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Mariya Khodakovskaya · Richard McAvoy · Jeanne Peters · Hao Wu · Yi Li

Enhanced cold tolerance in transgenic tobacco expressing a chloroplast ω -3 fatty acid desaturase gene under the control of a cold inducible promoter.

Abstract A new cold-inducible genetic construct was cloned using a chloroplast-specific omega-3-fatty acid desaturase gene (*FAD7*) under the control of a cold-inducible promoter (*cor15a*) from *Arabidopsis thaliana.* RT-PCR confirmed a marked increase in *FAD7* expression, in young *Nicotiana tabacum* (*cv. Havana*) plants harboring *cor15a-FAD7*, after a short-term exposure to cold. When young, cold-induced tobacco seedlings were exposed to low-temperature (0.5, 2 or 3.5^{o}C) for up to 44 days, survival within independent *cor15a-FAD7* transgenic lines (40.2 to 96%) was far superior to the wild type (6.7 to 10.2 %). In addition, the major trienoic fatty acid species remained stable in cold-induced *cor15a-FAD7 N. tabacum* plants under prolonged cold storage while the levels of hexadecatrienoic acid (16:3) and octadecatrienoic acid (18:3) declined in wild type plants under the same conditions (79% and 20.7% respectively). Electron microscopy showed chloroplast membrane ultrastructure in *cor15a-FAD7* transgenic plants was unaffected by prolonged exposure to cold temperatures. In contrast, wild type plants experienced a loss of granal stacking and disorganization of the thylakoid membrane under the same conditions. Changes in membrane integrity coincided with a precipitous decline in leaf chlorophyll concentration and low survival rates in wild type plants. Cold-induced double transgenic *Nicotiana alata* (*cv. Domino Mix*) plants, harboring both the *cor15a-FAD7* cold-tolerance gene and a *cor15a-IPT* dark-tolerance gene, exhibited dramatically higher survival rates (89 to 90%) than wild type plants (2%) under prolonged cold storage under dark conditions (2^{0} C for 50 days).

Key words Cold-induced expression · Transgenic tobacco · Desaturase · Cold tolerance · Fatty acid composition · Chloroplast ultrastructure · Tolerance to cold and dark environment

Abbreviations 16:0: hexadecanoic acid · 18:0: octadecanoic acid· 16:1: hexadecenoic acid· 18:1: octadecenoic acid· 16:2: hexadecadienoic acid · 16:3: hexadecatrienoic acid ·18:2: octadecadienoic (linoleic) acid · 18:3: octadecatrienoic (linolenic) acid · MS: Murashige-Skoog medium· ω -3 refers to position of the double bond from methyl end of a fatty acid.

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Introduction

In the past several decades, extensive attention has been paid to the molecular mechanisms of chilling sensitivity in plants because of the agricultural demands for improvements in chilling tolerance of horticultural crops (Graham and Patterson 1982; Nishida and Murata 1996; Iba 2002; Sakamoto and Murata 2002). When comparing the fatty acids in the cellular membranes of chilling resistant versus chilling-sensitive plants, it was found that the chilling resistant plants have a greater abundance of unsaturated fatty acids. During acclimation to cold temperature, the activity of desaturase enzymes increases and the proportion of unsaturated fatty acids rise (Williams et al. 1992; Palta et al. 1993). This modification allows membranes to remain fluid by lowering the temperature at which the membrane lipids experience a gradual phase change from fluid to semi-crystalline. Thus, desaturation of fatty acids provides protection against damage from chilling temperatures.

Several investigators have shown that it is possible to improve the cold resistance of plants by genetic modification. Saturated fatty acid level was greatly decreased in transgenic tobacco plants expressing a Δ9 cyanobacterial desaturase resulting in a significant increase in chilling resistance (Ishizaki-Nishizawa et al. 1996). The cyanobacterial desaturase introduces a *cis*-double bond at the Δ9-position of both 16- and 18-carbon saturated fatty acids that are linked to various membrane lipids.

In *Arabidopsis*, three gene products, *FAD3*, *FAD7* and *FAD8*, mediate the synthesis of trienoic fatty acids from 18:2 and 16:2. Browse and Somerville (1991) reported that a mutation in the *FAD7* gene resulted in a temperature-dependent reduction in the 18:3 and 16:3 content in thylakoid-specific lipids. Kodama et al. (1994; 1995) was able to induce a decrease in the dienoic fatty acids (16:2+18:2) and an increase in the trienoic fatty acids (16:3+18:3) in tobacco by overexpressing the *Arabidopsis FAD7* gene under the control of the *35S* promoter. Evaluation of transgenic and wild type plants showed differences in low-temperature tolerance in young seedlings but no discernible difference in the performance of mature plants. In young seedlings exposed to 1^{o}C for 7 days and then cultured at 25^{o}C, leaf growth recovered faster in transgenic plants with the *FAD7* gene than in wild-type plants. Low-temperature-induced chlorosis was also reduced in the plants transformed with this gene (Kodama et al. 1994). Conversely, tobacco plants with

the *FAD7* gene silenced had a lower trienoic fatty acid content than the wild type, and were better able to acclimate to higher temperatures (Murakami et al. 2000). While increased cold tolerance resulted from expression of the *35S-FAD7*, transgenic plants with a constitutively expressed desaturase enzyme were not particularly well suited for growth under warm temperatures.

An inducible cold-tolerance trait could have broad application in commercial agriculture, especially in the nursery industry. For instance by inducing the trait, young seedlings (transplants) or vegetative shoot tips (asexual propagules), could be stored at cold temperatures for a prolonged period without high mortality or loss of vigor when returned to normal growth conditions. Recently, Khodakovskaya et al. (2005) reported an increased tolerance to prolonged dark storage in transgenic petunia and chrysanthemum in which a cold-inducible promoter (*cor15a*) was used to up-regulate cytokinin production via *IPT* (isopentenyl transferase) gene expression. In that study, leaves and vegetative shoot tips from *IPT* plants expressed elevated cytokinin concentrations and retained high chlorophyll concentrations in prolonged dark storage if first exposed to cold-induction conditions. However, if the expression was not induced with a cold signal, the transgenic plants were no more tolerant to dark storage than the wild type plants. In the investigation reported herein, *FAD7* expression was regulated with a cold temperature signal by using the cold-inducible promoter from the *cor15a* gene (Baker et al. 1994). Transgenic and wild type plants were subjected to various low temperature stress conditions, and plant survival, chlorophyll concentration, changes in leaf fatty acid composition and chloroplast membrane organization were determined. In addition, double transgenic *Nicotiana alata* seedlings were generated, by crossing *cor15a-FAD7* and *cor15a-IPT* parent lines, and tolerance to prolonged exposure to combined cold and dark conditions was evaluated.

Materials and Methods

Plasmid construction

Molecular cloning procedures were carried out as described by Sambrook et al. (1989), The promoter sequence from the *cor15a* gene (0.98 kb) from *Arabidopsis thaliana* was synthesized from genomic *DNA* of *Arabidopsis* by PCR reaction. The initial construction, containing the *FAD7* gene sequence from *A. thaliana*, was provided by the Arabidopsis Biological Resources Center (DNA Stock Center) at The Ohio State University. The 1.5 kb *FAD7* sequence was released by *Sac*I and *Xho*I digestion and then subcloned into the same sites of the *pBluescript II SK* (+/-) vector. The c*or15a* promoter sequence was cloned via PCR and then subcloned in the *Sac*I site of the *pBluescript II SK* (+/-) vector, generating the *pBluescript II SK-cor15a-FAD7* construct. To establish the final binary vector with the *FAD7* gene, we used the *pBin19* vector containing the *NOS* terminator (the *pBin19* vector was provided by Dr. Li, University of Connecticut). The vector was cut by *Sac*I and then treated by *Klenow* fragment to generate a blunt end and then cut by *Sal*I. At the same time the *pBluescript II SK-cor15a-FAD7* vector (4.4 kb) was cut by *Apa*I, treated with *Klenow* fragment to generate a blunt end, and then cut by *Sal*I to release the *cor15a-FAD7* fragment. Finally, this fragment was introduced in pBin19-NOS vector by ligation of one blunt and one sticky end. Thus the binary *pBin19* vector containing the *FAD7* gene, under the control of the *cor15a* promoter and ending with the *nos* terminator, was generated (Fig. 1). The base sequences of the *cor15a* promoter and *FAD7* gene in the plasmid were confirmed by *DNA* sequence analysis (W.M. Keck Biotechnology Laboratory, Yale University, New Haven, CT, USA). The binary plasmid was transformed into *Agrobacterium tumefaciens* strain LBA 4404 by electroporation.

Transformation and regeneration of transgenic tobacco plants

Nicotiana tabacum cv. Havana was transformed using an *Agrobacterium*-mediated transformation protocol (An et al. 1988). Briefly, tobacco leaves were surface-sterilized,

cut into discs and co-cultivated with *Agrobacterium tumefaciens* LBA 4404 bearing the *cor15a-FAD7* construct. Following co-cultivation, the explants were transferred to the MS medium (Murashige and Skoog 1962) supplemented with 0.1 mg/l of α-naphthaleneacetic acid (NAA), 1 mg/l of 6-benzylaminopurine (BA), 300 mg/l of kanamycin for selection, and 400 mg/l of timentin. Explants were transferred to fresh medium every 2-3 weeks. Shoots developed from the transgenic calli were excised and transferred to hormone-free MS-selection medium (100 mg/l of kanamycin) to induce roots. Plants with roots were transferred in pots and acclimated to the greenhouse. Putative transgenic plants (T_0) were analyzed by PCR for foreign gene integration. T_0 transformants were allowed to self-fertilize in the glasshouse. Seeds of several generations were germinated on MS medium supplemented 100 mg/l kanamycin to determine the segregation patterns of the transgene. Progeny obtained from T_1 plants were analyzed and six homozygous lines (T_2) were used in future cold experiments. *Nicotiana alata cv. Domino* was transformed as described above with the following exceptions. Seedling hypocotyls segments (1 cm) were used as explant tissue and the regenerative MS medium was supplemented with 0.1 mg/l of NAA, 3 mg/l of kinetin and 3 mg/l of BA. Kanamycin for selection was used at a concentration 60 mg/l.

Plants DNA extraction and polymerase chain reaction (PCR) analysis

Total DNA was isolated from leaf tissue of primary putative transgenic plants of *N. tabacum*, as well as T_1 and T_2 generative plants, by DNeasy Plant Mini Kits (Qiagen Inc., Valencia, CA, USA) and 200 ng of DNA was subjected to PCR reaction. The primer pairs used for DNA amplification were 5'-GGAGCTCGTCGACAGATCTTGTCCGTTGAATTT-3'; and 5'-GGAGCTCGAGAGAGATCTTTAAGATGT- 3' for the 0.98 kb fragment of the *cor15a* promoter and 5'-GGTATACGACCTCTCCCC-3'; 5'-GGTCCAGACTTATCAGGC-3' for the 1.25 kb fragment of the *FAD7* gene (whole gene size – 1.5 kb). PCR amplification was performed using a thermocycler (GeneAmp PCR System 2700, Applied Biosystems, Inc., Foster City, CA, USA). Cycling conditions for *cor15a* promoter were: 3 min at 94^{o}C; 30 cycles of 1 min at 94^{o}C, 1 min at 54^{o} C and 1 min 30 sec at 72^{o}C; extension at

72°C for 5 min. Cycling conditions for 1.25 kb fragment of *FAD7* gene were: 3 min at 94°C; 30 cycles of 1 min at 94°C, 1 min at 57° C and 1 min 30 sec at 72°C; extension at 72°C for 5 min. The reactions involved 200 ng of DNA template, 0.2 mM of dNTPs, 0.5 mM of each primer, 1X*REDTaq* PCR Buffer and 1 u of *REDTaq* DNA polymerase (Sigma, Saint Louis, MI, USA) in a final reaction volume of 20 µl. PCR products were observed under UV after electrophoresis on a 1% agarose gel with ethidium bromide. A 1-kb DNA molecular marker was used as a reference (Gibco BRL, Carlsbad, CA, USA). Transgenic plants of *N. alata* containing the *cor15a-FAD7* construction were confirmed using the same procedure. Putative *cor15a-IPT* transgenic lines of *N. alata* were confirmed by PCR analysis according the protocol reported by Khodakovskaya et al. (2005). *Cor15a-IPT* and *cor15a-FAD7 N. alata* parent lines were crossed to produce double transgenic (*cor15a-IPT* x *cor15a-FAD7*) T_1 plants. Putative double transgenic (*cor15a-IPT* x *cor15a-FAD7*) and plants of T_1 generation plants were confirmed by PCR analysis.

Southern hybridization

Three kanamycin-resistant transgenic lines of the *N. tabacum* (T_2 generation) were analyzed by Southern blot. Total genomic DNA was isolated from transgenic plants using DNeasy Plant Maxi Kits (Qiagen Inc., Valencia, CA, USA) in accordance with the recommended protocol. Total genomic DNA from putative transgenic and non-transformed control plants (10 µg samples) was digested at 37° C overnight by double restriction with enzymes *Hind*III and *Eco*RI and the *cor15a-FAD7* fragment was released. Digested DNA from each line was separated through a 1% agarose gel prepared in TAE buffer pH 8.5 (Sambrook et al., 1989) and fragments were transferred from agarose gel to a nylon membrane (Amersham, Chalfont St Giles, UK) and cross-linked to the membrane under UV irradiation. The *FAD7* gene probe (for 1.25 kb fragment) was prepared with a PCR DIG Probe synthesis kit (Roche Molecular Biochemicals, Indianapolis, IN) in accordance with the recommended protocol. The DNA fixed on membranes was prehybridized using a prehybridization solution at 68°C for 3h, and then hybridized with the probe at 68° C overnight, and finally triple-washed with the post- hybridization

solution at 65°C in a hybridization oven (HB-2D, Techne Ltd., Duxford-Cambridge, UK). Solutions for sample hybridization, and pre- and post-hybridization, and the buffers for the following steps were prepared as previously reported by Mercier (1998). Membranes were washed for 5 min. in 50 ml of maleate buffer (0.1 M maleic acid, 3.0 M of NaCl, pH 8.0) at room temperature and then incubated for 1 hour in 50 ml of blocking solution consisting of maleate buffer plus 0.5% blocking reagent (Roche Molecular Biochemicals, Indianapolis, IN). Membranes were then incubated for 30 min. in 20 ml of blocking solution with anti-dioxigenin-AP, Fab fragments (Roche Molecular Biochemicals, Indianapolis, IN) diluted to 1:10,000 and then washed 4-times for 10 min. in 50 ml of the maleate buffer. As a final step, membranes were equilibrated for 5 min. in 50 ml of substrate buffer (100 mM of Tris-HCl; 100 mM of NaCl; 5mM of $MgCl_2$, pH 9.5) and then incubated at 37°C for 10 min in 2 ml (sandwiched between two translucent plastic pages) of substrate buffer plus chemiluminescent substrate at a 1:100 dilution (CSPD, Roche Molecular Biochemicals, Indianapolis, IN). Membranes were exposed to autoradiographic film (Kodak X-Omart AR) for 4 hours. X-ray films were developed with an automatic film processor.

Analysis of *FAD7* gene expression in leaves of tobacco

Total RNA was isolated from plants of wild type and transgenic tobacco line N2 (a line which was found to be cold tolerant in preliminary trials) by grinding previously frozen samples in a mortar with TRI reagent (Molecular Research Center, Inc., Cincinnati, OH, USA). For RT-PCR analysis, DNA contamination was removed from RNA samples by DNase treatment (DNA-free™, Ambion, Inc., Austin, TX, USA), and then first-strand cDNA was synthesized from 1 μg of total RNA using First Strand Synthesis Kit RETROscript™ (Ambion Inc., Austin, TX, USA) following the recommended protocol. For PCR, a 0.5μL aliquot of RT-mix was used in a final volume of 25μL. PCR reaction for the 1.25 kb fragment of the *FAD7* gene was conducted as previously described. PCR reaction products along with RT-mix and primers to 18S RNA were used as internal standards (QuantumRNA™ 18S Internal Standards from Ambion Inc., Austin, TX, USA). PCR products (10 μL) were run on a 1% agarose gel.

Cultivation and cold-resistance analysis of transgenic plants

Transgenic lines, which exhibited 'normal' morphology (no differences in growth or flowering from the wild type) and were found to be most cold tolerant in preliminary trials, were selected for use in this and subsequent experiments. Further the selected transgenic lines conformed to Mendelian principles of inheritance for a single genetic locus. Young T_2 seedlings from each of six homozygous transgenic lines and the wild type were arranged into identical groups of 12 (on 288 plug trays) when the seedlings reached the 4-true leaf stage. Seedlings were then exposed to a cold-induction signal (4^{o}C for 4 days) before returning to growth conditions (25^{o}C) for one week. Plants were then transferred to cold-stress conditions at 0.5, 2 or 3.5^{o}C. Seedlings under cold-stress were exposed to light at 10μM.m^{-2}.s^{-1} and watering as needed to prevent desiccation. After 44 days of exposure to cold, seedling survival was assessed by counting the number of dead seedlings and the number of live seedlings from each treatment group and calculating the percentage of surviving seedlings. Plants were considered dead when the entire shoot collapsed and the shoot apex appeared necrotic. Plants were considered alive, regardless of turgor status and leaf color, as long as the shoot apex was not necrotic and the shoot has not collapsed. The experiment was replicated in triplicate overtime. Data were analyzed as a two-way analysis of variance with genetic line and storage temperature as the independent variables.

Quantification of Chlorophyll

The specific chlorophyll concentration was determined as follows. The youngest, fully expanded leaf was sampled from individual plants in each sample group (the wild type and each transgenic line tested). Leaves were blotted dry, and a 100 mg sample leaf was placed in a 1.5-mL sample tube. The samples were resuspended in 80% acetone, ground with a disposable pestle, and incubated in the dark for 30 min. Total chlorophyll (Chl μg mL^{-1}) was determined using absorbance at 645 and 663 nm, according to the equation:

20.2 A_{645} + 8.02 A_{663} (Chory et al. 1994). The average of chlorophyll content was determined from 10 independent plants from each sample group both before and after cold.

Fatty Acid Analysis

The youngest fully expanded leaves were used for the fatty acid extraction. Leaf material (200 mg FW) from each line studied was lyophillized using a freeze dry system/freezone 4.5 (Labconco Inc., Kansas City, MO, USA). The extracts from the dried material were then prepared as described in Wang et al. (1996). Briefly, the lipids in the freeze-dried tissue were acidified with 1N H_2SO_4 and the fatty acids were methylated with heating at 80 °C for 90 min. A solution containg 0.9% NaCl and 200 μmol hexane was added to the sample and then vortexed. After centrifuging for 5 min at 250 x g, 3 μl of the sample was subjected to GC-MS analysis. Fatty acids were separated and identified with a HP GG-MS (HP 6890 GC and HP 5973 MS) (Hewlett Packard Co., Palo Alto, CA, USA). A 60-m HP-5MS capillary column with an ID of 0.25 mm was used. The GC was programmed to begin at 170 °C for 10 min, followed by a 10min ramp until 220 °C, at a flow rate of 1 ml/min. Leaf tissue was sampled from both wild type and transgenic (*cor15a-FAD7* line N2) seedlings, before and after exposure to 4^0 C for 40 days. Samples were collected from three independent plants from each test group. A standard *T*-test was used to compared changes in fatty acid composition before and after cold-induction and between wild type and transgenic plants.

Electron Microscopy

Both the transgenic line N2 and wild-type tobacco were used for microscopic analysis. Leaf samples were collected from five plants from each genetic source before cold treatment and then again after cold treatment. Leaves that appeared representative of the plant response in each treatment group were selected. Whole leaves were pinned onto Silgard-coated plastic petri dishes and overlaid with a fixing solution containing 2% paraformaldehyde, 2.5% glutaraldehyde, 1.5 mM calcium chloride ($CaCl_2$) and 1.5 mM

($MgCl_2$) IN 0.05 M PIPES buffer, pH 6.9. Small (1mm X 2 mm) pieces were then cut with a razor blade from the apical leaf tips and pinned in place to keep them submerged. Dishes were covered and fixation proceeded for 5.5 hrs at room temperature. Thereafter, leaf pieces were washed 3 times for 20 min each in 0.05 PIPES buffer containing 1.5 mM $CaCl_2$ and 1.5 $MgCl_2$ and placed at 4°C in the same solution overnight. Samples were washed one more time in the buffer rinse and then briefly postfixed at room temperature for 20 min in 1% osmium tetroxide, 0.8% potassium ferricyanide, 1.5 mM $CaCl_2$ and 1.5 mM $MgCl_2$ in 0.05 M PIPES buffer, pH 6.9, after which time Kodak Photo-flo was added (3.5% v/v) as a surfactant to reduce surface tension. After several minutes, pieces were unpinned from the petri dishes and transferred to small shell vials containing fresh fixative without Photo-flo. Post-fixation continued for an additional 2.25 hrs. After fixing, tissues were returned to 4°C by rinsing in cold distilled water, 3 times for 20 min each, and dehydrated in a ascending ethanol series from 10% to 70% ethanol (EtOH), in 10% increments for 20 min each. Tissues were then stained in 1% uranyl acetate in 70% EtOH for 1.5 hr at 4°C, followed by two 5 min rinses in 70% EtOH, with the temperature brought back to room temperature during the second rinse. Dehydration was continued by washing tissues once in 85% and 95% EtOH and twice in 100% EtOH, 15 to 20 min per step. Finally, two washes in propylene oxide for 10 min each preceded the embedment of material into Spurr's resin. Thin sections were cut from the embedded samples using an ultramicrotome equipped with a diamond knife. Sections were mounted on copper grids (200 mesh), stained with lead citrate, and examined under a transmission electron microscope (Philips EM 300, Eindhoven, The Netherlands) at the University of Connecticut Electron Microscope Laboratory. TEM images were viewed for ten thin sections prepared from each leaf sampled. Twelve chloroplasts of each section were observed and representative images for each treatment group were recorded.

Assessing double transgenic *N. alata* seedlings for tolerance to cold, dark conditions

Double transgenic *N. alata* seedlings were generated, by crossing *cor15a-FAD7* and *cor15a-IPT* parent lines. Seeds (T_1 generation) were germinated in Petri dishes on MS

media with 60mg/l kanamycin. Kanamycin resistant seedlings were transferred to a soil-less medium in 288 plug trays and acclimated to the greenhouse. A small number of plants from each genetic cross were exposed to cold-inductive temperatures and RT-PCR was used to confirm expression of both the *IPT* and *FAD7* genes. Double transgenic lines 33 and 41were found to express both genes in response to a cold signal and these lines were used to evaluate cold, dark tolerance as follows. Once seedlings reached the 4-true leaf stage and the roots were well established in the medium, 10 seedlings from each double transgenic line 33 and 41 and 10 wild type plants, were placed into a parallel rows on a fresh plug sheet. The plug sheet arrangement was repeated three times. Plants were then cold-induced at 4^{o}C for 3-days, returned to the greenhouse at 25^{o}C for 5 days and then placed in a dark, temperature controlled cooler at 2^{o}C for 50 days. Plants were checked regularly, watered as needed and assessed for visible signs of injury. At the end of the 50-day test period, plant survival was assessed as previously described. The entire experiment was repeated with fresh seedlings. Survival response data were analyzed as a one-way analysis of variance. Finally, the fatty acid profile from double transgenic *N. alata* lines and wild type plants was analyzed as previously described.

Results

Genetic construction and transgenic plant production

Agrobacterium transformation of tobacco with the new *cor15a-FAD7* construct resulted in more than 20 kanamycin-resistant putative transformants. PCR and Southern hybridization analysis confirmed recombinant DNA integration into the genome of individual putative-transgenic plants (Fig. 2) and plants of generations T_1 and T_2. PCR amplification of both plasmid DNA and the genomic DNA from tobacco lines produced the expected 0.98 kb fragment of the *cor15a* promoter (Fig. 2A) and the 1.25 kb fragment of the *FAD7* gene (Fig. 2B). No amplification of DNA was detected in non-transgenic plants. Southern blot analysis of genomic DNA revealed the integration of different copy numbers of *FAD7* gene into the genome of several T_2 lines; lines1 and 3 contained a

single T-DNA copy, line 2 carried two copies. No signal was detected in control plants (Fig. 2C).

Molecular analysis of transgenic plants expressing *FAD7* under the control of a cold-inducible promoter

Reverse transcription-PCR (RT-PCR) analysis wild type and selected transgenic tobacco lines revealed a strong *FAD7* gene transcription signal in *cor15a-FAD7* tobacco exposed to the 4^{o}C cold-induction treatment but the transcription signal was very weak in cor15a-*FAD7* tobacco plants (line N2) that were not exposed to the cold-induction treatment (Fig. 3). Wild type plants showed no evidence of *FAD7* gene expression regardless of temperature treatment. These data demonstrate that *FAD7* expression in *cor15a-FAD7* plants could be dramatically up regulated via a cold-induction signal.

Cold tolerance and survival rate of wild type and *cor15a-FAD7* transgenic plants

Although short-term cold-induction treatment (4 days at 4^{o} C) caused no visible injury in *cor15a-FAD7* transgenic or wild type tobacco seedlings, long-term exposure to cold (0,5 to 3.5^{0} C) caused visible injury and dramatically reduced survival in wild type plants. Chill injury first appeared as chlorosis and the degree and extent of injury became progressively more pronounced as exposure to the cold continued. After extended exposure to cold, most leaves on the transgenic *cor15a-FAD7* remained green and the plants appeared healthy, but in contrast, wild type plants exhibited leaf damage, whole plant loss of turgor, shoot collapse and death.

After 44 days exposure to 0.5^{o}C, 2^{o}C, or 3.5^{o}C, tobacco seedlings carrying the *cor15a-FAD7* construct were more resistant to injury than the wild type and enjoyed dramatically higher survival rates ($P \leq 0.001$). Survival rate for wild type plants averaged 8.3% (averaged over the entire temperature range), while average survival for individual

transgenic lines ranged from 54 to 79% (Fig. 4.). Survival rate varied with storage temperature ($P \leq 0.001$), with the highest survival rate observed at 2°C for the wild type and all *cor15a-FAD7* lines except N1 (which survived best at 3.5°C). At 2°C the survival rate of the wild type plants averaged 10.2% while the survival rate of *cor15a-FAD7* lines N 2 and N3 both averaged 96%.

Fatty acid composition in leaves of transgenic plants

The most abundant fatty acids detected in the leaves of wild type *N. tabacum* grown at ambient temperatures were 16:0, 16:3, 18:2, and 18:3 (Table 1). In wild type plants exposed to cold treatment, there was a decline in the trienoic species, 16:3 ($P \leq 0.05$) and 18:3 ($P \leq 0.05$) and an increase in the dienoic species, 18:2 ($P \leq 0.05$) and the saturated species, 18:0 ($P \leq 0.001$). The change observed in the other major fatty acid species, 16:0 was not significant ($P=0.07$). Before exposure to cold induction, the fatty acid profile of the wild type and the *FAD7* transgenic plants were similar with the exception of 18:0 ($P \leq 0.05$) which was >4 times higher in the *FAD7* plants. However after cold induction 16:3 and 18:3 were both higher in the *FAD7* transgenic plants than in wild type plants ($P \leq 0.01$ and $P \leq 0.001$, respectively), and 18:0, 18:1 and 18:2 were all lower in the *FAD7* transgenic plants than in wild type plants ($P \leq 0.01$, $P \leq 0.001$ and $P \leq 0.001$, respectively). In wild type plants exposed to cold induction the 16:3 levels declined 79% ($P \leq 0.05$) while remaining stable in the *FAD7* line N2 plants. In contrast, the level of 18:3 in wild type plants declined 20.6% after exposure to cold but increased 18.5% in *FAD7* plants after exposure to cold. Also, after cold induction 18:2 increased by 58% in wild type plants ($P \leq 0.05$) but declined to non-detectable levels in *FAD7* plants.

Effects of prolonged exposure to 4°C on chloroplast ultrastructure and chlorophyll concentration

Thylakoid structure and organization appeared similar in chloroplasts from both wild type and *cor15a-FAD7* transgenic prior to exposure to prolonged cold stress conditions (Fig. 5-I). However in wild type plants, exposure to cold stress resulted in extensive changes in chloroplast ultrastructure (Fig. 5-II a, b). After prolonged and continuous exposure to cold temperatures, micrographs of chloroplast from wild type plants revealed swelling, loss of granal stacking, and membrane disorganization typically associated with chloroplast death (Fig. 5-II a, b). In contrast, micrographs from *cor15a-FAD7* leaves revealed that chloroplasts retained normal thylakoid structure and organization even after 40 days at 4^{o}C (Fig. 5-I c, d; Fig. 5-II c, d).

Changes in leaf chlorophyll concentration coincided with changes in chloroplast ultrastructure. Prior to exposure to cold, leaf chlorophyll concentrations were initially similar in both wild type and *cor15a-FAD7* plants (919 and 916 *u*g/g fresh weight respectively). However after 40 days of exposure to cold (4^{o}C) leaf chlorophyll concentrations were dramatically lower ($P \leq 0.001$) in surviving wild type plants (144 *u*g/g FW) than in the *cor15a-FAD7* plants (677 *u*g/g FW). Compared to the concentrations observed prior to cold, chlorophyll concentrations declined by 80% in wild type plants but only declined by 28% in *cor15a-FAD7* plants exposed to the same conditions.

Enhanced tolerance to both cold and dark conditions.

Double transgenic *N. alata* seedlings were generated, by crossing *cor15a-FAD7* and *cor15a-IPT* parent lines, and the tolerance of the double transgenic seedlings to exposure to both dark and cold conditions was evaluated. RT-PCR analysis confirmed cold-induced expression of both the *IPT* and *FAD7* genes putative double transgenic seedlings (Fig. 6). No *IPT* expression was observed in either line 33 or line 41 in the absence of a cold-induction signal. No *FAD7* expression was observed in line 33 in the absence of a cold-induction signal, but a low level of expression was observed in line 41.

Both double transgenic T_1 generation lines 33 and 41 resisted injury under prolonged cold, dark conditions. On average, survival of double transgenic *N. alata*

plants was dramatically higher (90% for line 33 and 89% for line 41) than for wild type plants (2%) following prolonged exposure to cold, dark conditions ($P \leq 0.001$).

Fatty acid analysis of *N. alata* leaves revealed that 16:0, 16:3, 18:2, and 18:3 were the major fatty acid species detected (Table 2) in wild type plants, while 16:0, 16:3 and 18:3 were the major fatty acid species detected in the double transgenic lines. The level of 18:3 was higher in the double transgenic (lines 41 and 33) plants than in wild type regardless of cold induction treatment ($P \leq 0.01$ under non-inductive conditions and $P \leq 0.001$ under cold inductive conditions). In response to cold-induction temperatures, 18:3 in wild type plants showed a marginal decline ($P=0.08$) and the 16:0 content showed a marginal increase ($P=0.08$). In the double transgenic lines, the 18:3 content remained stable after exposure to cold-inductive temperatures and 16:3 increased 60% in line 41 ($P \leq 0.05$) and 49% in line 33 ($P \leq 0.01$), and the level of 16:0 decreased in both lines ($P \leq 0.05$). The fatty acid species 18:2 was detected only in wild type plants and was unaffected by cold inductive conditions.

Discussion

In our study, we tested a new genetic construct capable of regulating the degree of membrane fatty acid desaturation in response to a cold-induction signal. The *FAD7* chloroplast ω-3 fatty acid desaturase gene from *A. thaliana* was cloned under the control of the cold-inducible promoter from the *cor15a* gene from *Arabidopsis*, and the construct was introduced into *N. tabacum* and *N. alata*. In both species, transgenic plants showed superior tolerance to prolonged exposure to cold temperatures. Tolerance was characterized by increased plant survival that coincided with increased retention of chlorophyll, and stability in both the membrane trienoic fatty acid components (16:3 and 18:3) and the chloroplast thylakoid ultrastructure.

The importance of membrane fluidity in temperature tolerance has been delineated by mutation analysis, transgenic, and physiological studies (Sung et al. 2003; Orvar et al. 2000). A change in membrane fluidity is one of the immediate consequences of exposure

to low temperature, and in chilling-sensitive species, membrane lipids represent a potential site of temperature perception and/or injury. Although we do not report changes in membrane fluidity, this property is largely dictated by the composition of the lipid molecular species, the degree of membrane saturation and temperature.

The *FAD7* gene catalyzes desaturation of the 16- and 18-carbon lipid-linked dienoic fatty acids (16:2 and 18:2). The amino-terminal region of the *FAD7* gene product carries a chloroplast transit peptide so that the primary affect of *FAD7* gene expression is on chloroplast membranes (Iba et al.1993). The FAD7 ω-3 fatty acid desaturase enzyme acts on 16- and 18-carbon fatty acids in either the sn-1 or sn-2 position of all chloroplast lipids including monogalactosyl diacylglycerol, digalactosyl diacylglycerol, phosphatidylglycerol, and sulfolipids. However Iba et al. (1993) reported that mutants deficient in FAD7 activity also experienced changes in extra-chloroplastic lipid composition. This phenomenon was attributed to a change in the trienoic fatty acid flux from the chloroplast to extra-chloroplastic membranes. Ishizaki-Nishizawa et al. (1996) reported that transgenic plants expressing a cyanobacterial desaturase gene also had a higher level of unsaturated fatty acid content in most membrane lipids and exhibited a significant increase in chill resistance. Kodama et al. (1994) expressed *Arabidopsis* desaturase *FAD7* gene in tobacco controlled by the *35S* promoter and observed a reduction in the incidence of low-temperature-induced chlorosis found in wild-type plants exposed to the same stress conditions. These studies conclusively demonstrated that resistance to injury from chilling could be increased by selectively expressing of a fatty acid desaturation gene.

In our study, the *cor15a* gene promoter was selected to drive *FAD7* expression so that an increase in fatty acid desaturation would occur after the plants were exposed to a brief but specific environmental signal. The *cor15a* gene is a member of the *COR* (cold-regulated) gene family. *Arabidopsis cor15a* gene is cold regulated, has *CRT/DRE* regulatory elements, and is induced in response to the *CBF* transcriptional activators (Thomashow 1999; Thomashow 2001; Jaglo et al. 2001). *Cor15a* is inactive, or very weakly active, in most plant tissues and plant organs maintained under normal grown temperatures but becomes highly active in plant shoots in response to low temperature (Baker et al. 1994). Previously, Khodakovskaya et al. (2005) used the *cor15a* promoter

to drive expression of the *IPT* gene (resulting in increased tissue cytokinin concentrations) in petunia and chrysanthemum, and demonstrated that *IPT* expression could be regulated in response to a short (3-d) cold treatment. In that study, *IPT* expression did not affect plant morphology under normal growth temperatures (25^{o}C) but did produce increased cytokinin concentrations and delayed leaf senescence in cold induced shoots.

Chilling tolerance in plants represents an important agronomic trait. Young transplants or vegetative cuttings that can survive prolonged cold storage and then resume vigorous growth allow for greater productivity and greater flexibility for the commercial propagator. A molecular strategy whereby transgenic plants selectively express greater thylakoid membrane stability under cold stress conditions represents a novel approach to selective chill-tolerance in plants. On the basis of this scenario, we constructed the *cor15a-FAD7* fusion gene and tested the effects of this gene on membrane stability and long-term survival under cold conditions. Our data demonstrate that cold-induced expression of the trait gene for increased desaturation of chloroplastic membrane fatty acids effectively and dramatically increased seedling survival under prolonged cold storage and that greater thylakoid membrane stability was associated with survival.

In cor15a-FAD7 tobacco, RT-PCR analysis indicated that *FAD7* expression dramatically increased in response of cold treatment (4^0 C for 3 days), although some expression was noted at higher temperatures (25^0 C). The most striking affect of increased desaturase gene expression in cold-induced *cor15a-FAD7* plants was the increase survival under long-term cold storage. For example, survival of non-transgenic (wild-type) *N. tabacum* after 44 days in cold–storage (0.5^0 C, 2^0 C, 3.5^0 C) averaged only 8.3%. However, survival of plants from individual *cor15a-FAD7* lines ranged from 54 to 79% under the same conditions. Analyses of leaf fatty acid composition revealed that the trienoic fatty acids, 18:3 and 16:3 remained stable in *cor15a-FAD7* transgenic *N. tabacum* after exposure to cold while the saturated fatty acids (16:0, 18:0) decreased ($P \leq 0.01$). In contrast, the proportion of 16:3 and 18:3 in wild-type plants, decreased after exposure to cold-stress conditions ($P \leq 0.01$ and $P \leq 0.05$, respectively) whereas the saturated fatty acid species 18:0 increased ($P \leq 0.001$). The proportion (mol%) of 18:2 increased by 58% in wild type plants while decreasing to a non-detectable level in the

cor15a-FAD7 plants after exposure to cold. Kodama et al. (1994) reported a similar phenomenon in transgenic *35S-FAD7* tobacco seedlings with a decrease in 16:2 and 18:2 concomitant with an increase in the 16:3 and 18:3.

It may be that the decline in 16:3 and 18:3 in wild type plants is responsible for changes to the chloroplast ultrastructure that coincide with the loss of chlorophyll and poor survival under prolonged cold stress conditions. Routaboul et al. (2000) used the triple mutant *fad3-2 fad7-2 fad8*, which has no detectable levels of trienoic acids to demonstrate a correlation in chlorophyll content to prolonged cold exposure. In our study, electron microscopy confirmed that changes in chloroplast ultrastructure coincided with the increased incidence of visible injury (e.g. chlorosis, necrosis, loss of turgor and death) in wild-type tobacco plants. After 40 days at 4^{o}C, granal disorganization appeared in wild type plastids. In contrast, chloroplast membranes in *cor15a-FAD7* plants appeared well organized and unaffected by the cold stress, and both chlorophyll retention and survival were substantially higher. Studies have shown that *A. thaliana fad5* mutants which are devoid of 16:3, but have wild type levels of 18:3, became chlorotic, and experience reduced growth and chloroplast ultrastructure changes similar to those seen in our study with wild type plants (Fig 5-II a, b) exposed to cold for prolonged periods (Hugly et al. 1992). The cold effects seen in *fad 5* plants were dependent on the leaf development stage (Hugly et al. 1992). Routaboul et al. (2000) also observed similar damage to the chloroplast ultrastructure when *FAD7* deficient mutants were exposed to long-term cold storage (30 days at 4^{o}C). Thus, the use of the cold-inducible *FAD7* gene has obvious potential for producing cold tolerant plants that selectively express the trait only in response to a cold signal.

In commercial horticulture, plants and excised plant parts are typically stored under cool, dark conditions but the incidence of chilling injury and mortality increases with storage duration (Heins et al. 1995). Therefore it is important to improve both cold and dark storage tolerance in sensitive species. In our study, we established transgenic *N. alata* plants that expressed two independent transgenes, *IPT* (isopentenyl transferase) and *FAD* that were both controlled by the cold-inducible *cor15a* promoter. Previously, Khodakovskaya et al., (2005) reported that cold-induced plants carrying the *cor15a-IPT* construct retained high chlorophyll concentrations, coincident with increased cytokinin

concentrations, after prolonged exposure to dark conditions. In the study herein, *N. alata* plants carrying both the *cor15a-IPT* gene for dark tolerance and the *cor15a-FAD7* gene for cold tolerance, were tested under continuous cold, dark storage. As expected the double transgenic plants showed superior tolerance to prolonged dark, cold storage and the trienoic fatty acid (16:3+18:3) components were higher in the cold-tolerance double transgenic lines and lower in the cold-sensitive wild-type line.

How plants perceive temperature in order to regulate membrane fatty acid desaturation remains an open question (Sakamoto and Murata 2002). However, regulation of fatty acid composition via a molecular genetic approach can be immediately beneficial in commercial agriculture.

In summary, our experiments indicated that deasaturase activity in transgenic plants could be regulated by a cold signal by using the cold inducible *cor15a* promoter to drive *FAD7* expression. The proportion of trienoic fatty acids in leaves of *cor15a-FAD7* plants was higher than in wild-type leaves after long-term exposure to cold, and the fatty profile was correlated with a more stable thylakoid ultrastructure and increased survival under prolonged exposure to cold. Further, combining multiple trait genes (such as the isopentenyl transferase (*IPT*) gene for dark-tolerance and the *FAD7* gene for cold-tolerance, under the control of the same cold-inducible promoter can be used to confer selective tolerance to multiple stress conditions.

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Table.1. Fatty acid profile from leaves of wild type (WT) and transgenic lines of *Nicotiana tabacum* (line N2) containing the fatty acid desaturase (*FAD7*) gene under the control of a cold-inducible promoter (*cor15a*). The major fatty acid components were isolated from total lipids extracted from young mature leaves. Leaf samples were obtained from plants grown under both normal greenhouse conditions (25 ^{o}C) and from similar plants after exposure to cold-inductive temperatures (4^{o}C). Each value represents the mean of three independent experiments.

		Fatty acid (mol%)							
Line	**Cold-induction condition**	**16:0**	**16:1**	**16:2**	**16:3**	**18:0**	**18:1**	**18:2**	**18:3**
WT	Non-inductive	13.5±2.2	0.7±0.6	0.4±0.2	10.9±1.8	0.9±0.5	1.1±0.7	14.6±1.6	58±3.3
WT	After cold-induction	19.5±0.4	nd	nd	2.3±0.4	5.3±0.8	3.8±0.3	23±1.4	46±1.4
cor15a-FAD7 N2	Non-inductive	17.5±0.4	0.2±0.1	0.4±0.2	4.9±0.9	3.8±0.07	2.2±0.4	6.8± 4.8	65±6
cor15a-FAD7 N2	After cold-induction	16±0	0.23±0.02	nd	4.1±0.18	2.2±0.07	1.2±0	nd	77±0.7

Table 2. Fatty acid profile from leaves of wild type (WT) and double transgenic *Nicotiana alata* (lines 33 & 41) containing both the *FAD7* and *IPT* genes under the control of a cold inducible promoter (*cor15a*). The major fatty acid components were isolated from total lipids extracted from young mature leaves. Leaf samples were obtained from plants grown under both normal greenhouse conditions (non-inducing temperatures) and from similar plants after exposure to cold-inductive temperatures. Each value represents the mean of three independent experiments.

		Fatty acids (mol%)							
Line	**Cold-induction condition**	**16:0**	**16:1**	**16:2**	**16:3**	**18:0**	**18:1**	**18:2**	**18:3**
WT	Non-inductive	23.5±0.4	nd	nd	5.1±0.3	2.6 ±0.1	0.9±0	12.6±0.3	52±2.1
WT	After cold-induction	27.6±1.4	1.5±0.2	nd	4.7±0.3	5.5±1.2	4.4±0.7	12.7±1	44±2.6
41	Non-inductive	19.6±1.2	0.61±0.03	nd	5.2±0.5	3.7±1.1	0.9±0.4	nd	70±2.8
41	After cold-induction	16.1±0.4	0.36±0.09	nd	8.3±0.5	2.5±0.4	0.75±0.1	nd	72±0
33	Non-inductive	20.4±1.1	0.70±0.4	nd	4.7±0.3	2.5±0.04	0.65±0.02	nd	71.5±1.1
33	After cold-induction	15.1±0.8	0.4±0.06	0.47±0.02	7.0±0.3	1.6±0.2	0.72±0.1	nd	75±0.7

Figures to paper:

Figure 1. Scheme showing the structure of the *cor15a-FAD7-nos* construct. From left to right: *RB*-right border of *pBin19*; P-*nos*-nopaline synthase promoter; NPTII -neomycin phosphotransferase (*nptII*) gene from Tn5; T-*nos* -nopaline synthase terminator; cor15a -promoter from the cor15a gene; FAD7 -gene from *Arabidopsis thaliana*; T-*nos* -nopaline synthase terminator; *LB* -left border of *pBin19*.

Figure 2. PCR analysis of DNA from putative transgenic tobacco lines and Southern blot analysis of genomic DNA isolated from T_2 generation of tobacco plants. **a** PCR analysis showing the presence of the expected 0.98 kb fragment of the *cor15a* promoter from putative-transgenic tobacco lines. *Lane 1* 1 kb molecular marker; *Lane 2* Positive control (plasmid *cor15a-FAD7-nos*); *Lane 3* Negative control (untransformed tobacco plants); *Lanes 4-8* putative-transgenic tobacco plants. **b** PCR analysis showing the presence of the expected 1.25 kb fragment of *FAD7* gene from putative-transgenic tobacco lines. *Lane 1* 1 kb molecular marker; *Lane 2* Positive control (plasmid cor15a-*FAD7-nos*); *Lane 3* Negative control (untransformed tobacco plants); Lane 4-8: putative-transgenic tobacco plants. **c** Southern blot analysis of genomic DNA from *cor15a-FAD7* tobacco plants (generation T_2). A DIG-labeled fragment of the FAD7 gene from the *cor15a-FAD7-nos* plasmid DNA was used as a probe. An *FAD7* fragment was detected from each of the transgenic lines analyzed (*Lanes 1-3*) but not from wild-type tobacco DNA (*Lane 4*). Plasmid DNA was used as the positive control (*Lane 5*).

Figure 3. RT-PCR analysis of *FAD7* expression in *cor15a-FAD7* and wild type *Nicotiana tabacum* plants exposed to normal growing temperatures (non-inductive conditions) or a cold-induction treatment (4^0C for 3 days). Lanes: *1* wild type (control) tobacco under non-inductive conditions, *2* wild type tobacco after 3 days exposure to a cold-induction treatment, *3 cor15a-FAD7* transgenic tobacco under non-inductive conditions, *4 cor15a-FAD7* transgenic tobacco after 3 days of a cold-induction treatment.

Figure 4. Young seedlings of wild type (*WT*) and six independent *cor15a-FAD7* transgenic tobacco lines after 44 days in cold storage at 0.5, 2 or 3.5^{o}C. Over the entire temperature range survival of wild type plants averaged just 8.3% but survival of individual *cor15a-FAD7* transgenic tobacco lines was dramatically higher ($P \leq 0.001$). Bars represent standard error (*SE*) of the mean survival rate.

Figure 5. Transmission electron micrographs of chloroplast ultrastructure in wild-type (**a, b**) and *cor15a-FAD7* transgenic tobacco leaves (**c, d**) under normal greenhouse conditions (I) and after 40 days at 4^{o}C (II). I-Prior to exposure to cold stress conditions the chloroplast ultrastructure appeared similar in micrographs obtained from both wild type (**a** and **b**) and *cor15a-FAD7* (**c** and **d**) plants. II-After prolonged and continuous exposure to cold temperatures, micrographs of chloroplast from wild type plants revealed

swelling, loss of granal stacking, and membrane disorganization typically associated with chloroplast death while chloroplast membranes of *cor15a-FAD7* plants appear normal. Scale bars equal to a length of 0.5 µ m in (**a**) and (**c**) and 0.1 µ m in (**b**) and (**d**).

Figure 6. RT-PCR analysis of *FAD7* (a) and *IPT* (b) gene expression in double transgenic *Nicotiana alata* lines 33 and 41 containing both genes under the control of a cold-inducible (*cor15a*) promoter. Gene expression was assessed in plants exposed to both non-inductive temperatures (normal growing temperatures) and cold-inductive temperatures. For both a and b, lanes: *1* double transgenic line 33 under non-inductive temperatures (25^0 C), *2* double transgenic line 41 under non-inductive temperatures (25^oC), *3* double transgenic line 33 exposed to cold-inductive temperatures (5 d at 4^o C), *4* double transgenic line 41 exposed to cold-inductive temperatures (5 d at 4^o C).