Changes in ABA and gene expression in cold-acclimated sugar maple

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Summary To determine if cold acclimation of sugar maple (Acer saccharum Marsh.) is associated with specific changes in gene expression under natural hardening conditions, we compared bud and root translatable mRNAs of potted maple seedlings after cold acclimation under natural conditions and following spring dehardening. Cold-hardened roots and buds were sampled in January when tissues reached their maximum hardiness. Freezing tolerance, expressed as the lethal temperature for 50% of the tissues (LT50), was estimated at −17 °C for roots, and at lower than −36 °C for buds. Approximately ten transcripts were specifically synthesized in cold-acclimated buds, or were more abundant in cold-acclimated buds than in unhardened buds. Cold hardening was also associated with changes in translation. At least five translation products were more abundant in cold-acclimated buds and roots compared with unhardened tissues. Abscisic acid (ABA) concentration increased approximately tenfold in the xylem sap following winter acclimation, and the maximum concentration was reached just before maximal acclimation. We discuss the potential involvement of ABA in the observed modification of gene expression during cold hardening.

Keywords: Acer saccharum, cold hardening, freezing tolerance, in vitro translation.

Introduction

Woody species native to the northern temperate zone have an annual cycle of cold hardening and dehardening. In many plants, acclimation to cold with major biochemical and physiological changes (Sakai and Larcher 1987), and altered gene expression (Lee and Chen 1993). However, little is known about the relationship between the biochemical changes and the changes in gene expression, particularly in woody species.

The extent of freezing tolerance of roots of sugar maple (Acer saccharum Marsh.) has been related to the capacity of the species to survive harsh winter conditions (Bertrand et al. 1994a). Calmé et al. (1994) found that a portion of the roots can survive temperatures as low as −30 °C in December, but are killed by a −6 °C frost in August, indicating that major changes in freezing tolerance occur during the year. Despite the importance of this phenomenon, little is known about the cold-hardening process in sugar maple. The identification of gene products that specifically accumulate in cold-acclimated tissues could provide a tool to study cold-hardening processes and to identify cold-tolerant families of trees.

Several studies have shown that changes in abscisic acid (ABA) concentration are related to the cold-hardening process in Acer species (Irving 1969, Dumbroff et al. 1979). Recently, we demonstrated that the concentration of ABA in xylem sap is a useful indicator of physiological changes occurring in sugar maple (Bertrand et al. 1994a). Thus, it is possible that ABA not only plays a direct role in response to cell desiccation (Hartung and Davies 1991) and as a modulator of growth (Saab et al. 1990), but is also involved in the control of gene expression during cold hardening (Chandler and Robertson 1994). The observation of simultaneous changes in endogenous concentrations of ABA and of particular gene products after exposure to a stress, such as cold, further supports the idea that ABA regulates the expression of certain genes. These observations led to the hypothesis that low temperatures induce increased synthesis of ABA in plants, which in turn triggers the expression of genes involved in freezing tolerance.

We analyzed changes in the populations of translatable mRNAs isolated from sugar maple buds and roots to determine if cold hardening induces changes in genetic expression. Specifically, we characterized the freezing tolerance of sugar maple buds and roots during a cycle of hardening and dehardening under natural conditions and compared the patterns of in vitro translation products in unhardened and cold-hardened tissues. In parallel, we measured the ABA concentration in the xylem sap to establish the relationships among acquisition of cold tolerance, changes in gene expression, and accumulation of ABA.

Materials and methods

Plant material

On May 25, 1994, two-year-old sugar maple seedlings (height 50–90 cm and provenance 46°10′ N, 70°37′ W) were planted in Styroblock containers (15 cavities per container, 750 cm³ per cavity). The substrate was a 2:1:1 (v/v/v) mix of compost/peat moss/sand. Seedlings were grown in the field at the Centre de recherches acéricoles (Tingwick, Québec) from June
1 to September 19. Seedlings were watered to saturation weekly and fertilized on June 28, July 21, and August 15 and 30 with a soluble commercial preparation (CIL, N,P,K, 20,20,20, 3 g l⁻¹). On September 19, the seedlings were transferred to the Laurentian Forestry Centre, Sainte-Foy, Québec, and kept outdoors during the winter. Substrate temperature (starting on November 15, at a depth of one-third of the cavity) and air temperature (starting on November 1, 2.0 m above the containers) were recorded continuously with copper-constantan thermocouples connected to a data logger (Model CR10, Campbell Scientific Inc., Logan, UT), and averaged hourly.

Freezing tolerance determination

Freezing tests were performed on seedlings in a modified programmable (MIC 6000 control, Partlow Corp., New Hartford, NY) cold room (dimensions: 2.7 × 3.9 × 2.1 m, minimum temperature −40 °C), as described by Calmé et al. (1994). The seedlings were placed individually in plastic bags, and the interior temperatures of the bags were recorded during the test using copper-constantan thermocouples connected to a data logger (Model CR21X, Campbell Scientific Inc.). Plants were equilibrated at 3 °C for 2 h before the temperature was lowered at a rate of 3 °C h⁻¹, followed by a 1-h plateau. Seedlings were sampled at ten temperatures at 3 °C intervals; the range of test temperatures varied according to the physiological state of the seedlings. Before damage assessment, the seedlings sampled at each test temperature were placed in a cold room at 3 °C for 2 days, and then in a dark room at 20 °C for 15 days.

Root systems were excised just above the primary lateral roots and examined using a stereo microscope. Dead and live roots were separated with dissecting forceps and a razor blade, roots and examined using a stereo microscope. Dead and live at each test temperature were placed in a cold room at 3 °C for 15 days.

Xylem sap extraction and ABA quantification

Root systems were gently washed in tap water to remove substrate. Stems were cut 12 cm above the primary lateral root and the basal part was placed immediately in a pressure chamber (PMS Instruments Co., Corvallis, OR). Roots were covered with a damp paper towel. The pressure in the chamber was gradually increased, and then maintained at 0.8–1.2 MPa for approximately 5 min for the collection of 100 ml of xylem sap. The vials containing the sap were placed on ice and frozen at −80 °C within 1 h. After a 30-min incubation with polyvinylpolypyrrolidone (PVPP, 1 mg ml⁻¹), xylem sap was assayed for free ABA concentration by radioimmunoassay (RIA), as described by Bertrand et al. (1994a). Antibodies specific to free (+) cis-trans ABA were prepared according to Weiler (1980). The antibody showed a linear response from 0.1 to 10.0 pmol under RIA conditions. The antiserum was highly specific with a cross-reactivity of 53% for (+) cis-trans ABA, 32% for (±) racemic ABA and 7% for methylated (±) ABA. The affinity constant for the antiserum was 2.47×10⁻⁹ mol⁻¹. All standards and samples were assayed in triplicate. Depending on their initial concentration, samples were diluted 1/20 or 1/200 in phosphate buffered saline (Weiler 1980).

Extraction of RNA

Buds were collected for RNA extraction at their maximum hardness on January 18, 1995 (cold-hardened) and when they were swelled, on May 15, 1995 (unhardened). Roots were collected on January 8, 1995 (cold-hardened) and on June 4, 1995 (unhardened).

The lateral and terminal buds of approximately ten seedlings and the fine roots of three seedlings were excised, frozen immediately in liquid nitrogen and kept at −80 °C until extraction. Six grams of buds or roots were ground to a fine powder with a pestle in a mortar containing liquid nitrogen, and total RNA was extracted as described by De Vries et al. (1988). Twelve milliliters of a preheated (90 °C) 1/1 mixture of RNA extraction buffer (100 mM Tris- NaOH (pH 9.0), 100 mM LiCl, 10 mM EDTA and 1% (w/v) SDS) and phenol equilibrated with TLE (200 mM Tris-HCl (pH 8.0), 100 mM LiCl, 5 mM EDTA) containing 0.1% (w/v) 8-hydroxyquinoxoline, were added to each sample. After mixing at room temperature for 5 min on a rotary shaker at 300 rpm, chloroform (1 ml g⁻¹ fresh weight of sample) was added. The mixture was shaken at 300 rpm for 20 min, and then centrifuged at 20,000 g for 30 min. The aqueous phase was recovered and the chloroform extraction repeated. The final aqueous phase was adjusted to 2 M LiCl and allowed to precipitate for 16 h at 4.0 °C. Samples were then centrifuged at 12,000 g for 30 min. The resulting pellet was washed once with 2 M LiCl, twice with 80% ethanol and vacuum dried. The final pellet was dissolved in 0.5 ml Tris-EDTA (10 mM Tris-HCl and 1 mM EDTA, pH 7.4). The color of the samples indicated the presence of phenolic compounds. Further chromatographic purification was performed by two separations on Sephadex G-50 exclusion matrix (20% (w/v) Sephadex G-50 equilibrated with Tris-EDTA (10 mM Tris-HCl, 1 mM EDTA and 100 mM NaCl, pH 8.0)). Two ml of the Sephadex G-50 preparation was placed in a Poly-Prep column (Bio-Rad, Hercules, CA) and washed twice with H₂O treated with 0.1% (v/v) diethylpyrocarbonate (DEPC). Tris-HCl EDTA was added to bring sample volumes to 0.2 ml, and the entire volume was loaded on a column and centrifuged at 1500 g for 4 min. Purified samples were precipitated for 2 h at −20 °C in an ice-cold ethanol mix (2.5 ml cold ethanol, 0.1 ml sample buffer (10 mM TrisHCl, 1 mM EDTA, 3.0 M NaCl, pH 7.4) and 0.01 ml glycojen) (Pharmacia mRNA purification kit), centrifuged at 12,000 g for 15 min, washed with ethanol, vacuum dried and stored at −80 °C.

Four mg of nucleic acids was purified by oligo (dT) cellulose chromatography (mRNA purification kit; Pharmacia,
Baie d’Urfé, Québec, Canada). Total and poly A-tailed RNA were quantified by spectrophotometry at 260 nm, and the integrity of the RNA was verified by size distribution analysis on denaturing 1% agarose formaldehyde gels (Fourney et al. 1988).

In vitro translation

In vitro translation was performed with 1 mg poly A-tailed RNA using a wheat germ extract system (Promega Co., Madison, WI). The standard reaction mixture had a final volume of 50 μl and contained 25 μl of wheat germ extract, 1 μl (40 units μl⁻¹) of placental ribonuclease inhibitor (RNasin, Promega), 4 μl of 1 mM amino acid mixture (lacking methionine), 3.75 μl of 1 M potassium acetate and 1 μg of poly A-tailed RNA heated at 65°C for 10 min. Forty μCi of [35S]-methionine (1,000 Ci mmol⁻¹; Amersham, Oakville, ON, Canada) was added to each reaction, and DEPC-treated water was added to attain final volume. After a 60-min incubation at 25 °C, in vitro translation reactions were stopped by placing the reaction tubes on ice. Five μl of the translation mixture was precipitated with trichloroacetic acid (TCA) to determine the extent of label incorporation. The remaining 45 μl was thoroughly mixed with 55 μl of O’Farrell’s lysis buffer (O’Farrell et al. 1977) (9.5 M urea, 5% (v/v) 2-mercaptoethanol, 2% (v/v) Nonidet P-40, 2% (w/v) LKB ampholines (pH 3.5–10; LKB, Baie d’Urfé, Québec, Canada), and briefly centrifuged at 13,000 g before analysis of translation products by two-dimensional polyacrylamide gel electrophoresis (PAGE).

Electrophoresis

In vitro translation products were analyzed by two-dimensional PAGE as described by Bertrand et al. (1994b). Gels were prepared for fluorography according to Bonner and Laskey (1974), dried and exposed to Kodak X-Omat AR5 X-ray film at −80 °C. To give equivalent overall intensities on the fluorograms, exposure times were normalized according to the total dpm of [35S]-methionine incorporated during in vitro translation. Exposure times varied from 3 to 30 days. Fluorograms were scanned with a Bio-Rad Video Densitometer Model 620, and the images were processed, analyzed and compared using the Bio-Rad 2D- Analyst II computer program.

Experimental design and statistical analysis

The experimental design was a randomized complete block with sampling dates as the main effect, completely randomized within each of the three blocks. The experiment included a total of 396 seedlings: 3 blocks × 11 sampling dates × 12 seedlings. One seedling was sampled for ABA quantification for each block and date, and one seedling was sampled for each of the 10 temperatures tested during the freezing test. Buds and roots of the remaining seedlings were sampled twice for RNA extraction (January 18 and May 15, 1995 for buds and January 18 and June 4, 1995 for roots). Sampling dates for LT50 and ABA determination were August 11, September 23, October 14, November 2, November 16, December 14, January 18, March 22, April 12, April 26 and May 15. Data were evaluated by analysis of variance using the SAS software package (SAS Institute Inc.) after the confirmation of homogeneity of variances by residual analysis.

Results

Freezing tolerance

Seedlings became cold hardened under fall and winter conditions. The lowest air temperature of −32 °C was reached on January 10 (Figure 1). The lowest soil temperatures were reached in November and December, before a sufficient snow cover stabilized the temperature slightly below the freezing point. Freezing tolerance (LT50) of the roots and shoots differed significantly between sampling dates (P < 0.0001) (Figure 2). Freezing tolerance of the roots increased markedly from August to mid-January (LT50 = −3.6 °C and −17.0 °C, respectively). Root freezing tolerance then decreased, and the tissues reached an LT50 of −11.4 °C on May 15. Shoot LT50 decreased rapidly from −5 °C on August 11 to −36 °C on November 16. Between November 16 and March 22, the shoot LT50 was lower than −36 °C, but could not be precisely evaluated because the lowest temperature reached in the cold room was −40 °C.

Xylem sap ABA concentration

The ABA concentration in the xylem sap varied significantly (P < 0.0001) during the experiment (Figure 3). It increased from 200 pmol ml⁻¹ of xylem sap on September 23 to reach a maximum concentration of 1600 pmol ml⁻¹ on December 14. The highest concentration of ABA, 500 pmol ml⁻¹, preceded the maximum hardened state of roots, which was attained on January 18. From March 22 to May 15, ABA remained at a stable and low concentration of approximately 200 pmol ml⁻¹, comparable to the concentration measured in August of the preceding summer.

Figure 1. Minimum daily air and soil temperatures at the outdoor site.
Changes in the populations of translatable mRNAs in buds and roots

Analysis of the in vitro translation products by two-dimensional PAGE revealed between 150 and 200 polypeptides, depending on the tissue and the state of hardening. Comparison of in vitro translation products from RNA extracted from cold-hardened and from unhardened buds revealed major modifications in translatable mRNA populations during cold acclimation. Although the abundance of nine transcripts or groups of transcripts encoding low molecular weight proteins was lower in cold-hardened than in unhardened buds (Figure 4), in vitro translation of mRNA from cold-hardened buds showed that ten transcripts or groups of transcripts were newly synthesized or were present in higher amounts in cold-hardened than in unhardened buds (Figure 4).

Many transcripts or groups of transcripts encoding polypeptides of different molecular weight or charge were more abundant in unhardened than in cold-hardened roots (Figure 4); however, cold hardening increased the abundance of twelve transcripts or groups of transcripts in the roots (Figure 4). Among the transcripts associated with the cold-hardened state, five were common to both buds and roots (Figure 4).

Discussion

Freezing tolerance of sugar maple buds and roots increased markedly following fall acclimation and was associated with significant modifications in gene expression. Some in vitro translation products were down-regulated by natural cold hardening, whereas the relative abundance of approximately ten transcripts increased in both bud and root tissues. Among these, five transcripts or groups of transcripts were more abundant in both cold-hardened buds and roots of sugar maple. Our observation of the down- and up-regulation of certain genes of sugar maple buds is similar to the observations of Hance and Bevington (1992), who reported an increase in protein synthesis in sugar maple embryos after a 20-day acclimation at 4 °C and a concomitant repression of protein synthesis in cotyledons. These authors concluded that, during stratification, the growth potential of the embryonic axes increases because of the greater capacity for translation of mRNA, whereas the inhibitory effect that the cotyledons exert on the growth of the embryo is removed by repression of the expression of specific cotyledon genes. There seems to be an interesting parallel between these observations and our results, because most of the down-regulated transcripts encoding low molecular weight proteins that we observed were specific to buds, but were not found in roots of sugar maple. Conversely, a majority of the up-regulated transcription products were common to buds and roots and may be part of the general acclimation response of seedlings to cold.

Other reports of changes in gene expression that increase cold hardiness in woody species are mainly quantitative and do not provide details on the nature of the modifications (Clapham et al. 1994, Lloyd et al. 1996). However, changes in population of in vitro translation products in response to cold have been documented for many other plants that show cold hardening (Sitbon et al. 1993). In alfalfa, the abundance of some in vitro translation products is related to the degree of hardiness of cultivars, indicating that the genes encoding these polypeptides may constitute genetic markers for cold tolerance (Mohapatra et al. 1989, Newton et al. 1991, Castonguay et al. 1993). The cold-induced in vitro translation products could play structural, regulatory or protective roles in the cellular modifications that occur during winter.
Freezing has a major effect on cellular water relations (Guy 1990). Tolerance to cold-induced dehydration is a key survival strategy in most cold-hardy plant species (Lee and Chen 1993). A characteristic of cold hardening in sugar maple is a modification of the concentration of ABA in the xylem sap. We observed a marked increase in ABA concentration in mid-November, at the time when air and soil temperatures were below freezing. The ABA concentration increased tenfold and reached a maximum before maximum hardiness was reached in buds and roots. This could be a direct effect of cold-induced dehydration because desiccation results in an accumulation of ABA (Hartung and Davies 1991). The increased apoplastic ABA concentration may be involved in the modulation of sugar maple gene expression during cold acclimation. Exogenous application of ABA to many species, including *Acer negundo* L., increases freezing tolerance in the absence of low temperature exposure, and also induces changes in gene expression that are, in some cases, similar to those elicited by low temperatures (Thomashow 1990). Our finding that the highest apoplastic ABA concentration preceded the state of maximum cold hardiness of the tissues is in accordance with the proposition that ABA is involved in the cold-hardening process through its effect on gene expression (Zeevart and Creelman 1988).

In both cold-hardened buds and roots, we have identified an increase in the abundance of a group of transcripts encoding proteins with an apparent molecular weight of approximately 40 kDa. There may be a relationship between our findings and the previous reports of the accumulation of a 42 kDa protein that accumulated in both ABA- and low-temperature-treated cells of alfalfa (Robertson and Gusta 1986), plantlets of *Solanum commersonii* Dun (Tseng and Li 1991) and other plants (Close et al. 1993). This protein could be one of the dehydrins, a family of proteins that is synthesized in response to any

![Figure 4. Two-dimensional PAGE analysis of $\text{^{35}S}$-methionine-labeled *in vitro* translation products from unhardened and cold-hardened buds and roots of sugar maple. Triangles identify peptides or groups of peptides that were less abundant in cold-hardened tissues. Squares and rectangles identify peptides or groups of peptides that were newly-induced or more abundant in cold-hardened tissues. Mr = Molecular weight.](image)
environmental stress that has a dehydration component, or in response to ABA (Close et al. 1993). Based on their amino acid sequence and cellular localization, it has been suggested that dehydrins play a structural role in membrane stabilization. Genetic changes induced by ABA are generally similar but not identical to changes induced by cold. Some polypeptides are common to both treatments, but others are specific (Robertson and Gusta 1986, Tseng and Li 1991). Furthermore, low temperature tolerance in ABA-treated potato cells is transient when cells are not exposed to cold, but cells remain cold-tolerant if they are kept at low temperatures (Lee et al. 1992). Amzallag and Lerner (1995) proposed that the stressing factor must be present in order for the adaptive response to a stress to be maintained. In sugar maple, the tenfold increase in ABA concentration in the xylem sap might be required to trigger the initial acclimation response involving the synthesis of certain stress proteins; however, continuous exposure to low temperatures, as occurs under natural hardening conditions, may be essential to maintain cold hardiness.

The identification of genes expressed during cold acclimation could provide an important basis for future genetic improvement, however few studies have related cold acclimation to the accumulation of specific transcripts in woody plants. We have now identified gene products that specifically accumulate in cold-acclimated sugar maple tissues. If kinetic studies link the accumulation of these proteins to increasing cold tolerance, they could be used as indicators of the physiological state of the tree. In addition, identification of the genes encoding these proteins would allow for a description of cold-tolerant genotypes of sugar maple.

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References


