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BRIEF COMMUNICATION

# Transformation with a gene for myo-inositol *O*-methyltransferase enhances the cold tolerance of *Arabidopsis thaliana*

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## Abstract

In this study, we report a function of myo-inositol-*O*-methyltransferase (Imt1) in response to low temperature stress using transgenic *Arabidopsis thaliana*. *Imt1* gene was constructed identical to the *Imt1* gene from a halophyte *Mesembryanthemum crystallinum*. After cold stress, the *Imt1* transgenic plants exhibited stronger growth than the wild type plants. The elevated cold tolerance of the *Imt1* over-expressing plants was confirmed by the lower electrolyte leakage and accumulation of malondialdehyde, but higher proline and soluble sugar contents in transgenic than wild type plants.

*Additional key words*: electrolyte leakage, malondialdehyde, proline, soluble sugars.

Cold is an environmental factor that limits the geographical distribution and growing season of many plant species, and it often adversely affects crop quality and productivity (Thomashow 1999). In order to survive, plants have evolved intricate mechanisms to respond and adapt to cold stress at the molecular, cellular and whole-plant level (Tsutsui *et al*. 2009). Plant genes regulated by cold (Thomashow 1998, 1999, Seki *et al*. 2002) are involved in respiration, metabolism of sugars, lipids, antioxidants, production of chaperones, antifreeze proteins and osmolytes.

*Imt1* gene, encoding myo-inositol-*O*-methyltransferase (Imt1) that uses *S*-adenosyl-*L*-methionine to methylate myo-inositol to form *D*-ononitol, was identified from the halophyte ice plant (*Mesembryanthemum crystallinum)*. The molecular and physiological functions of this gene have been firstly studied in ice plant and *Escherichia coli* (Vernon and Bohnert 1992, Rammesmayer *et al* 1995). Transgenic tobacco plants carrying a cDNA encoding Imt1 accumulated *D*-ononitol and as a result, acquired enhanced photosynthesis protection and increased recovery under drought and salt stress (Sheveleva *et al*. 1997). In the current study, we generate transgenic *A. thaliana* plants expressing the ice plant *Imt1* to investigate the contribution of this gene to cold stress tolerance.

We synthesized the *Imt1* gene by PCR-based two-step DNA synthesis (PTDS) method (Xiong *et al*. 2004). Errors in the synthetic gene were corrected by the overlap extension polymerase chain reaction (OE-PCR) method (Peng *et al*. 2006, Xiong *et al.* 2006). Twenty-six 60 nt oligonucleotides and one 75 nt oligonucleotide were used to synthesize the recombinant gene (Table 1). *BLAST* search showed that the synthesized recombinant *Imt1* gene was 100 % identical to the wild type *Imt1* gene (acc. No. GI 167261). The condition of this PCR-mediated

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*Abbreviations*: Imt1 - myo-inositol *O*-methyltransferase; MDA - malondialdehyde; nt - nucleotide; OE-PCR - overlap extension polymerase chain reaction; PTDS - PCR-based two-step DNA synthesis.
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Table 1 Primers for enzymatic synthesis of the *ImtI* gene. Odd number: forward primer; Even number: reverse primer. *Shadow box*: *Bam* HI and *Sac* I sites.

| Primer | Oligonucleotides |
|---|---|
| Imt1-1 | GAAGGATCCATGACCACCTATACTAATGGTAACTACACTCAACCTAAGACTCTGGACAAG |
| Imt1-2 | CATTTGCCAGAGTGACAGCCAATCCAGCAAGCTGCTCGTCCTTGTCCAGAGTCTTAGGTT |
| Imt1-3 | GGCTGTCACTCTGGCAAATGCAGCTGCATTCCCAATGATCTTGAAGAGTGCATTCGAGCT |
| Imt1-4 | GACACCTTCACCAGCCTTGGAGAAGATGTCCAGGATCTTCAGCTCGAATGCACTCTTCAA |
| Imt1-5 | CCAAGGCTGGTGAAGGTGTCTTCGTCAGTACCAGCGAGATCGCTTCTCAGATTGGTGCTA |
| Imt1-6 | CGAAGCATACGGTCCAGCAGGACTGGTGCATTTGGATTCTTAGCACCAATCTGAGAAGCG |
| Imt1-7 | CTGCTGGACCGTATGCTTCGTCTTCTGGCATCTCACAGCGTCCTGACCTGTAAACTTCAG |
| Imt1-8 | GAGCTGGACCATAGACCCTCTGACTACCACCTTCACCCTTCTGAAGTTTACAGGTCAGGA |
| Imt1-9 | GAGGGTCTATGGTCCAGCTCCACTGTGTAACTACCTGGCATCCAACGACGGTCAAGGAAG |
| Imt1-10 | CATGACCTTGTCATGGTGCAGGACGAGCAGTGGTCCAAGGCTTCCTTGACCGTCGTTGGA |
| Imt1-11 | TGCACCATGACAAGGTCATGATGGAGTCCTGGTTCCACCTGAACGACTACATCCTGGAAG |
| Imt1-12 | AACTGGATCATTCCATGTGCTCTCTTGAATGGAACACCTCCTTCCAGGATGTAGTCGTTC |
| Imt1-13 | GCACATGGAATGATCCAGTTTGACTACACTGGTACTGACGAGAGGTTCAACCACGTCTTC |
| Imt1-14 | TCTTCATGACCAAGATGGTATGATGAGCCATACCTTGGTTGAAGACGTGGTTGAACCTCT |
| Imt1-15 | TACCATCTTGGTCATGAAGAAGCTCCTTGACAACTACAATGGTTTCAACGACGTCAAGGT |
| Imt1-16 | AGAGACGTTGACACCAATGTTACCACCAACATCGACCAGGACCTTGACGTCGTTGAAACC |
| Imt1-17 | ACATTGGTGTCAACGTCTCTATGATCGTTGCTAAGCACACCCATATCAAGGGTATCAACT |
| Imt1-18 | GGATAGCTTGGAGCGTCTGCGATGACGTGTGGAAGGTCGTAGTTGATACCCTTGATATGG |
| Imt1-19 | GCAGACGCTCCAAGCTATCCTGGTGTTGAGCATGTTGGTGGTAACATGTTCGAGTCTATC |
| Imt1-20 | CATGCAAGACCCACTTCATGAAGATTGCATCTGCTTGTGGGATAGACTCGAACATGTTAC |
| Imt1-21 | CATGAAGTGGGTCTTGCATGACTGGTCTGACGAACACTGCGTCAAGATCCTGAACAAATG |
| Imt1-22 | AACCAGGATGATCTTACCTCCCTTAGCCAAGGACTCGTAGCATTTGTTCAGGATCTTGAC |
| Imt1-23 | GAGGTAAGATCATCCTGGTTGAGTCTCTGATCCCAGTCATTCCAGAAGACAACCTTGAGT |
| Imt1-24 | TGAACAAGAGTATGACAGTCAAGAGAGAAGACCATGTGAGACTCAAGGTTGTCTTCTGGA |
| Imt1-25 | GACTGTCATACTCTTGTTCACAACCAAGGTGGTAAGGAGAGATCCAAGGAGGACTTCGAA |
| Imt1-26 | TGACGTCAACAGTGCTGAATCCAGTCTTACTTGCCAGTGCTTCGAAGTCCTCCTTGGATC |
| Imt1-27 | AAGGAGCTCTTACTTCTTATACAACTCCATAACCCAGGTATCGTAGCGCAACAGATGACGTCAACAGTGCTGAAT |

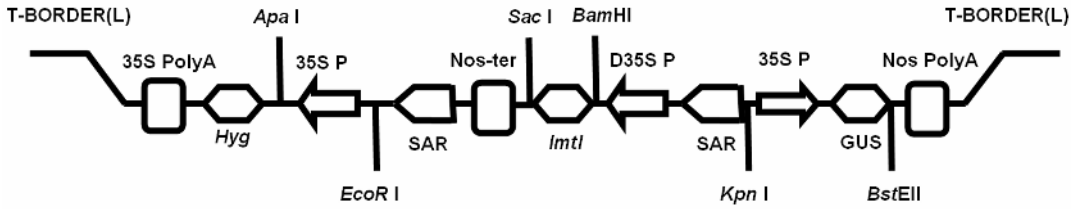


Fig. 1. Schematic diagram of the vector used in this study. The vector contains the double CaMV 35S (DCaMV35S) promoter and the tobacco mosaic virus (TMV) sequence fused to the *ImtI* gene. For steady transmission of the *ImtI* gene, two Scaffold attachment regions (SAR) were fused upstream of the DCaMV35S promoter and downstream of the Nos-terminator (Nos-T).

assembly was 30 s at 94 °C, 30 s at 45 °C and 30 s at 72 °C for each cycle followed by an additional 10 min at 72 °C to ensure complete extension for all PCR reactions.

*Arabidopsis thaliana* L. cv. Columbia was transformed with the *Agrobacterium tumefaciens* strain GV3101 using the floral dip method as described previously (Clough and Bent 1998). The plasmid used in the transformation was derived from pYF7716 (Peng *et al*. 2001). The synthesized *Imt1* cDNA was digested with *Bam* HI and *Sac* I, and cloned into the binary vector under the control of an enhanced double CaMV 35S promoter and the tobacco mosaic virus TMV *Omega* leader sequence (Fig. 1).

Eleven transgenic plants with *Imt1* were obtained, and transgenic plants of the $T_2$ generation were confirmed by β-glucuronidase (GUS) stain and PCR detection similarly as Xue *et al*. (2010). We selected three transgenic lines (named N1, N2, and N3, corresponding to lanes 2, 3, and 4, respectively, in Fig. 2) and used them for further freezing tolerance and physiological experiments. It was shown that *Imt1* gene has been highly expressed in these three transgenic lines (Fig. 2). These *A. thaliana* lines were grown on germination medium in pots for about 10 d. In freezing tests, the plants were incubated at 4 °C for 2 d to induce cold acclimation under either long- or short-day photoperiods. The plants were then placed in

-20 °C for 30 min and then transferred to 4 °C for one night in order to acclimate. The next day, all plants were returned to growth chamber (23 ± 2 °C). The survival rates of lines N1, N2, and N3 were 40.0 - 72.0 %, which were significantly higher (especially N1 and N2) than those of wild type plants (28.0 %).

Electrolyte leakage was measured as described (Alferez et al. 2006) and the malondialdehyde (MDA) content was estimated by the reaction of thiobarbituric acid (TBA) as described by Cakmak and Horst (1991). Proline content was assayed on water-extracted seedlings using the ninhydrin assay of Bates *et al*. (1973). Soluble sugars were determinated by the anthrone colorimetry (Yemm and Willis 1954, Sanchez *et al*. 1998). All these physiological experiments were repeated for three times and data are the means ± SD of three replicates.

Low temperature stress caused significant increases in electrolyte leakage and MDA content in both wild type and transgenic lines (Fig. 3*A*,*B*). The electrolyte leakage content of transgenic plants was significantly lower than that of wild-type plants after freezing stress (Fig 3*A*), demonstrating that less membrane damage occurred in the transgenic plants. James *et al*. (1997) studied the relationship between a synthetic antifreeze protein and a decrease of electrolyte leakage in potato at freezing temperatures. Our findings are consistent with their results that low temperature stress caused a significant increase in electrolyte leakage. Transgenic plants had lower MDA content than wild type plants, not only in normal conditions but also after stress. Lower MDA content was especially observed in N3 line. Similar results were also obtained by over-expression of At-ALDH3 in *A. thaliana* and MsALR from *Medicago sativa* in tobacco (Oberschall *et al*. 2000, Sunkar *et al*. 2003). Less MDA accumulation in transgenic lines means lower membrane damage because MDA can act as an indicator of the production of ROS, which can induce direct damage to cellular components. This implies that the *Imt1* gene could protect the cell membrane integrity under low temperature stress.

We tested whether cold stress induced changes in the proline contents of wild type and transgenic plants at

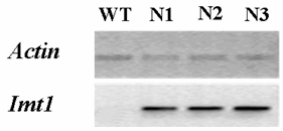


Fig. 2. Confirmation of *Imt1* genomic integration in transgenic lines by RT-PCR. Wild type (*lane* WT) and transgenic lines (*lanes* N1, N2, N3) of *A. thaliana* were used as PCR templates.

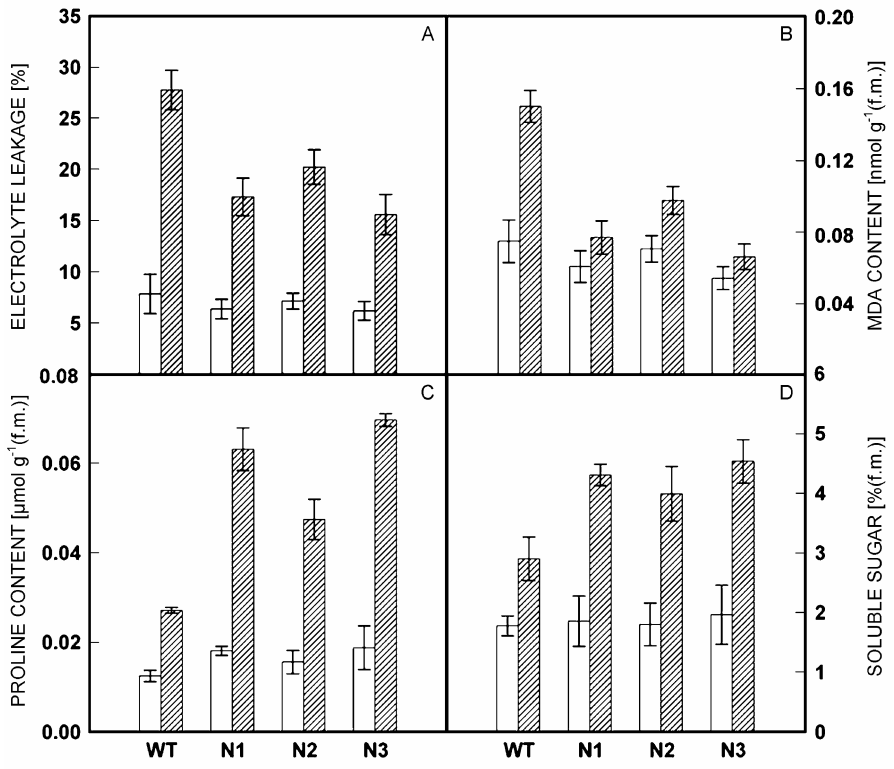


Fig. 3. Effects of low temperature stress on the physiological properties in leaves of wild type and transgenic plants. Data are the means ± SD of three replicates. The wild type plants (WT) and transgenic lines (N1 to N3) were grown in the same conditions. *White columns* - 3-week-old wild type (WT) and transgenic lines (N1 to N3) growing under normal conditions; *stripped columns* - wild type and transgenic plants exposed to the temperature of - 4 °C for 2 d. *A* - electrolyte leakage, *B* - MDA content, *C* - proline content, *D* - soluble sugar content.

different temperatures. The proline contents of transgenic plants were almost the same to that of wild type plants in normal conditions. Freezing stress caused an increase in the proline content of the wild type and transgenic plants. The increase after freezing stress in the transgenic plants was higher than that in wild type plants (Fig. 3*C*).

There was no distinct different between the transgenic lines and wild type plants for the total soluble sugar content in normal conditions. However, the total soluble sugar content of transgenic lines was higher than wild type plants after freezing. Soluble sugar contents increased markedly after cold acclimation, suggesting that the increase in soluble sugar content during cold acclimation could be related to the acquisition of freezing tolerance. Accumulation of sugars might make a greater contribution to osmotic adjustment than proline (Watanabe *et al*. 2000). Similar to the results seen in proline content, the total soluble sugar contents were significantly increased after low temperature stress. The level of freezing tolerance and the accumulation of proline and soluble sugars paralleled each other after freezing stress, indicating that proline and sugar accumulation is a fundamental component of enhanced freezing tolerance.

We also preliminarily examined tolerance to heat, dehydration and salt stresses in the transgenic plants. The plants revealed tolerance to NaCl stress, but an analogous enhancement of tolerance to heat and dehydration stress were not observed (data not shown).

In conclusion, it has been determined that over-expression of *Imt1* gene in *A. thaliana* displays tolerance to low temperature stress and it is suggested that the accumulation of proline and total soluble sugars in leaves maybe related to low temperature stress. Further investigation should be done to gain more information about the function of *Imt1* gene in stress responses.

## References


Alferez, F., Pozo, L., Burns, J.K.: Physiological changes associated with senescence and abscission in mature citrus fruit induced by 5-chloro-3-methyl-4-nitro-1H-pyrazole and ethephon application. - Physiol. Plant. **127**: 66-73, 2006.

Bates, L.S., Waldren, R.P., Teare, I.D.: Rapid determination of free proline in water-stress studies. - Plant Soil **39**: 205-207, 1973.

Cakmak, I., Horst, W.J.: Effect of aluminium on lipid peroxidation, superoxide dismutase, catalase, and peroxidase activities in root tips of soybean (*Glycine max*). - Physiol. Plant. **83**: 463-468, 1991.

Clough, S.J., Bent, A.F.: Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. - Plant J. **16**: 735-743, 1998.

James, G.W., Wang, H.Y., Daniel, J.G.: Expression of a synthetic antifreeze protein in potato reduces electrolyte release at freezing temperatures. - Plant mol. Biol. **35**: 323-330, 1997.

Oberschall, A., Deak, M., Torok, K., Sass, L., Vass, I., Kovacs, I., Feher, A., Dudits, D., Horvath, G.V.: A novel aldose/ aldehyde reductase protects transgenic plants against lipid peroxidation under chemical and drought stresses. - Plant J. **24**: 437-446, 2000.

Peng, R.H., Huang, X.M., Li, X., Sun, A.J., Yao, Q.H., Peng, Y.L.: Construction of a plant binary expression vector containing intron-kanamycin gene and transformation in *Nicotiana tabacum*. - Acta phytophysiol. sin. **27**: 55-60, 2001.

Peng, R.H., Xiong, A.S., Yao, Q.H.: A direct and efficient PAGE-mediated overlap extension method for gene multiple-site mutagenesis. - Appl. Microbiol. Biotechnol. **73**: 234-240, 2006.

Rammesmayer, G., Pichorner, H.D., Adams, P., Jensen, R.G., Bohnert, H.J.: Characterization of IMT1, myo-Inositol O-methyltransferase, from *Mesembryanthemum crystallinum*. - Arch. Biochem. Biophys. **322**: 183-188, 1995.

Sanchez, F.J., Manzaranes, M., De Andres, E.F., Tenorio, J.L., Ayerbe, L.: Turgor maintenance, osmotic adjustment and soluble sugar and proline accumulation in 49 pea cultivars in response to water stress. - Field Crops Res. **59**: 225-235, 1998.

Seki, M., Narusaka, M., Ishida, J., Nanjo, T., Fujita, M., Oono, Y., Kamiya, A., Nakajima, M, Enju, A., Sakurai, T., Satou, M, Akiyama, K., Taji, T., Yamaguchi-Shinozaki, K., Carninci, P., Kawai, J., Hayashizaki, Y., Shinozaki, K.: Monitoring the expression profiles of 7000 *Arabidopsis* genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. - Plant J. **31**: 279-292, 2002.

Sheveleva, E., Chmara, W., Bohnert, H.J., Jensen, R.G.: Increased salt and drought tolerance by D-ononitol production in transgenic *Nicotiana tabacum* L. - Plant Physiol. **115**: 1211-1219, 1997.

Sunkar, P., Bartels, D., Kirch, H.H.: Overexpression of a stress-inducible aldehyde dehydrogenase gene from *Arabidopsis thaliana* in transgenic plants improves stress tolerance. - Plant J. **35**: 452-464, 2003.

Thomashow, M.F.: Role of cold-responsive genes in plant freezing tolerance. - Plant Physiol. **118**: 1-7, 1998.

Thomashow, M.F.: Plant cold acclimation, freezing tolerance genes and regulatory mechanisms. - Annu. Rev. Plant. Physiol. Plant. mol. Biol. **50**: 571–599, 1999.

Tsutsui, T., Kato, W., Asada, Y., Sako, K., Sako, K., Sonoda, Y., Kidokoro, S., Yamaguchi-Shinozaki, K., Tamaoki, M., Arakawa, K., Ichikawa, T., Nakazawa, M., Seki, M., Shinozaki, K., Matsui, M., Ikeda, A., Yamaguchi, J.: DEAR1, a transcriptional repressor of DREB protein that mediates plant defense and freezing stress responses in *Arabidopsis*. - J. Plant Res. **122**: 633- 643, 2009.

Vernon, D.M., Bohnert, H.J.: A novel methyl transferase induced by osmotic stress in the facultative halophyte *Mesembryanthemum cytstallinum*. - The EMBO J. **11**: 2077-2085, 1992.

Watanabe, S., Kojima, K., Ide, Y., Sasaki, S.: Effects of saline and osmotic stress on proline and sugar accumulation in

*Populus euphratica in vitro*. - Plant Cell Tissue Organ Cult. **63**: 199-206, 2000.

Xiong, A.S., Yao, Q.H., Peng, R.H., Li, X., Fan, H.Q., Cheng, Z.M., Li, Y.: A simple, rapid, high-fidelity and cost-effective PCR-based two-step DNA synthesis method for long gene sequence. - Nucl. Acids Res. **32** (Suppl.): e98, 2004.

Xiong, A.S., Yao, Q.H., Peng, R.H., Duan, H., Li, X., Fan, H.Q., Cheng, Z.M., Li, Y.: PCR-based accurate synthesis of long DNA sequences. - Nat. Protocols **1:** 791-797, 2006.

Xue, Y., Peng, R., Xiong, A., Li, X., Zha, D., Yao, Q.: Over-expression of heat shock protein gene hsp26 in *Arabidopsis thaliana* enhances heat tolerance. - Biol. Plant. **54**: 105-111, 2010.

Yemm, E.W., Willis, A.J.: The estimation of carbohydrates in plant extracts by anthrone. - Biochem. J. **57**: 508-514, 1954.