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**Mechanisms of Chilling Injury and Resistance in
Ornamental Plants**

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Mechanisms of chilling injury and resistance in ornamental plants

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C. Abstract

This study was aimed at finding ways to reduce chilling damage in ornamental pot plants, while gaining a better understanding of the metabolic and molecular events occurring during and following chilling.

Using clerodendrum (Clerodendrum speciosum), gloxinia (Sinningia speciosa) and dracaena (Dracaena marginata), applicable methods were developed to harden chilling-sensitive plants. These include a single application of growth retardant (triazoles) or growing the plants for three days to few weeks at moderately decreased temperatures (12-17°C).

Following membrane analyses, lipid and protein metabolism was studied. Possible damage to enzymes responsible for phospholipid synthesis was indicated. A decline in the amount of protein following chilling was found to be accompanied by an increase in ubiquitin conjugation, especially to the membrane proteins. In addition, a new polypeptide appeared following chilling. This latter finding, together with studies involving catalase in tomatoes, promoted us to pursue research into the molecular biology of the chilling response. Another line of research currently being followed is the effect of chilling on components of the cellular signal transduction system: we have found significant declines in the content and activities of GTP-binding proteins following chilling. We assume that this change is related to the modified ability of chilled plants to respond to exogenous stimuli.

D. Objectives of the Original Research Proposal

The goals of this project were twofold: to define and optimize conditions leading to the hardening of selected ornamental plants to chilling, and to gain a better understanding of events occurring at the molecular level during chilling and hardening.

The research dealt with these general objectives, and deviations from the original plan involved placing more emphasis on two molecular mechanisms which were not to be dealt with originally. One was a study of the possible role of ubiquitin in the chilling-related enhancement of protein degradation, and the second of modifications of the cellular signal transduction mechanism following chilling.

E. Body of the Report

Chilling and hardening

The first part of the research dealt with detailed analyses of the chilling injuries occurring in commercially important pot plants. These studies included gloxinia (Sinningia speciosa) and clerodendrum (C. speciosum) as well as a less thorough analysis of dracaena (D. marginata).

Gloxinia was studied (Borochoy and Shahar, 1989, Scientia Hortic. 39:331-339, Appendix I) with respect to the effects of two growth regulators, mefluidide and paclobutrazol (PP333) on growth and flowering. Following soil-drench treatments with the growth retardants, mefluidide, at 0.1 mg per plant, had very little effect on plant growth, but damaged it significantly at 1.0 mg per plant. A single application of PP333 at 0.5, 1.0 or 2.0 mg per plant resulted in reduced canopy growth and increased tuber size, with lower water contents in both. Flowering was only slightly affected. Gloxinia was found to be sensitive to chilling, with typical symptoms of injury following exposure to 2°C for 15h. The most convenient objective measure of chilling damage was electrolyte leakage from the tissue. Plants pretreated with 0.5 or 1.0 mg PP333 showed less chilling injury than untreated or mefluidide-treated plants. It was concluded that PP333, at concentrations of 0.5-1.0 mg per plant, can be used to control chilling sensitivity and growth of gloxinia.

The sensitivity of gloxinia to chilling could also be lowered by

moderately decreasing growth temperatures (12/17, night/day) for a few weeks, before exposure to chilling (Table 1).

Table 1. Effect of growth temperature on chilling-mediated wilting of gloxinia leaves. Plants grown at the lowest temperatures showed no visual symptoms of chilling injury.

Growth temperature (day/night)	Water content following chilling (g H ₂ O/g DW)
17/12	6.3±0.3
22/17	4.1±0.2
27/22	3.6±0.6
32/27	2.9±0.2

The flowering pot plant clerodendrum (Tandler, Borochoy and Halevy, 1989, Acta Hortic. 261:333-336, Appendix II; Tamari, Tandler and Borochoy, 1992, Scientia Hortic. in press, Appendix III) also exhibits typical symptoms of chilling injury following transient exposure (48 h or more) to 2°C. Symptoms include decreased leaf water content and increased electrolyte leakage. Plants grown for three days or longer at day temperatures of 17-22°C displayed much slower growth rates than plants grown at 32°C, and were hardened to chilling for 48h at 2°C. Similar results were obtained in plants treated at least 10 days prior to

chilling with various triazoles, including PP333 and uniconazole as a single foliar spray application. Both treatments inhibited the chilling-induced degradation of membrane proteins and phospholipids.

We propose that at least some elements of the mechanisms by which both of these hardening techniques act in gloxinia and clerodendrum are analogous.

Further support for the general applicability of these findings was achieved in studies involving the foliage pot plant dracaena. This plant is also sensitive to low temperatures: whereas transport at temperatures above 14°C leads to undesirable growth characteristics, including curved development and of etiolation, exposure to lower temperatures resulted in chilling injuries.

Table 2. Effects of horizontal, low temperature storage (6°C for 14 days) on chilling damage and etiolated growth of *D. marginata* plants. Results are means of 4 replicates $\pm$ SE.

Storage temp.	Pre-storage growth temp.	Chlorophyll (A665/g FW)	Electrolyte leakage (%)	Water content (g H ₂ O/g DW)
14°C	28°C	0.35 $\pm$ 0.05	23.3 $\pm$ 1.0	7.23 $\pm$ 0.18
6°C	28°C	1.33 $\pm$ 0.26	50.6 $\pm$ 11.5	3.94 $\pm$ 0.38
6°C	14°C	1.35 $\pm$ 0.53	27.1 $\pm$ 1.7	n.d

As with gloxinia, a hardening treatment, in this case two weeks at 14°C, eliminated the injuries and facilitated long-term shipment at low temperatures (6°C) without loss of quality (Table 2).

Membrane composition

In order to facilitate an in-depth study of membrane-related events, a protocol was optimized for the isolation of a relatively pure plasma-membrane fraction from clerodendrum leaves. It was based on the two-phase method which separates membranes on the basis of surface charge differences between dextran and PEG, used to prepare plasma membranes from carnation petals (Drory, Borochoy and Mayak, 1992, J. Plant Physiol., in press, Appendix IV). Table 3 indicates that a polymer concentration of 6.3% produces an upper-phase fraction enriched with plasma membranes. These are characterized by vanadate-sensitive ATPase activity, a high sterol:phospholipid ratio, and depletion of mitochondria, ER and Golgi apparatus enzyme activity.

Table 3. Effects of polymer concentration on characteristics of membrane fraction isolated from clerodendrum leaves. The membranes were either microsomal or in the upper phase following two-polymer centrifugation. Enzyme activities are expressed as $\mu\text{mol product} / \text{mg protein} \times \text{min}$.

Characteristic	Microsomal fraction	Upper phase of dextran/PEG at		
		6.0%	6.3%	6.5%
Protein (mg)	6.44	0.31	0.47	0.35
Sterol/PL (mM/M)	7.68	*	20.38	*
Cyt c oxidase	4.88	2.42	**	**
Cyt c reductase	3.52	10.92	3.15	6.29
Latent IDPase	5988.00	**	**	**
Nitrate-sensitive				
ATPase	**	**	**	**
Vanadate-sensitive				
ATPase	**	0.93	1.47	0.11

* Not determined

** Below detection level

Membrane studies were focused on clerodendrum leaf microsomal and plasma-membrane fractions. A sharp decline in the content of both protein and phospholipid (PL) was found in the membranes.

(Table 4). Concomitantly, the sterol (ST):PL ratio was determined; a change in this ratio is usually leading also to physical changes in the membrane lipids. Surprisingly, this parameter was not modified by the chilling treatment.

Table 4. Effect of chilling on composition of microsomal and plasma membrane fractions isolated from clerodendrum leaves. Results are means of 3 replicates $\pm$ SE.

Treatment	Membrane fraction	Protein (mg/g DW)	Phospholipid (μ mol/g DW)	ST/PL (mM/M)
Control	Microsomal	2.27 $\pm$ 0.02	1.05 $\pm$ 0.02	7.68
Control	Plasma membrane	0.23 $\pm$ 0.00	0.17 $\pm$ 0.14	20.38
Chilled	Microsomal	1.39 $\pm$ 0.02	0.67 $\pm$ 0.02	7.69
Chilled	Plasma membrane	0.12 $\pm$ 0.00	0.09 $\pm$ 0.01	19.31

Lipid metabolism

The decrease in PL content suggested that enzymes involved in PL-synthesis were being affected by low temperatures. Results of another study, dealing with the metabolism of phosphatidylcholine in hardened and freeze-stressed canola plants support this notion: that study (Itzhaki, Pauls and Borochoy, 1991, J. Plant

Physiol. 138:75-79, Appendix V), the properties of cytoplasmic membranes isolated from control and acclimated canola (Brassica napus) plants were compared. Growth at 2°C for two weeks reduced the LT₅₀ of the plants from -6.5°C to -14°C. Microsomal membranes from acclimated leaf tissue exhibited a lower microviscosity, higher phospholipid content and lower ST:PL ratios than membranes from control plants. Choline (¹⁴C-methyl) incorporation into leaf disks from acclimated plants was significantly higher than into leaf disks from control plants both before and after freezing to -5°C. The rate of incorporation into leaf disks peaked at 20°C for both acclimated and control tissue. The levels of labelled phosphorylcholine, CDP-choline and lipid were all higher in the acclimated tissue in the temperature range of 5 to 37°C. CDP-choline phosphotransferase specific activity was significantly higher in membrane preparations from acclimated tissue than from control tissue. Total CDP-choline phosphotransferase activity declined after freezing of the control tissue to less than one-half the level measured in non-frozen samples, but was not significantly reduced by freezing in acclimated tissue. The results indicate that acclimation increases the capacity of canola tissue to synthesize phosphatidylcholine rendering the biosynthetic pathway less sensitive to freezing.

Analyses of this enzyme in chilling-sensitive plants, such as clerodendrum, are planned.

Protein metabolism

The decrease in membrane protein content led us to look for potentially responsible mechanisms. This decrease results either from decreased synthesis of membrane proteins or increased degradation. In recent years, evidence has accumulated pointing to ubiquitin conjugation as a key process in the control of cellular protein metabolism. Ubiquitin is a highly conserved, 76-amino-acid polypeptide which is involved in ATP-dependent protein degradation in eukaryotic cells, including plants. Ubiquitination may also be involved in the response of many eukaryotic cells to environmental stresses, including those related to extreme temperatures. An increase in ubiquitin conjugation to wheat root proteins in response to high temperature stress has recently been reported. In our work (Gindin and Borochoy, 1992, submitted to Plant Physiol., Appendix VI) we found enhanced ubiquitin-protein conjugation in clerodendrum leaves following exposure to chilling.

Molecular biology of the chilling response

Several approaches were taken to study the possible modifications in protein synthesis occurring during chilling. In the first approach we used clerodendrum plants. The soluble fractions of extracts from leaves of control and chilled plants were separated on a Sephacryl S-200 column, and proteins were eluted according to size. We found a new polypeptide, with a MW of

ca. 36 kDa following chilling (Figure 1). Anti serum to this fraction was prepared rabbits, and we are currently testing it's properties (titer, etc.). We plan to use to further analyze protein modifications related to chilling.

A B

Figure 1. Coomassie blue stained non-denatured PAGE of fractions eluted from a Sephacryl S-200 column. A - control, B - chilled.

The second approach was performed using tomato as the model system. Tomato was primarily because of the ease with which it can be transformed and its sensitivity to low, nonfreezing temperatures. In preliminary experiments we found that treatment of tomato seedlings with the growth regulating chemical paclobutrazol at 0.5 mg/plant resulted in the induction of

resistance to chilling stress. In this system we assessed the damage to the photosynthetic apparatus due to chilling by measuring their leaf fluorescence at different time points following the chilling stress and during recovery. Particular attention was paid to the Fv/Fm value, which describes the photochemical efficiency of PSII in the leaves. Using this parameter it was found that 24 h at 2-4°C caused a drastic reduction in PSII efficiency which recovered upon returning the plant to 20°C. Pretreatment with paclobutrazol eliminated this decrease in PSII efficiency during chilling stress.

In preliminary experiments we found that treatment of tomato seedlings for 24 h with 2-4°C resulted in a significant decrease (40%) in the activity of catalase and concomitant increase in the content of hydrogen peroxide. It was also noted that the activity of superoxide dismutase did not change during this treatment. Clearly, an increase in the content of hydrogen peroxide as a result of insufficient degradation capacity could have profound implications for the tissues ability to maintain a favorable oxidative environment. Therefore, using molecular cloning techniques we set out to isolate a cDNA clone for tomato catalase and study its regulation during chilling stress and induction of chilling resistance in tomato.

~~Molecular cloning of tomato catalase.~~ Catalase (EC 1.11.1.6) is a peroxysomal enzyme involved in the degradation of hydrogen peroxide in aerobic cells. In plants, catalase plays an important role in the scavenging of hydrogen peroxide released during the β -oxidation of fatty acids particularly in the cotyledons of

germinating seedlings. In addition, the enzyme is an important component of the system involved in detoxification of reactive oxygen species which increase following biotic and abiotic stresses such as chilling injury. In this system, superoxide dismutase reacts with superoxide radicals yielding hydrogen peroxide, which is subsequently converted to water and oxygen.

Our strategy for cloning catalase from tomato was to use polymerase chain reaction to amplify a cDNA using RNA derived cDNA as a template. Two oligonucleotide primers were synthesized based on the sequence of sweet potato catalase. Total RNA extracted from mature green tomato fruit was used as a template for cDNA synthesis and the resulting cDNA used in a PCR reaction with the synthesized primers. The resulting product was subcloned into pGEM7Zf(+) to serve as a template for double stranded sequencing. Sequence analysis confirmed we had amplified a 1068 bp fragment encoding catalase. This partial cDNA clone was used to screen a cDNA library prepared in lambda ZAPII from poly(A)+RNA isolated from young tomato leaves. Several putative catalase cDNAs were identified and the longest insert (pTOMCAT1) sequenced. The sequence of pTOMCAT1 is 1822 bp and contains an open reading frame of 492 amino acids (Figure 2).

The nucleotide sequence of pTOMCAT1 is 73%, 69%, 67% and 66% identical with catalases from cottonseed (subunit 1), pea, sweet potato and maize (CAT1), respectively. The predicted protein of pTOMCAT1 shares extensive homology with other catalases. Of the 10 amino acids reported to be involved in catalytic activity (His-74, Ser-113, and Asn-147) and direct association with the heme group


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1 CTCTCTTCAATCTTCTTTTCATTTCCATCACCATGGATCCCTCTAAGTATCGCCCATCAA 10
M D P S K Y R P S S
61 GGCATACGACACCCCTTTCTTGACAACAATGCTGGTGGTCTGTGTACAACAATGTTT 30
A Y D T P F L T T N A G G P V Y N N V S
121 CTTCTTGTGACTGTGACCTAGAGGGCCTGTTCTGCTTGAGGATTACTATCTAATTGAGA 50
S L T V G P R G P V L L E D Y Y L I E K
181 AGCTCGGACATTGATCGCGAGAAGATACCTGAACGTTGTTTCATGCTAGAGGTGCTA 70
L A T P D R E K I P E R V V H A R G A S
241 GTGCTAAGGATTTCTTTGAAGTTACTCATGACATTTCTCATCTTACTTGTGCTGATTTT 90
A K G F F E V T H D I S H L T C A D F L
301 TCCGAGCTCCCTGGCGCTCAAACGCTGTTATTTGTCGATTCTCTACTGTTGTCCATGAAC 110
R A P G A Q T P V I C R F S T V V H E R
361 GTGGAGCCCCGAGTCTATCAGGGACATTCGTGGTTTTGCTGTCAAGTTCTACACCAGAG 130
G S P E S I R D I R G F A V K F Y T R E
421 AGGGTAACCTTTGATCTTTGTTGGAACAATGTCCCGTGTCTTTAATCGTGATGCTAAGT 150
G N F D L V G N N V P V F F N R D A K S
481 CGTTCCTGACACGATTGTCGATTGAAACCAATCCAAAGTCACACATTCAGGAGAAT 170
F P D T I R A L K P N P K S H I Q E N W
541 GGAGGATCTTGATTTCTTCTCGTTCCCTCGAGAGTTTGCATACATTCGCCTTCTTCT 190
R I L D T F F S F L P E S L H T F A F F Y
601 ACGATGATTTGTCTCCCAACGGATTACAGACACATGGAAGGTTTTGGCGTTACGCGT 210
D D V C L P T D Y R H M E G F G V H A Y
661 ATCAATTGATTAACAAAGAGGGGAAAGCACATTATGTGAAGTTCCACTGGAAGCCAATT 230
Q L I N K E G K A H Y V K F H W K P T C
721 GTGGTGTGAATGTATGTCTGAGGAAGAAGTATTAGAGTCGGTGGTACTAATCATAGCC 250
G V K C M S E E A I R V G G T N H S H
781 ACGCGACCAAGGATCTTTACGATTCAATTGCTGCTGGAAGTATCCTGAGTGAAGCTTT 270
A T K D L Y D S I A A G N Y P E W K L F
841 TTATCCAAACAATGACCCCCGAGGATGTAGACAAGTTCGATTTTGATCCTCTGGATGTAA 290
I Q T M D P E D V D K F D F D P L D V T
901 CCAAGACATGGCCTGAGGATCTCTTGGCGTTGATCCCAAGTGGTGCATTGGTGTGAACA 310
K T W P E D L L P L I P V G R L V L N R
961 GGAACATTGATAACTTCTTCGACAGAGAATGAACAACCTCGCGTTAACCCCTGGACATATT 330
N I D N F F A E N E Q L A F N P G H I V
1021 TCCTGTTATTTACTATTCCGAGGATAAGCTTCCAGACTAGGATATTCCGCTATGCTG 350
P G I Y Y S E D K L L Q T R I F A Y A D
1081 ATACTCAGACACCGGATTGACCAAACTATATGCAGCTCCCAAGTAAATGCTCCCAAGT 370
T Q R H R I G P N Y M Q L P V N A P K C
1141 GTGGTCATCACAACAATCATCGGATGGTGTCTATGAACATGACACATCGCGATGAAGAGG 390
G H H N N H R D G A M N M T H R D E E V
1201 TGGATTATTTGCCCTCGAGGTTTGATCCTTGTCTGCTGCTGAGCAGTACCCGATTCTCT 410
D Y L P S R F D P C R P A E Q Y P I P S
1261 CTTGTGTCTTGAATGGAAGGCGTACAATTTGTGTCTATCCGAAAGAAAACAACCTTCAAAC 430
C V L N G R R T N C V I P K E N N F K Q
1321 AGGCAGGGGAGAGGTACAGATCATGGGAACCTGACAGGCAAGACAGATACATCAACAAAT 450
A G E R Y R S W E P D R Q D R Y I N K W
1381 GGGTTGAGTCTTTATCCGATCCAGAGTCACTCATGAGATTCCGAGCATATGGATATCAT 470
V E S L S D P R V T H E I R S I W I S Y
1441 ACTTGTCTCAGGCTGACAAGTCTGTGGTCAGAAGGTCGCTTCTCGTCTCACTGTGAAGC 490
L S Q A D K S C G Q K V A S R L T V K P
1501 CTACAATGTGAAAAATCAATGAAAATAGTTGAAATGCTTCAAGCTGCAATGTTGAAGG 492
T M
1561 ACTAATGCAAAAAAAGCTCCGCGTTGTGCTATAAACTGTACTTCTTTTTCAATCGTAATG
1621 TTGTATTTTGTATCGAATTTTCGATGTCTTGTGTTTTTACTATAATGATGTTGGAACCTGA
1681 ATAAGTTCACAGTTGTATGTTCAATGTTTCACTTTCTAAAGTTATGTAATTATGTTGAGT
1741 TCTTGTTCACTTTGGTGCTTGAAGAACACACTCTCAATTTCAATAATATCACTTTTCATT
1801 CAGTAAAAA

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Figure 2. The sequence of pTOMCAT1.

(Val-73, Arg-11, Tyr-114, Phe-150, Pro-335, Arg-353 and Tyr-357), all are conserved in the predicted protein of pTOMCAT1.

Therefore, we conclude pTOMCAT1 encodes a subunit of tomato catalase. Studies are nearing completion in which we have assessed the expression patterns of catalase and chloroplastic superoxide dismutase following chilling stress and recovery of control and paclobutrazol treated plants. In addition, we have constructed chimeric genes of catalase in order to transform tomato and overexpress this enzyme. These transgenic plants will be valuable in subsequent studies on the molecular genetics of low temperature stress physiology.

Signal transduction

In addition to the typical chilling induced injuries, clerodendrum exhibits a decrease in its capacity to respond to exogenous stimuli: for example, normal responses to gibberellin disappear. This could indicate an impaired signal transduction pathway. GTP-binding proteins (G-proteins) are considered essential to such pathways and an elucidation of their fate following chilling was the aim of this study. Antisera raised against a highly conserved amino acid sequence (G-alpha_{common} peptide) of mammalian GTP-binding proteins cross-reacted with proteins of microsomal membranes isolated from leaves clerodendrum. The labeled sets of proteins were of M_r =112, 48 and 29 kDa.

Proteins of microsomal membranes isolated from leaves bound

GTP- γ -S with a high affinity, following the sequence GTP>GTP- γ -S>GDP>>ATP. The K_d of GTP- γ -S binding to leaf membranes was ca. 19 nM. The number of high affinity binding sites was ca. 2 nmol/mg total membrane protein. Purified plasmalemma was relatively rich in GTP- γ -S binding sites. Following chilling treatment, membranes isolated from clerodendrum leaves exhibited a reduction in the number of GTP binding sites and the amount of protein labeled with anti-G- α -common antibody was less than in the control. The findings indicate that plant membranes, preferentially the plasmalemma, contain GTP-binding proteins with a sequence homology as well as molecular weight and nucleotide specificity comparable to those of mammalian GTP-binding proteins. The expression of these proteins seems to be modulated by environmental conditions, suggesting that GTP-binding proteins play a role in the physiological response of plants to the environment.

F. Description of the Collaboration

During the research period, continuous contact was maintained via regular and electronic mail between the Israeli and U.S. laboratories. In addition, several visits between labs were made: Prof. Woodson visited Israel in 1988, and Prof. Mayak spent four months in Purdue in 1989. Dr. Borochoy also visited Prof. Woodson in 1991. During these visits, results and experimental design were discussed. Also, two former graduate students from the Israeli laboratory (H. Itzhaki and A. Drory) are engaged in postdoctoral work in Prof. Woodson's laboratory, further facilitating communications.

G. Evaluation of the Research Achievements

Our research achievements are twofold: immediate, industry-applicable results and those which are related to a basic understanding of plant responses to low temperatures. At the applied level, we found means to harden commercially important pot plants such as clerodendrum, gloxinia and dracaena, to temperatures that would otherwise cause significant injury. The growers can use either available chemicals (triazoles) or moderately lowered growth temperatures for few weeks, to achieve the required hardening.

As for elucidating the basic mechanisms involved in the plant's response to chilling, we gained knowledge concerning the lipid and protein modifications taking place following chilling. These include changes in the membrane, in the activity of enzymes involved in lipid synthesis and degradation, quantitative and qualitative changes in the proteins and alterations in the leaf's signal transduction systems. Our findings, especially those relating to basic metabolic mechanisms, need to be pursued to obtain clearer overall picture of the chilling process in plants.

List of Publications

1. Borochoy A. and Shahar T. (1989) Effects of mefluidide and paclobutrazol on growth, flowering and chilling response of gloxinia. *Scientia Hort.* 39:331-339.
2. Tandler J., Borochoy A. and Halevy A.H. (1989) Chilling effects on clerodendrum: interactions of water balance and ethylene. *Acta Hort.* 261:333-336.
3. Goszczynska D.M., Itzhaki H., Borochoy A. and Halevy A.H. (1990) Effects of sugars on physical and compositional properties of rose petal membranes. *Scientia Hort.* 43:313-320.
4. Itzhaki H., Pauls P.K. and Borochoy A. (1991) Effects of hardening on microsomal membrane properties and phosphatidylcholine biosynthesis in Brassica napus leaf tissue. *J. Plant Physiol.* 138:75-79. 12
5. Borochoy-Neory H. and Borochoy A. (1991) Response of melon plants to salt: 1. Growth, morphology and root membrane properties. *J. Plant Physiol.* 139:100-105.
6. Tamari G., Tandler J. and Borochoy A. (1992) Hardening of clerodendrum to chilling: chemical treatments and growth at low temperatures. *Scientia Hort.* (in press).

7. Drory A., Borochoy A. and Mayak S. (1992) Transient water stress and phospholipid turnover in carnation flowers. J. Plant Physiol. (in press).

8. Drory a., Thompson J.E., Mayak S. and Borochoy A. (1992) Carnation petal membranes: a study of surface properties using dansyl-pyrrolidine, a new fluorescent probe. J. Plant Physiol. (in press).

9. Drory, A. and Woodson W.R. (1992) Molecular cloning and nucleotide sequence of a cDNA encoding catalase from tomato. Plant Physiol. (in press).

10. Gindin E. and Borochoy A. Ubiquitin involvement in the response of clerodendrum to chilling. (submitted to Plant Physiol.).

11. Borochoy-Neori H, Tamari G., Gindin E. and Borochoy A. GTP-binding proteins in leaf and root membranes: characteristics and modifications by environmental conditions. (submitted to Plant Physiol.).

Ubiquitin conjugation to proteins increases following chilling of *Clerodendrum* leaves¹.

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Manuscript accepted: _____

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Footnotes

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² Abbreviations: FIA, fluorescence immunoassay;

ABSTRACT

The protein content of clerodendrum (C. speciosum) leaves declines following chilling (48 h, 3°C). Using Western and dot blots and fluorescence immunoassays we found that isolated leaf proteins had more conjugated ubiquitin following chilling. The increase in ubiquitin conjugation was greater in the membrane fraction than in the soluble fraction.

INTRODUCTION

Chilling injury occurs in many tropical and sub-tropical plants following exposure to low temperatures which are above freezing, usually in the range of 0-15°C (9).

With clerodendrum, as with most other chilling sensitive plants, the first visual symptom of chilling injury is leaf wilting. Wilting is accompanied by a massive leakage of cellular electrolytes (10), indicating chilling-induced changes in the cell membranes. Indeed, we have reported recently on a decrease in the content of leaf membrane phospholipids and proteins, following a transient exposure of clerodendrum plants to chilling (10).

The decrease in membrane proteins is either the result of a decreased synthesis or an increased degradation. In recent years, evidence has accumulated which point to ubiquitin conjugation as a key process in the control of cellular protein metabolism. (For a recent review, see ref 4). Ubiquitin is a highly conserved, 76-amino acid polypeptide which is involved in ATP-dependent protein degradation in eukaryotic cells including plants (3,4,11). Ubiquitination also might be involved in the response of many eukaryotic cells to various environmental stresses, including those related to extreme temperatures (4). As for plants, an increase in ubiquitin conjugation to wheat root proteins in response to high temperature stress was recently reported (3). In this work we present results showing enhanced ubiquitin-protein conjugation in clerodendrum leaves following exposure to chilling.

MATERIALS AND METHODS

Plant material and environmental conditions

Clerodendrum (*C. speciosum*) plants were produced from single node cuttings and grown in 1.5-L pots in peat and volcanic scoria (1:1 v/v) and watered twice a day. Plants were grown at 27/18°C (day/night) under long day conditions as described before (10). Chilling was induced by exposing the plants to 3°C for 48 h in a temperature-controlled chamber. Analyses of fully grown leaves were done within 1 h after the end of the treatments.

Membrane preparation

Microsomal membranes were isolated from the leaves in the presence of 2 mM DTT and 2 mM PMSF using differential centrifugation as described previously (7). The membrane pellets were resuspended in 20 mM Tris-HCl at pH 7.4. During all the isolation steps the temperature was kept at 0-4°C. Immediately following isolation, protein content was determined according to Bradford (2) and the various analyses were conducted.

Fluorescence immunoassay

Quantitative analysis of the ubiquitin was conducted by FIA² according to a manufacturer (Sigma) protocol: ubiquitin-flourescein conjugate (100 uL) at 20.4 pmole/mL (Sigma U-5540) was added to all test tubes except the blanks. A sample (100 uL

sample of a 1 mg protein per mL of protein to be tested was added to all tubes. Finally, 100 uL rabbit anti-ubiquitin IgG (Sigma U-5379) was added to all samples. The samples were mixed by vortexing and incubated for 60 min at room temperature, in the dark. Then, 1 mL of immuno-precipitation reagent (goat anti-rabbit IgG in 0.1M PBS) was added to each test tube and the samples were centrifuged at 2000g for 15 min at 4°C. The supernatants were aspirated and discarded and the pellets were resuspended in 3 mL of 0.1 N NaOH in 2% SDS. The samples were excited with 494 nm and the fluorescence was measured at 516 nm using an SLM spectrofluorimeter (model 4600). A decrease in the immunoprecipitated fluorescein labeled ubiquitin resulted in a decrease in the fluorescence intensity and indicated higher level of ubiquitin in the tested sample. The amount of the ubiquitin in the samples was calculated according to a calibration curve which was prepared with purified ubiquitin (Sigma) (Fig. 1).

Blot analysis

For Western blots, proteins were resolved on 16% SDS-PAGE and transferred to nitrocellulose as described previously (11). The Western blots were blocked with a blocking solution of 5% milk in 10 mM Tris-HCl, pH 7.4, at room temperature for 4 h, followed by an overnight incubation with rabbit anti-ubiquitin IgG (Sigma U-5379) diluted 1:500 with the same buffer. The Western blots were washed four times for 5 min in the above buffer and incubated for 2 h in [125 I]-protein A (Amersham

IM144). Autoradiograms of the blots were prepared with X-ray film (AGFA, Curix RP2) with an exposure time of 2-4 d at -70°C.

Dot blot analyses were done using 100 ug protein samples, bound to nitrocellulose at one spot. The nitrocellulose was blocked with 5% BSA in 10 mM Tris-HCl, pH 7.4, and probed with two different rabbit anti-ubiquitin IgG: a commercial preparation against free ubiquitin (Sigma U-5379) and an anti-conjugated-ubiquitin IgG (6). The blots were washed 5 times for 5 min with the blocking solution and incubated with [¹²⁵I]-Protein A (Amersham IM144), washed 5 times for 5 min in the above buffer and 3 times for 5 min in 10 mM Tris-HCl, pH 7.4. The dots were then cut out and counted in a gamma-counter.

RESULTS AND DISCUSSION

Exposure of clerodendrum plants to 3°C for 48 h resulted in a decrease in the content of all the cellular protein fractions (Table I). Membrane proteins declined by more than 80%, and the content of the water-soluble proteins declined by a somewhat lesser extent.

In order to examine the possibility that ubiquitination of protein increased as a result of chilling, we measured the ubiquitin-conjugated proteins extracted from control and chilled plants. The results of a representative Western blot are presented in Fig. 2. As with other plant systems studied (3,8,11), proteins conjugated to ubiquitin are evident in the extracts from both control and chilled leaves. This is to be expected, assuming that ubiquitin is playing a role in the normal control of protein degradation. However, from these results it is clear that there are significant quantitative differences between control and chilled leaf proteins: there was more ubiquitin associated with two major proteins, at about 28 kda and 80 kda, in samples from chilled tissue.

Quantitation of the effect of chilling on the ubiquitin-protein conjugation was carried out using two additional immunochemical approaches. In the first we used a commercial kit for the FIA of ubiquitin, which was introduced recently by Sigma. This technique is similar to the radio immunoassay which was used recently by Ferguson et al. (3) to quantitate ubiquitin in wheat roots. Using a purified ubiquitin as a standard in the range of 10-500 pmol, the log of the

fluorescence values obtained with FIA were negatively and linearly related to the ubiquitin content (Fig. 1). It should be emphasized that using this assay, one has always to include proper blanks (standards and samples without antisera), since the auto-fluorescence from samples made of green leaves contributes as much as 20% to the total fluorescence (data not shown). The specific amount of the ubiquitin bound to the total leaf proteins (Table II) was similar to that measured before in wheat roots (3), and much larger than that bound to the membrane proteins.

In plants exposed to chilling, the content of the ubiquitin conjugated to membrane proteins increased almost 5-fold, while the chilling-induced increase in the ubiquitin bound to the total protein was relatively small (Table II). The results are reported as specific binding (per mg protein). Because the membrane proteins comprise only a small fraction of the total cellular proteins (Table I), most of the ubiquitin is in fact associated with soluble proteins. Nevertheless, if the rate of ubiquitin-related proteolysis is directly related to its specific binding, then ubiquitin binding could contribute to the loss of membrane proteins following chilling.

The anti-ubiquitin which was used in the FIA measurements was developed against free ubiquitin, while our analyses were aimed at ubiquitin-protein conjugates. However, the results were similar whether or not antisera which was developed against free ubiquitin and also antisera which was developed against ubiquitin-protein conjugates (6) were compared using

the dot-blot technique (Table III).

To summarize, our findings indicate that chilling stress induces an increase in ubiquitin conjugation to chlorodendron leaf proteins, mainly to the membrane proteins. This increase could contribute to the loss in soluble and membrane proteins following chilling (Table I; ref 5). One possibility is that leaf proteins are modified, directly or indirectly, by the chilling and the ubiquitination is a way for the tissue to get rid of these proteins by enhanced specific proteolysis. A recent report (1) on stress induction of the ubiquitin gene in rice cells raises the possibility that de-novo synthesis of ubiquitin is involved.

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Figure captions

Figure 1. Calibration curve, prepared for the fluorescence immunoassay using free ubiquitin. The values were obtained following subtraction of blanks and are the means of 3 replicates $\pm$ SE.

Figure 2. Western blot analysis of total proteins from leaves of control (A) and chilled (B) clerodendrum plants. Blots were probed with an anti-ubiquitin followed by [125 I]-protein A.

Table I. The effect of Chilling on Protein Content of Clerodendrum Leaves.

Plants were held at 3°C for 48 h. The leaves were extracted and the protein content of the crude homogenate, the microsomal membranes and the soluble fraction was measured.

Treatment	Protein (mg x g DW ⁻¹)		
	Total homogenate	Microsomal membranes	Soluble fraction
Control	92.3 ^a	6.3 ^b	86.0 ^b
Chilling	24.6 ^a	1.2 ^a	23.4 ^a

^a Values, in the same column, which are not followed by the same letter are significantly different at P = 0.05 (Duncan's multiple range test).

Table II. The effect of Chilling on the Ubiquitin Content of Clerodendrum Leaves as Measured by Fluorescence Immunoassay.

Plants were held at 3°C for 48 h. The leaves were homogenized and microsomal membranes were prepared. Measurements were conducted as described in the "Materials and Methods" and the amounts of ubiquitin were calculated according to the calibration curve presented in Fig. 1.

Treatment	Ubiquitin (pmol x mg protein ⁻¹)	
	Total proteins	Microsomal proteins
Control	224 ^a	59 ^a
Chilling	294 ^b	282 ^b

^a Values, in the same column, which are not followed by the same letter are significantly different at P = 0.05 (Duncan's multiple range test).

Table III. The effect of Chilling on the Relative Ubiquitin Content of Membranes Isolated From Clerodendrum Leaves as Measured by Dot-blot Technique.

Plants were chilled at 3°C for 48 h. The leaves were homogenized and microsomal membranes were prepared. Dot-blots were as detailed in the "Materials and Methods". The results are counts of [¹²⁵I]-protein A reacted with anti-free- or anti-conjugated-ubiquitin.

Treatment	Bound [¹²⁵ I]-protein A (cpm x mg protein ⁻¹)	
	Anti-free ubiquitin	Anti-conjugated ubiquitin
Control	4670	3100
Chilling	9460	7730

Fig. 1
1000000
100000
10000
1000
100
10
1
0.1

Fig. 2
Gindin & Borochov