by diffusion.

3. Results and Discussion

The present investigation demonstrated that the calli of *L. caerulea* developed freezing tolerance in response to low temperatures (**Fig. 1**). The survival rate of the calli at -7.5 ℃ began to rise after 8 days of incubation at 5 ℃ and reached 61% on day 12. This level was maintained thereafter. Subsequently,

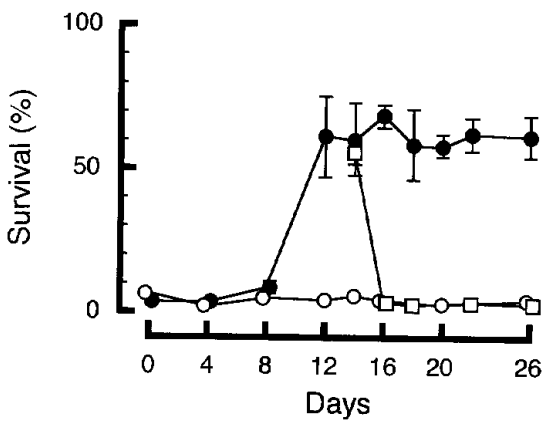

Fig. 1 Changes in the freezing tolerance of *L. caerulea* calli during cold acclimatization or de-acclimatization. The segments excised from the calli that had been incubated for the indicated number of days at 5 ℃ (●), 25 ℃ (○), and 25 ℃ following incubation at 5 ℃ for 14 days (□). After cold acclimatization or de-acclimatization, the explants were cooled to -7.5 ℃, thawed, and re-cultured. The survival rate of the tissues was evaluated as described in Materials and Methods. Mean ± SE of three replications (n=12).

returning the calli that were cold-acclimatized at 5 ℃ for 14 days to the regular growth temperature (25 ℃) resulted in a rapid decrease of the elevated freezing tolerance. In contrast, the survival rate of the segments excised from the calli which had been maintained at 25 ℃ was consistently low (**Fig. 1**). **Fig. 2** shows the callus grown from the segments which had been acclimatized to the low temperature and survived at -7.5 ℃ (**Fig. 2A**) and also a non-acclimated callus which was damaged by freezing and failed to grow in culture (**Fig. 2B**).

The freezing tolerance of the calli of *L. caerulea*, as evaluated by the regrowth test in culture, is shown in **Fig. 3**. Approximately 100% of the calli survived at 0 ℃ after cold-acclimatization at 5 ℃ for 14 days, and more than 54% of the calli were viable after freezing even at -10 ℃. On the other hand, although 82% of the calli that had been maintained at 25 ℃ survived at 0 ℃, their viability was decreased to 18% when the temperature was lowered to -5 ℃. The viability was also compared with those of de-acclimatized tissues. As a result, no difference in freezing tolerance was detected between the calli before being cold-acclimatized and those after being de- acclimatized for 4 days. It has been reported that increased freezing tolerance of the calli was comparable with that of plantlets grown and cold-acclimated *in vitro* (Guy *et al.*, 1987). Similarly, our results indicated that the calli of *L. caerulea* were acclimated to the same level of freezing tolerance which having been determined for *in vitro* grown plantlets of *L. caerulea* [LT_{50} = -7 ℃ in stem; LT_{50} = -12 ℃ in apical shoots, the data shown in Imanishi *et al.* (1999)]. Thus, it was concluded that the calli of *L. caerulea* might be usable for studies of cold acclimation as an alternative to intact plants. As the callus tissue of *L. caerulea* appeared to be homogeneous, the difference in response may be small from cell to cell.

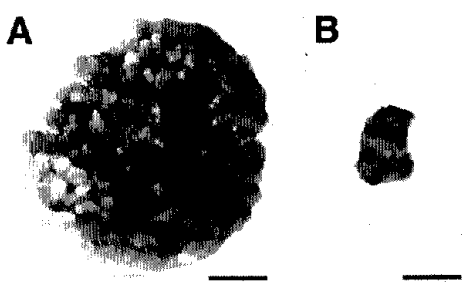

Fig. 2 The typical appearance of calli grown from callus segments of *L. caerulea* that had been incubated at 5 ℃ (A) and 25 ℃ (B) for 14 days before freezing. Bar = 2 mm.

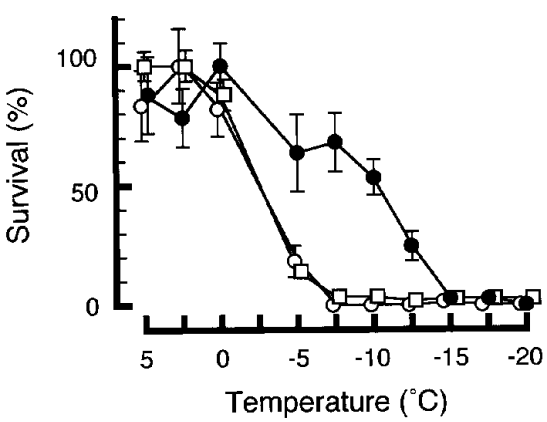

Fig. 3 The freezing tolerance of *L. caerulea* calli after cold acclimatization or de-acclimatization. The segments of calli that had been incubated at 5 ℃ (●), and 25 ℃ (○), for 14 days and, at 5 ℃ for 4 days following 25 ℃ for 14 days (□), were cooled to the indicated temperature, thawed, and re-cultured. The survival of the callus was estimated by the regrowth of the calli. Mean ± SE of three replications (n=12).

In order to investigate proteins induced by cold treatment, protein was extracted from the calli of *L. caerulea* and analyzed electrophoretically. **Fig. 4** shows the two-dimensional electrophoretogram of proteins extracted from calli grown under non-acclimatized (**Fig. 4A and 4C**), acclimatized (**Fig. 4B and 4D**) and de-acclimatized (**Fig. 4E**) conditions. We found differences in the intensity of several spots among protein samples, depending on the temperature of the cultures. Of these, polypeptide having molecular weight of 42 kDa (*pI* 5.4) was remarkably increased in association with transferring calli to a low temperature condition (**Fig. 4B**

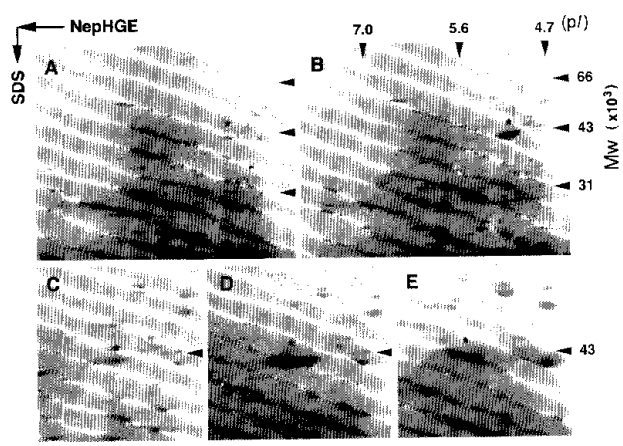

Fig. 4 Electrophoretograms of proteins extracted from cold-acclimatized and de-acclimatized *L. caerulea* callus. Proteins were extracted from calli incubated for 14 days at 25 ℃ under 16 h daily light (A), at 5 ℃ under 8 h daily light (B), and 25 ℃ under 8 h daily light (C). Otherwise, the calli cold-acclimatized for 14 days at 5 ℃ under 8 h daily light (D), were de-acclimatized at 25 ℃ for 8 days under 16 h daily light (E).

and 4D, spots indicated by asterisks). This spot gradually decreased after returning the de-acclimatized callus to the culture condition at 25 ℃. Thus, the 42 kDa polypeptide seemed to be changed quantitatively, associated with cold-acclimation and de-acclimation. Since the polypeptide disappeared gradually from the calli following the rapid decrease in freezing tolerance, it is thought that the synthesis was down-regulated and moderately decomposed under growing conditions at 25 ℃. The spot was not found consistently in the calli grown at 25 ℃ during the culture period. It did not increase appreciably during incubation at 25 ℃ for 14 days under 8 h daily light. The spot also increased during incubation at 5 ℃ under 16 h daily light to the approximately the same level contained in calli grown at 5 ℃ under the short day condition. Accordingly, it is likely that the accumulation depended only on the temperature in culture.

It has been shown that several protein species were newly synthesized in *Arabidopsis* and spinach (Welin *et al.*, 1994; Guy *et al.*, 1985) in response to low temperature. In blueberry, the relative amount of the three major polypeptides increased in response to chilling unit accumulation in floral buds (Muthalif and Rowland, 1994). These polypeptides have been shown to be dehydrins or dehydrin-like proteins. The presence of proteins showing a potentially cryoprotective function has also been suggested for *Arabidopsis* (Lin *et al.*, 1990). The 42 kDa protein of *L. caerulea* may play a role for protecting intracellular structures and molecules against freezing injury and/or for increasing tolerance against desiccation under low temperature. In this matter, it may be necessary to understand the relationship between *L. caerulea* 42 kDa protein and antifreeze proteins (Griffith *et al.*, 1992), cryoprotective protein (Sieg *et al.*, 1996) and late embryogenesis abundant-like (LEA-like) proteins of plants (Gilmour *et al.*, 1992; Lin and Thomashow 1992; Salzman *et al.*, 1996). For this reason, we are now trying to obtain the sequence data of the 42 kDa protein. In future, we will examine this protein using plants grown in fields.

Acknowledgements

We are grateful to Mr. N. Kasai (Hokkaido University) for his excellent technical assistance.

References

Arora, R., Wisniewski, M. E., Scorza, R., 1992. Cold acclimation in genetically related (sibling) deciduous and evergreen peach (*Prunus persica* [L.] Batsch). Plant Physiol., **99:** 1562-1568.

Gilmour, S. J., Artus, N. N., Thomashow, M. F., 1992. cDNA sequence analysis and expression of two cold-regulated genes of *Arabidopsis thariana*. Plant Mol. Biol., **18:** 13-21.

Griffith, M., Ala, P., Yang, D. S. C., Hon, W-C., Moffatt, B. A., 1992. Antifreeze protein produced endogenously in winter rye leaves. Plant Physiol., **100:** 593-596.

Guy, C. L., Haskell, D., 1987. Induction of freezing tolerance in spinach is associated with the synthesis of cold acclimation induced proteins. Plant Physiol., **84:** 872-878.

Guy, C. L., Hummel, R. L., Haskell, D., 1987. Induction of freezing tolerance in spinach during cold acclimation. Plant Physiol., **84:** 868-871.

Guy, C. L., Niemi, K. J., Brambl, R., 1985. Altered gene expression during cold acclimation of spinach. Proc. Nat. Acd. Sci. USA., **82:** 3673-3677.

Imanishi, H. T., Suzuki, T., Masuda, K., Harada, T., 1998. Accumulation of raffinose and stachyose in shoot apices of *Lonicera caerulea* L. during cold acclimation. Scientia Horticulturae., **72:** 255-263.

Imanishi, H., Suzuki, T., Masuda, K., Harada, T., 1999. Accumulation of raffinose during cold acclimation in *Lonicera caerulea* L. plantlets cultured *in vitro*. Cryo-Letters., in press.

Kurkela, S., Franck, M., 1990. Cloning and characterization of a cold- and ABA-inducible *Arabidopsis* gene. Plant Mol. Biol., **15:** 137-144.

Laemmli, U. K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 680-685.

Levitt, J., 1980. "Responses of plants to environmental stresses. I. Chilling, freezing and high temperature stresses", Ed 2. Academic Press. Orlando.

Lin, C. H., Guo, W. W., Everson, E., Thomashow, M. F., 1990. Cold acclimation in *Arabidopsis* and wheat. A response associated with expression of related genes encoding 'boiling-stable' polypeptides. Plant Physiol., **94:** 1078-1083.

Lin, C., Thomashow, M. F., 1992. DNA sequence analysis of a complementary DNA for cold-regulated *Arabidopsis* gene *cor*15 and characterization of the COR15 polypeptide. Plant Physiol., **99:** 519-525.

Meza-Basso, L., Alberti, M., Raynal, M., Ferrero-Cadinanos, M. L., Delseny, M., 1986. Changes in protein synthesis in rapeseed (*Brassica napus*) seedlings during a low temperature treatment. Plant Physiol., **82:** 733-738.

Mohapatra, S. S., Wolfraim, L., Poole, R. J., Dhindsa, R. S., 1989. Molecular cloning and relation to freezing tolerance of cold-acclimation-specific genes of alfalfa. Plant Physiol., **89:** 375-380.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant., **15:** 473-497.

Muthalif, M. M., Rowland, L. J., 1994. Identification of dehydrin-like proteins responsive to chilling in floral buds of blueberry (*Vaccinium*, section *Cyanococcus*). Plant Physiol., **104:** 1439-1447.

Nordin, K., Heino, P., Palva, E. T., 1991. Separate signal

pathways regulate the expression of a low-temperature-induced gene in *Arabidopsis thaliana* (L.) Heyhn. Plant Mol. Biol., **16:** 1061-1071.

O'Farrell, P. Z., Goodman, H. M., O'Farrell, P. H., 1977. High resolution two-dimensional electrophoresis of basic as well as acidic proteins. Cell., **12:** 11 33-1142.

Perras, M., Sarhan, F., 1989. Synthesis of freezing tolerance proteins in leaves, crown, and roots during cold acclimation of wheat. Plant Physiol., **89:** 577-585.

Sakai, A., Larcher, W., 1987. Frost survival of plants. Responses and adaptation to freezing stress, Springer-Verlag. Berlin.

Salzman, R. A., Bressan, R. A., Hasegawa, P. M., Ashworth E. N., Bordelon, B. P., 1996. Programmed accumulation of LEA-like proteins during desiccation and cold acclimation of overwintering grape buds. Plant Cell Environ., **19:** 713-720.

Sieg, F., Schroder, W., Schmitt, J. M., Hincha, D. K., 1996. Purification and characterization of a cryoprotective protein (cryoprotectin) from the leaves of cold-acclimated cabbage. Plant Physiol., **111:** 215-221.

Welin, B. V., Olson, Å., Palva, E. T., 1994. Characterization and differential expression of dhn/lea/rab-like genes during cold acclimation and drought stress in *Arabidopsis thaliana*. Plant Mol. Biol., **26:** 131 -144.

Weretilnyk, E., Orr, W., White, T. C., Iu, B., Singh, J., 1993. Characterization of three related low-temperature-regulated cDNAs from winter *Brassica napus*. Plant Physiol., **101:** 171-177.