Ann. Bot. Fennici 45: 173–185 ISSN 0003-3847 (print) ISSN 1797-2442 (online)
Helsinki 27 June 2008

Molecular and physiological changes in maize (*Zea mays*) induced by exogenous NAA, ABA and MeJa during cold stress

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Received 2 Mar. 2007, revised version received 19 Apr. 2007, accepted 9 May 2007

Battal, P., Erez, M. E., Turker, M. & Berber, I. 2008: Molecular and physiological changes in maize (*Zea mays*) induced by exogenous NAA, ABA and MeJa during cold stress. — *Ann. Bot. Fennici* 45: 173–185.

Different concentrations of abscisic acid (ABA), naphthalene acetic acid (NAA) and methyl jasmonate (MeJa) were applied to maize (*Zea mays*) plants exposed to cold stress. ABA enhanced plant resistance against the damaging effects of freezing. ABA-treated plants produced some specific proteins when exposed to cold. Prominent changes were observed in the size and number of stomata with the application of ABA during cold stress. MeJa inhibited root growth, but increased root dry weight during cold acclimation. The chlorophyll content was lower at 100 μg ml^{-1} NAA and 50 μg ml^{-1} MeJa and higher in the residual applications when compared with that in the control plants. Carotenoid levels increased in all NAA and some ABA treatments, decreased however in MeJa treatment. Exogenous MeJa treatments increased endogenous *trans*-zeatin (*t*-Z) and indole-3-acetic acide (IAA) in contrast decreased ABA levels. Exogenous NAA also increased endogenous IAA, ABA and zeatin riboside (ZR) levels, while exogenous ABA decreased endogenous IAA and ZR levels.

Key words: cold stress, HPLC, maize, phytohormones, SDS PAGE

Introduction

The maize (*Zea mays*) is a major source of food products as well as of industrial raw materials and animal fodder. Adaptation of maize plants to early annual planting dates requires improvement of cold tolerance, which implies high percentage germination and vigorous seedling growth under cold temperatures (Revilla *et al*. 2000). At freezing temperatures, ice nucleation is initiated in extracellular spaces because of their lower solute concentration (Guy 1990). This causes water flow from the cells to the extracellular ice crystals, resulting in the loss of vital intracellular water, impairments in cellular processes including alterations in membrane properties, especially irreversibly rupture of membrane, interactions between macromolecules and enzymatic reactions, and cell death. Destabilization of cell membrane also obstructs gas exchange (Burke *et al*. 1976, Steponkus 1984, Warren 1998, Pearce 2001). Frost-tolerant plants have evolved mechanisms that allow them to avoid freezing (Atici & Nalbantoglu 2003). Resistance or sensitivity to

stress depends on the species, the genotype, and the developmental stage of the plant (Revilla *et al*. 2000).

The ability of plants to withstand frost is acquired by cold-hardening, a complex process resulting in increased freezing tolerance and involving many biochemical and physiological changes (Levitt 1980). The changes include alterations in gene expression, changes in hormone levels, increase of soluble sugars, amino acids and organic acids, accumulation of osmoprotectants and protective proteins, as well as modification of the membrane lipid composition (Palva 1994, Hughes & Dunn 1996, Thomashow 1999).

Comparative analysis of proteins from wild-type and ABA deficient flacca-mutants of tomato by 2-dimensional electrophoresis indicate that several polypeptides are specifically synthesized during adaptation to drought when ABA is exogenously applied (Swamy & Smith 1999).

Phytohormones regulate many physiological processes in plants (Shakirova *et al*. 2003). Cold-hardening involves some phytohormones, especially abscisic acid (ABA), which is generally considered a stress hormone. The role of ABA in this process was deduced from various findings (Macháckova *et al*. 1989, Taylor *et al*. 1990, Bravo *et al*. 1998). Although ABA is reported to reduce cold stress, a few reports have indicated that ABA did not play an essential role in the development of frost tolerance (Dallaire *et al*. 1994, Murelli *et al*. 1995). A number of interacting signal pathways appear to control the activation of the low temperature responsive genes, some of which are mediated through the action of ABA. The literature reports indicate that cold acclimation can be triggered by exposing the plants to exogenously applied ABA (Lång *et al*. 1989, Guy *et al*. 1992, Mäntylä *et al*. 1995). ABA plays an important role in many cellular processes including seed development, dormancy, germination, vegetative growth, environmental stress responses (Xiong & Zhu 2003) and stomatal control (Hetherington 2001). ABA-pretreatment decreases stomatal conductance and, in consequence, transpiration rates and net photosynthetic rates become lower (Pospíšilová 2003), whereas it improves chill tolerance by increasing root water flow in maize (Aroca *et al*. 2003).

Freezing is a major environmental stress, inflicting economic damage on crops and limiting the distribution of both wild and crop species. Therefore, understanding freezing and how it damages plants is of outstanding practical importance.

Plants synthesize a spectrum of new proteins on exposure to different environmental stresses. Among those, cold-stress related proteins are well documented. It is evident that many of the stress-responsive proteins are induced by ABA and several groups of homologous proteins. The proteins are thought to play a role in protecting the cells from water deficit and low temperatures (Swamy & Smith 1999).

The aim of the present study was to determine the protein profile, chlorophyll, carotenoid and endogenous phytohormone levels, stomatal growth and developmental changes in maize resulting from exogenously applied plant growth-regulators during cold stress. The effect of ABA on cold acclimation has been studied extensively, but MeJa and NAA have received much less attention.

Material and methods

The OSSK 644 variety of maize (*Zea mays*) seeds was provided by the Department of Field Crops, University of Yuzuncu Yil, Van, Turkey. Seeds were germinated in pots (120 × 90 mm) containing soil and sand (2:1). Three samples were chosen for each PGR (plant growth regulator) treatment. For each treatment, pots were divided into five groups consisting of internal and external controls, 50, 100 and 150 μg ml^{-1} of abscisic acid (ABA), naphthalene acetic acid (NAA) and methyl jasmonate (MeJa) treatments at +4 °C and –4 °C temperatures. External controls were not exposed to cold or PGR treatments. The plants in external controls were grown in ambient conditions, whereas internal controls were exposed to cold stress at +4 °C and –4 °C without exogenous PGR treatments. Experimental plants were also exposed to cold stress at +4 °C and –4 °C with 50, 100 and 150 μg ml^{-1} of ABA, NAA and MeJa. Healthy and vigorous seeds were chosen. Seeds were washed in sterile, distilled water and kept in the dark for

24 h. Ten to twelve seeds were sown in each pot and left to germinate at room temperature. The seeds were kept overnight in the same concentration of exogenously applied PGRs.

When the plants started to germinate, cold stress treatments were applied in different ways. The first group was kept in a phytotron (Sanyo, Fitotron, SGC097.CFX.F) at +4 °C for 24 h (8 h daylight/16 h moonlight), the second at 0 °C for 8 h (daylight) and at +4 °C for 16 h (moonlight), the third at –4 °C for 4 h (daylight) and at 0 °C for 20 h (4 h daylight/16 h moonlight). Phytohormones were sprayed onto the leaves over four weeks except for the controls. Plants were irrigated every day with 150 ml of water. At the end of the growing period, plants were harvested for analysis and samples were kept in deep freezer at –80 °C until analyses were performed.

Determination of chlorophyll and carotenoid content

Fresh leaves were homogenized with a pestle in a mortar. Chlorophyll and carotenoids were extracted from the leaves of 15-day-old plants with 80% acetone and absorbance values were measured at 663 and 645 nm wavelengths for chlorophyll *a* and *b* and at 450 nm for carotenoids in a UV-160 Shimadzu spectrophotometer. The amount of chlorophyll *a*, *b*, total chlorophyll and carotenoids was calculated according to Witham *et al*. (1971).

Determination of phytohormones

Extraction, purification, and isocratic HPLC (High Performance Liquid Chromatography) analyses were performed according to the modified methods of Battal *et al*. (2004) and Kuraishi *et al*. (1991). Two grams of leaves frozen in liquid nitrogen were ground into powder. Cold methanol was added and tissue samples were homogenized in an Ultra Tissue Lyser (Ultrasonic Processor Jenway Ltd.) at 4 °C for 1 h. The homogenization process was continued at +4 °C for 24 h in the dark. The samples were filtered through filter paper (Whatman no. 1) and the supernatant was transferred into clean vials. The residues were reprocessed and combined with the former supernatant. The supernatants were filtered through PTFE filters (0.45 μm) and methanol was removed under reduced pressure. The extracts were redissolved in 0.1 M potassium dihydrogen phosphate KH_2PO_4 pH 8.5) buffer and centrifuged at 10 000 rpm for 1 hour at +4 °C. The extracts were passed through polyvinylpolypyrrolidone (PVPP, Sigma Chemical Co. UK) and Sep-Pak C18 (Waters) cartridges. The hormones absorbed by the cartridge were eluted with 80% methanol-water (v/v) and the extracts were collected in vials. Phytohormones were separated in an isocratic HPLC-system consisting of 6000A pump (Waters), UV detector (Unicam), and μ-Bondapack C18 (Waters) column.

Examination of stomata samples

When plants were harvested from the field, leaves were preserved in formalin–acetic acid–ethanol (FAA; 1:1:9) for microscopic examination (Zwieniecki *et al*. 2004).

Preparation of whole-cell proteins

Five grams of fresh sample was ground into powder in liquid nitrogen and transferred into an eppendorf tube. After adding 25 μl of denaturing buffer containing 0.06 M Tris-HCl, 2.5% glycerol, 0.5% SDS and 1.25% β-mercaptoethanol (pH 6.8), the cells were stirred, and the proteins were denatured in boiling water for 5 min. After centrifugation for 3 min at 10 000 *g*, the supernatant was put into an eppendorf tube and kept at –50 °C until electrophoresis was carried out.

SDS-PAGE analysis

Solubilized proteins were subjected to SDS-PAGE in gel slabs 1 mm thick(3.5 cm, 4% stacking and 15.5 cm, 12% resolving gels) as described by Laemmli (1970). Electrophoresis was performed with a discontinuous buffer system in a UVP Vertical Electrophoresis Unit (Cambridge, UK). The gel was run at 30 mA

until the bromophenol blue marker had reached the bottom of the gel. Protein molecular masses were estimated by comparison with the following standards (Prestained SDS-PAGE Standards, Broad Range, BIO-RAD): myosin (201 kDa), β-galactosidase (120 kDa), bovine serum albumin (100 kDa), ovalbumin (55 kDa), carbonic anhydrase (38 kDa), soybean trypsin inhibitor (29 kDa), lysozime (20 kDa) and aprotinin (6 kDa). After electrophoresis the gels were rinsed for 20 min with an isopropanol–acetic acid–water (1:3:6) solution, then for 5 min with a methanol–acetic acid–water (3:1:6) solution. The gels were stained for 6 h in 0.01% (w/v) Coomassie Brilliant Blue R-250. Afterwards, the gels were destained in a methanol–acetic acid–water (3:1:6) mixture until the protein bands became clearly visible.

The gels were scanned with a densitometer (Desaga CD-60 Densitometer, Germany) and the molecular weight of each band was determined using the one-dimensional analysis software (Lab Image ver. 2.6, Halle, Germany).

Statistical analysis

The data are given as means ± standard errors (SE). For statistical analysis the SPSS/PC package (SPSS/PC+, Chicago, IL, USA) was used. For all parameters, means and SEs were calculated according to standard methods. The significance level was defined at $p < 0.05$. All analyses were carried out in triplicates (three different plants).

Results

Morphological observations

In all experiments, external-control plants grown in ambient conditions developed morphologically better than those of internal controls and PGR-treated plants. The comparisons were made with reference to the internal-control plants, which were exposed to cold stress without PGRs treatments. The internal control and experimental plants were exposed to +4, 0 and –4 °C. The plants treated with ABA showed weak development at –4 and 0 °C as compared with that of the control plants, whereas their morphological development was better than that of the plants treated with MeJa and NAA. However, at +4 °C, 100 μg ml^{-1} ABA gave clearly better results than did MeJa and NAA treatments, and as compared with the control plants. Besides, chlorosis, necrosis and folding were observed on the leaf margins.

Stoma size and numbers per area did not change in MeJa and NAA treatments, but as compared with the control plants, stoma size decreased and the number of stomata per area increased at higher ABA concentrations and reduced temperatures (Fig. 1).

As compared with the control plants, root growth was more prevalent with the application of 50–100 μg ml^{-1} ABA at +4 °C and 0 °C and 50–150 μg ml^{-1} MeJa at +4 °C. Interestingly, the best root growth was observed in 150 μg ml^{-1} ABA application at –4 °C. The residual applications on the other hand reduced root growth (Fig. 2).

Stem growth was also better in the applications of 50 μg ml^{-1} ABA and MeJa at +4°C, 100 μg ml^{-1} ABA and NAA at +4 °C, 150 μg ml^{-1} ABA, NAA and MeJa at +4 °C and 150 μg ml^{-1} MeJa at –4 °C as compared with the control plants (Fig. 2).

Leafs were longer in 50 μg ml^{-1} ABA and MeJa at +4 0 °C, and 150 μg ml^{-1} ABA at +4 °C and shorter in the rest as compared with the control plants. However, growth inhibition is clear at decreasing temperature in spite of the PGR treatments (Fig. 2).

Chlorophyll and carotenoid contents

The total chlorophyll content was reduced at all temperatures with 50 μg ml^{-1} MeJa, at +4 and –4 °C of 100 μg ml^{-1} NAA and at 0 °C of 150 μg ml^{-1} ABA applications. The other applications increased the total chlorophyll levels (Fig. 3).

The carotenoid level was lower at +4 °C with 50 and 100 μg ml^{-1} ABA, –4 °C with 100 μg ml^{-1} ABA and in all of the MeJa applications. The other treatments increased carotenoid levels (Fig. 4).

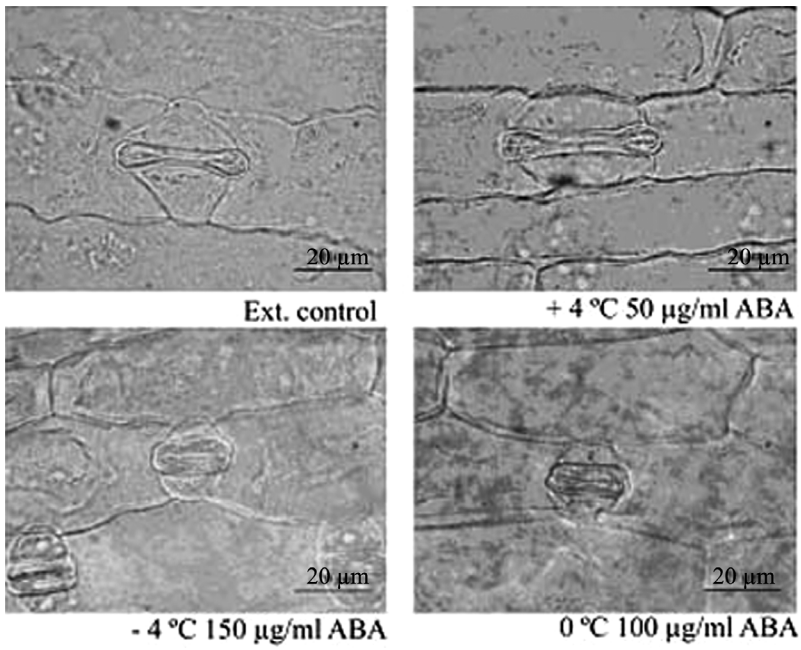

Fig. 1. The effect of exogenous ABA on stoma size and number in *Zea mays* during cold application.

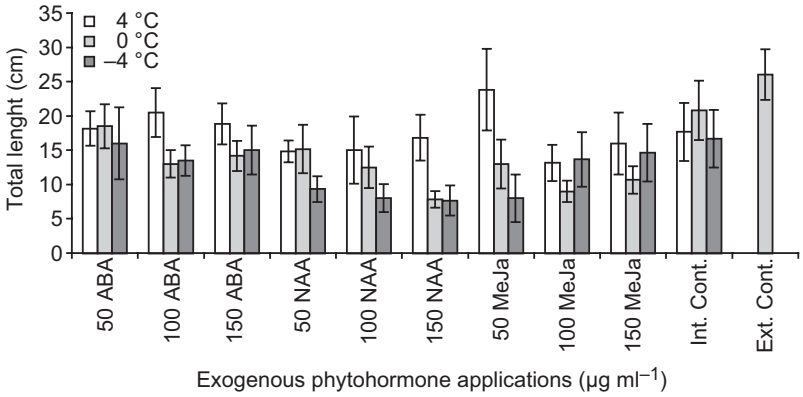

Fig. 2. The effect of exogenous PGRs applications on total lengths of *Zea mays* stem, leaf and root.

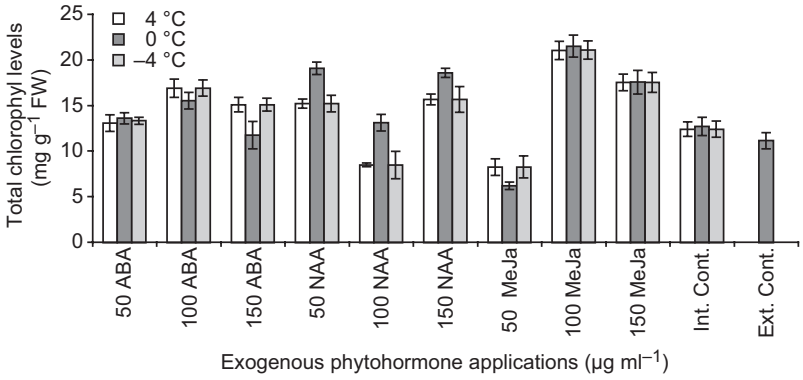

Fig. 3. The effect of exogenous PGRs applications on total chlorophyll concentration in *Zea mays*.

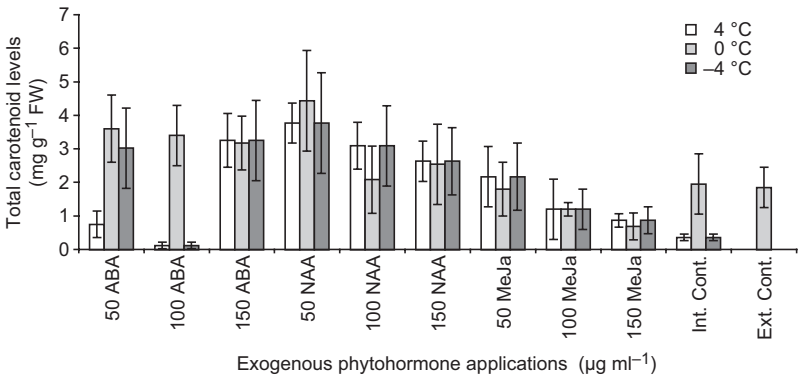

Fig. 4. The effect of exogenous PGRs applications on carotenoid levels in *Zea mays*.

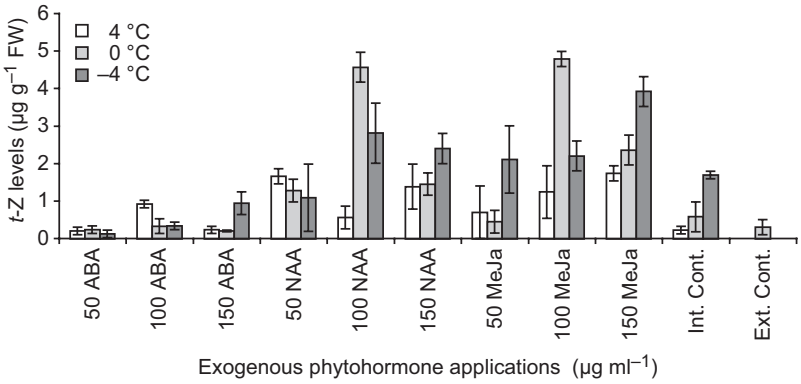

Fig. 5. The effect of exogenous PGRs applications on endogenous *t*-Z in *Zea mays* exposed to cold stress.

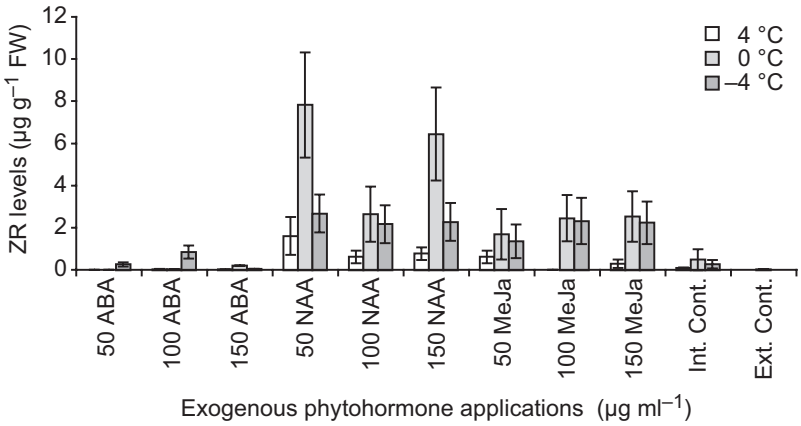

Fig. 6. The effect of exogenous PGRs applications on endogenous ZR levels in *Zea mays* exposed to cold stress.

The effect of ABA, NAA and MeJa applications on *t*-Z level

The *t*-Z level decreased significantly with ABA applications, but increased with NAA and MeJa application, especially at 0 °C, 100 μg ml^{-1} NAA, and MeJa application gave the most prominent rise in *t*-Z levels (Fig. 5).

The effect of ABA, NAA and MeJa applications on ZR levels

The ZR level was reduced for all applications of ABA, except for 100 μg ml^{-1} ABA at –4°C. In contrast, the most significant increases were observed in particular with 50 and 150 μg ml^{-1} of NAA applications at 0 °C (Fig. 6).

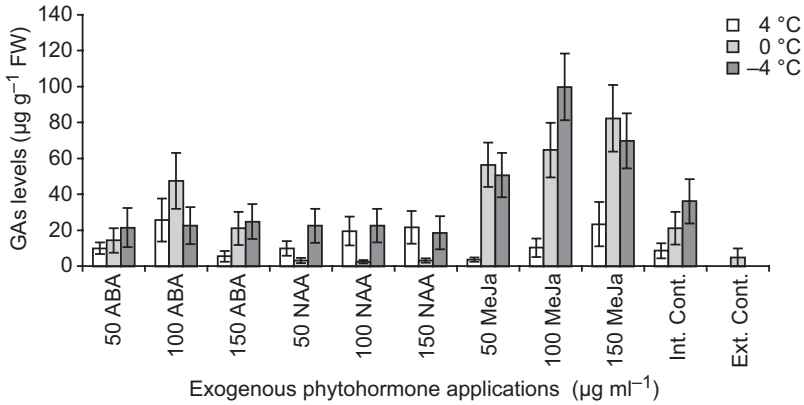

Fig. 7. The effect of exogenous PGRs applications on endogenous GAs levels in *Zea mays* exposed to cold stress.

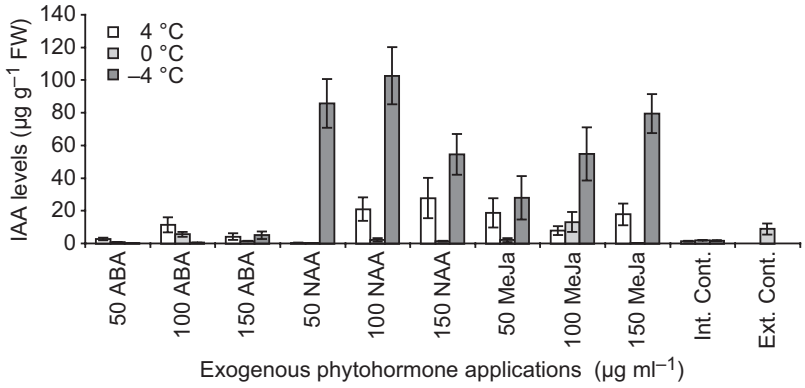

Fig. 8. The effect of exogenous PGRs applications on endogenous IAA levels in *Zea mays* exposed to cold stress.

The effect of ABA, NAA and MeJa applications on GAs levels

GAs levels increased in all MeJa applications with decreasing temperatures. They were higher in 100 μg ml^{-1} ABA application at 0 °C. The other applications reduced GAs levels (Fig. 7).

The effect of ABA, NAA and MeJa applications on the IAA level

ABA applications decreased the IAA level, while NAA and MeJa applications at –4 °C significantly increased it (Fig. 8).

The effect of ABA, NAA and MeJa applications on ABA levels

The applications of 150 μg ml^{-1} ABA, 100–150 μg ml^{-1} NAA and 50 μg ml^{-1} MeJa at +4 °C, 50 μg ml^{-1} ABA and 50 μg ml^{-1} NAA at 0 °C and 100 μg ml^{-1} NAA at all temperatures increased the endogenous ABA level. The highest ABA level was observed with 50 μg ml^{-1} NAA applications at 0 °C. The changes in the residual experiments were not statistically significant (Fig. 9).

The effects of exogenously applied phytohormones on protein synthesis during cold stress

The protein bands at molecular weights of 155, 105, 83, 48, 39, 32 and 28 kDa (kilo Dalton) — numbered as 1, 4, 5, 7, 8, 9 and 10, respectively — were found in the treated plants at +4 and 0 °C and in the control plants (Figs. 10 and 11). The residual bands of either structural or stress-induced proteins were not visible in the external-control plants. The levels of the seven bands considered as structural proteins were

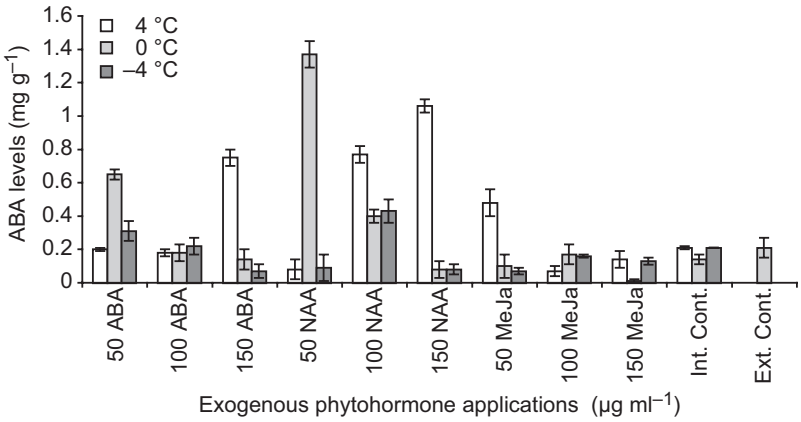

Fig. 9. The effect of exogenous PGRs applications on endogenous ABA levels in *Zea mays* exposed to cold stress.

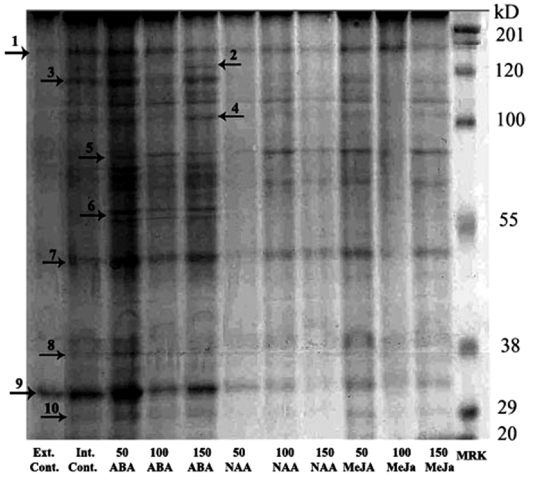

Fig. 10. The protein profiles in *Zea mays* exposed to +4 °C cold stress with exogenous PGR applications in different concentrations.

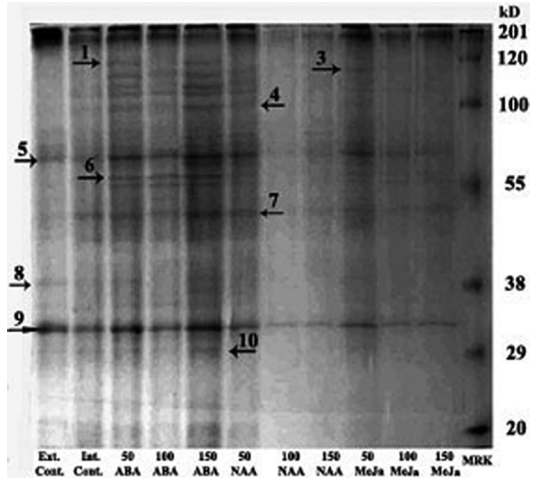

Fig. 11. The protein profiles in *Zea mays* exposed to 0 °C cold stress with exogenous PGR applications in different concentrations.

lower in NAA than in ABA and MeJa applications. The protein band in molecular weight 119 kDa, numbered as 3, was determined only in the internal-control plants and ABA applications, but not in the external-control plants or NAA and MeJa applications at +4 °C (Fig. 10). Some proteins in molecular weights between 55 and 100 kDa (number not given) were not detected in control plants although they were synthesized in PGR applications in different amounts and were more prominent particularly in ABA treatments (Fig. 10). The double protein bands in molecular weights 58/59 kDa, numbered as 6, were observed in all ABA treatments at +4 and 0 °C (Figs. 10 and 11), and 50 μg ml^{-1} NAA and MeJa applications at 0 °C (Fig. 11). The band patterns between 100–120 kDa were found to be different at 0 °C to those at +4 °C (Fig. 10 and 11).

The protein profile changed significantly at –4 °C as compared with the protein profiles obtained from the application at +4 and 0 °C. The bands between 100–120 kDa could not be distinguished from each other in internal-control plants and PGR applications and also were not visible in the external-control plants. However, some dominant protein bands were observed in PGR applications at –4 °C at changing concentrations. The protein bands determined in previous applications at +4 and 0 °C and interpreted as structural and stress-inducing proteins were

not synthesized in the present application at –4 °C. For example the bands numbered as 1, 2, 3, 4, 6 and 8 were not seen at –4 °C. No additional different band pattern apart from the previous two applications was observed at –4 °C. The band patterns of 50–100 μg ml^{-1} MeJa applications at –4 °C were quite different from the other applications (Fig. 12).

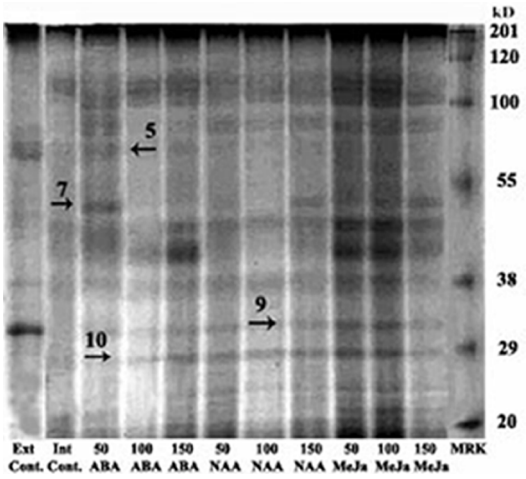

Fig. 12. The protein profiles in *Zea mays* exposed to –4 °C cold stress with exogenous PGR applications in different concentrations.

Discussion

Growth and development of plants were slower under cold stress when compared with those of external-control plants. Necrotic zones on the margins and yellowing were seen on the leaves. Similar effects were already reported by Kinaci and Kun (1996).

Morphological observations

The effect of ABA on maize plants during cold acclimation was more prominent than that of NAA and MeJa. Moreover, plants exposed to ABA at +4 °C showed better development than internal-control plants, and NAA and MeJa treatments. These findings are in full agreement with Kadlecová *et al*. (2000), who described an important relationship between cold stress and ABA, indicating that ABA inhibited cold stress and prolonged plant development during cold stress. NAA treatments, however, reduced plant development as compared with the control and MeJa treatments under cold stress. The most pronounced effect of MeJa treatments was seen at +4 °C. However, plant development was suppressed at decreasing temperature with increasing MeJa concentrations. As a consequence, it can be postulated that ABA diminishes the injurious effect of cold stress and renders plants resistant towards an adverse environment.

Plants exposed to cold stress, especially at temperatures below 0 °C, undergo osmotic drought resulting in weakness of stem, root and leaf development. MeJa treatment decreased root development, whereas ABA increased it. Aroca *et al*. (2003) reported that water uptake changed and root development was initiated upon the application of ABA during cold treatment. On the other hand, JA treatment was reported to inhibit root and meristem cell development (Swiatek *et al*. 2003). Similar results were obtained In the present study. The changes observed in stem and leaf development may be explained by a different response of phytohormones in different organs. Therefore, it can be concluded that phytohormones have different effects on the mechanisms of cold acclimation and adaptation in plants (Faltusová-Kadlecová *et al*. 2002). Systemic signals, which are elevated in stress response, such as JA and ABA, can directly influence vegetative growth. Possibly, because the stress reaction mobilizes plant metabolism into different pathways, the limited metabolite and energy supply is re-directed towards restructuring of the tissue. Root growth inhibition is a typical plant reaction to exogenous jasmonate. JA caused root growth inhibition by decreasing the cell size and meristematic activity (Swiatek *et al*. 2003).

A remarkable difference was seen in the dry weight with ABA treatments when compared with the internal-control plants, and with MeJa and NAA treatments. The most prominent changes appeared in the leaves and roots. Kadlecová *et al*. (2000) reported an increase in dry weight of barley (*Hordeum vulgare*) from 12% to 23% with ABA application during cold stress. The level of proline and betain, which are osmolites, was reported to increase parallel to cold stress by Nanjo *et al*. (1999). Moreover, Gusta

et al. (1999) reported increased sugar levels with decreasing water loss during cold treatment. The increase in dry weight of maize under cold stress may be connected to the accumulation of osmolites and sugars counteracting cold stress. Similarly, in the process of plant hardening by low temperature, an accumulation of dry weight takes place and is accompanied by a lowering osmotic potential, as described by Lalk and Dörffling (1985) for a wheat cultivar (*Triticum aestivum*).

Chlorophyll and carotenoid contents

The chlorophyll content was lower in 100 μg ml^{-1} of NAA and 50 μg ml^{-1} of MeJa applications, whereas it was higher in the rest. The most significant increase was seen in MeJa and NAA treatments. However, opposite results related to the process of thylakoid membrane were reported in resistant types of rye (*Secale cereale)* (Krupa *et al*. 1987). This contradictory finding may be attributed to the difference in type and concentration of phytohormones, the period and temperature of cold treatment, growth conditions and the variety of plant (Faltusová-Kadlecová *et al*. 2002).

Carotenoid content of maize at +4 and –4 °C after 100 μg ml^{-1} ABA applications was lower than that of the controls. In most of the treatments, carotenoid levels were higher than in the control plants. This suggests that carotenoids might eliminate the destructive effects of free radicals in plants (Everett *et al*. 1996).

Stomata

The stomatal aperture, size and form were quite different in the experimental plants than in the control plants. In ABA treatments, the number of stomata per area was higher, the size was smaller, and the shape was more irregular and closed than in the control, and in the MeJa and NAA treatments. These findings are in accordance with the results of Franks and Farquhar (2001), who reported that the ABA application during cold stress caused an increase in stomata number and a decrease in the size and permeability of stomata in *Tradescantia virginia*. Similar results were observed in MeJa treatment, but it was not as pronounced as in the ABA treatment. Fedina and Tsonev (1997) reported that the MeJa application locked the stomatal apertures. Our results along with literature records indicate that the stoma is an organelle that controls plant development in adverse conditions and develops an adaptation mechanism against cold stress.

Phytohormone levels

The levels of *t*-Z and ZR — which initiate growth and development — were observed to be lower with ABA application, but higher with MeJa and NAA applications than in the internal-control plants. ABA is a senescence-promoting phytohormone; ABA and cytokinins function antagonistically. Therefore ABA may prevent the biosynthesis of *t*-Z (Buchanan-Wollaston 1997, Nooden 1988). Cold may affect the endogenous level of plant growth substances or the activities of senescence-retarding substances such as cytokinins, since senescence-associated genes have been reported to be affected by abscisic acid (ABA) and jasmonates (Reinbothe *et al*. 1994, Buchanan-Wollaston 1997, Weaver *at al*. 1998).

The IAA levels were lower in ABA application than in the control plants, and in the MeJa and NAA application. The low level of IAA was statistically significant and IAA levels decreased with increasing ABA concentrations. Chang and Jacobs (1973) reported decreased IAA levels in ABA application with increasing abscission and slowing down of IAA transportation.

We observed that some applications of exogenous ABA and NAA increased the endogenous ABA levels, or at least prevented a decrease of endogenous ABA levels, whereas most MeJa applications decreased it. NAA may also initiate ABA synthesis resulting in slower metabolism and growth as a response to cold stress. MeJa acts like ABA, therefore, in the presence of MeJa the plants might not need ABA which leads to a decrease in the endogenous ABA levels (Thaler & Bostock 2004).

This finding is in agreement with Pospisilova's (2002) result that auxin and cytokinin levels increased with ABA, but decreased with JA and MeJa applications.

GAs levels increased with MeJa application. The differences of phytohormone levels in different applications of exogenous phytohormones with low temperature may indicate that plants develop different adaptation mechanisms to resist an adverse environment and some phytohormones may help them to survive. MeJa is known as a growth-inhibiting substance (Swiatek *et al*. 2003). From our results it can be postulated that when MeJa inhibited the growth, GAs was synthesized to remove the imbalance of some metabolic function in plant metabolism and ion transportation.

As a conclusion, ABA may support plant resistance against cold stress by inhibiting phytohormone synthesis, resulting in decreased growth and development rate via low levels of these growth-supporting substances.

Protein profiles

SDS-PAGE analysis at +4 and 0 °C revealed that the protein bands with molecular weights of 155, 105, 83, 48, 32 and 28 kDa present in all applications and controls are probably involved in structure, general metabolism or transcriptional regulation. The quantity of the proteins synthesized changes with changing temperature and with the concentration of different PGR applications. The most negative effect on the protein synthesis was observed in NAA applications. The quantity of structural proteins was found to be lower in NAA than in ABA and JA applications. Tarrchesky *et al*. (2002) obtained a similar result, indicating that ABA and JA application increased the polypeptide synthesis of the proteins in adverse environmental conditions. The protein band with a molecular weight of 135 kDa found in the internal-control plants and in the ABA application, but not in the external-control plants or In the NAA and MeJa applications at +4 °C, indicates that the gene which synthesizes this protein might be suppressed by the application of NAA and MeJa. Some proteins in the molecular weight range between 55–100 kDa not detected in the controls, although they were synthesized in PGR applications in different amounts and especially more prominent in ABA treatment, might be the products of the genes activated by PGRs. Some of these proteins might demonstrate anti-freeze activity. The double protein bands with molecular weight of 58/59 kDa observed in all ABA applications and in 50 μg ml^{-1} NAA and MeJa applications at 0 °C indicate that those might be anti-freeze proteins (Olave-Concha *et al*. 2004). ABA and low concentration of NAA and MeJa at 0 °C may regulate gene transcription for some specific regulators, which code for anti-freeze proteins. Brovski *et al*. (2002) worked with maize under cold stress and found the same bands presumed to represent dehydrine proteins. The anti-freeze effect of the proteins in cell membranes and some cell structures has been well documented (Thomashow 1997, Warren 1998, Swamy and Smith 1999, Atici and Nalbantoglu 2003). The band patterns between 100–120 kDa determined to be different at 0 °C from at +4 °C may be attributed to the varying effect of temperature.

The protein profile of PGRs applications at –4 °C was considerably changed, although the protein profile at +4 and 0 °C showed similar patterns. The plants may not tolerate the cold application and metabolism is impaired at –4 °C. Some protein bands observed at changing concentrations of PGR applications at –4 °C may be structural or associated with general metabolism and transcriptional regulation. Most of the structural, transcriptional and stress-induced proteins determined in previous applications were not seen at –4 °C. The finding might be attributed to the severity of cold stress. The gene/genes encoding the proteins might be suppressed by cold. The expressions of band concentration are prominent in the application of 50–100 μg ml^{-1} MeJa at –4 °C (Fig. 12). This may indicate that MeJa stimulates protein production better than ABA and NAA at low temperatures.

Conclusions

Our results obtained from anatomical, physiological and molecular studies were congruent with each other. PGRs were also seen to have different effects on the parameters depending on the concentrations and temperature. The most pronounced results among PGRs were seen with ABA, which caused plants to gain resistance against cold stress via regulating the metabolism

for adaptation towards adverse effects. It was also observed that maize synthesized anti-freeze proteins and elevated levels of structural, transcriptional and general metabolism related proteins to resist cold stress. Up to date, the effect of ABA on cold stress is well documented in the literature. The present study showed that MeJa may boost plant's resistance against freezing via elevating the levels of some proteins. Although the positive effect of NAA application on protein synthesis during cold acclimation was not seen in the present study; indirect beneficial results of NAA on plant metabolism against cold treatment via elevated chlorophyll and ABA synthesis were observed.

Acknowledgements

This study was supported by a grant from the Scientific Research Projects Presidency of Yuzuncu Yil University (2003-FED-051).

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