A Chilling Sensitive Mutant of Arabidopsis with Altered Steryl-Ester Metabolism

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ABSTRACT

A chilling-sensitive mutant of Arabidopsis thaliana was isolated and subjected to genetic, physiological, and biochemical analysis. The chilling-sensitive nature of the mutant line is due to a single recessive nuclear mutation at a locus designated chsl. In contrast to wild-type plants, which are not adversely affected by low temperatures, the chsl mutant is killed by several days of exposure to temperatures below 18°C. Following exposure to chilling temperatures, the mutant displays two common symptoms of chilling injury—leaf chlorosis and electrolyte leakage. In these respects, the physiological response of the mutant to low temperatures mimics the response observed in some naturally occurring chilling sensitive species. The biochemical basis of chilling sensitivity was explored by examining the pattern of incorporation of 14C02 into soluble metabolites and lipids in wild-type and mutant plants. The only difference observed between the mutant and wild type was that following low temperature treatment, the mutant accumulated 10-fold more radioactivity in a specific class of neutral lipids which were identified by a variety of criteria to be steryl-esters. The accumulation of radioactivity in the steryl-ester fraction occurs 24 hours before there is any visible evidence of chilling injury. These results suggest one of two possible explanations: either the mutation directly affects sterol metabolism, which in turn leads to chilling sensitivity, or the mutation affects another unidentified function and the accumulation of radioactivity in steryl-esters is a secondary consequence of chilling injury.

Many plants of tropical origin are injured or killed by exposure to low temperatures below 20°C (6), whereas chilling-resistant species can survive and maintain growth at temperatures between 0°C and 20°C. A variety of symptoms such as root injury, wilting and leaf electrolyte leakage, reduced or retarded germination and seedling emergence, leaf and tissue necrosis, and leaf chlorosis are associated with chilling injury. In attempts to determine the primary cause of chilling injury, the biochemical differences between various chilling-resistant and -sensitive plant species at various temperatures have been analyzed. Because of the difficulty in distinguishing which, if any, of the differences observed specifically relate to low temperature fitness, the molecular basis of chilling resistance or sensitivity has not been established.

The ideal experimental material for investigating the mechanisms associated with chilling tolerance would be a pair of isogenic lines in which quantitative or qualitative differences in one or a few gene products were responsible for a significant difference in chilling sensitivity. Therefore, we have explored the possibility that variability in chilling tolerance could be induced by mutagenic treatment. Our working hypothesis was that survival at low temperature requires gene products that are not required at higher temperatures. Thus, we sought to convert the chilling-resistant species Arabidopsis thaliana into a chilling-sensitive species by the inactivation of gene products that were specifically required for growth at low temperature. We isolated a number of mutants of Arabidopsis that exhibited symptoms associated with chilling sensitivity when exposed to low nonfreezing temperatures, but that have no visible phenotype at standard growth conditions. Here, we describe the genetic, biochemical, and physiological characterization of one such chilling-sensitive mutant of Arabidopsis designated PM11.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The lines of Arabidopsis thaliana (L.) Henyg, described here were descended from the Columbia wild type. The mutant line PM11 was isolated from an M2 population of plants which had been mutagenized with ethyl methane sulfonate as described previously (11). The mutant line carries a defective allele of a locus designated chsl which is required for growth below 18°C. It was backcrossed to the wild type three times before use in the experiments described here. The line MK1 (an, cer2, gll1, py, msl2) was obtained from M. Koornneef (17). Plants were grown at 22°C under continuous fluorescent illumination (100–150 μE m−2 s−1) on a perlite:vermiculite:sphagnum mixture (1:1:1) irrigated with mineral nutrients. Chilling treatments were carried out at 13°C for 2 weeks old plants or as otherwise noted in the text.

1 This work was supported in part by grants from the U.S. Department of Agriculture (87-RCR-1-2507) and the U.S. Department of Energy (DE-AC02-76ER01338).

2 Abbreviations: The following symbols for genetic loci were used: an, angustifolia; cer2, eceriferum; chsl, chilling sensitive; ga4, gibberelin requiring; gll1, glabra; msl1, male sterile; py, pyrimidine requiring; thl1, thiamine requiring.
were grown on agar medium in Petri dishes which were positioned vertically such that root growth occurred along the surface of the medium. The position of the root tip of 10 d old seedlings was recorded and the plants were incubated at 22°C or 13°C. After 6 days, root growth was measured for 10 plants of each genotype at both temperatures. Callus was initiated from seeds as previously described (11). Uniform portions of calli were weighed and treated at normal or chilling temperature for 10 d. The net increase in mg fresh weight was measured for four calli of each genotype at both temperatures.

Electrolyte Leakage

Leaf electrolyte leakage was determined using a conductivity meter. Plants were grown at 22°C for 2 weeks and then transferred to 13°C. At 1 d intervals two leaves of each genotype were placed in 2 mL distilled water at 22°C, illuminated (150 μE m⁻² s⁻¹), and the conductivity of the bathing solutions was monitored over a 4 d period. At the end of the experiment total ion content of each sample was determined by heating each leaf sample to 100°C for 2 min, cooling to room temperature, and measuring conductivity. The conductivity, which is expressed as the percent of conductivity of the 100°C-treated samples, was determined for three separate leaf samples.

Leaf Fatty Acid, Lipid, and Sterol Analysis

Fatty acid methyl esters were prepared from plants grown at 22°C and from plants chilled at 13°C for 2 d. Single leaves were harvested and refluxed at 80°C for 1 h in 1 mL of 1.0 M methanolic HCl in Teflon-sealed tubes. One mL of 0.9% NaCl was added and the fatty acid methyl esters were extracted into 1 mL of hexane. GLC was performed as described.

Table I. Genetic Analysis of PM11

The last five lines of the table indicate the frequency of the various homozygous mutant phenotypes from the multiply marked line MK1 in the F2 progeny of the MK1 × PM11 cross.

<table>
<thead>
<tr>
<th>Cross</th>
<th>Cold-Resistant Plants</th>
<th>Cold-Sensitive Plants</th>
<th>χ²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observed</td>
<td>Expected</td>
<td>Observed</td>
</tr>
<tr>
<td>PM11 (Selfed)</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>WT (Selfed)</td>
<td>100</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>MK1 (Selfed)</td>
<td>25</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PM11 × WT (F1)</td>
<td>25</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MK1 × PM11 (F2)</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PM11 × WT (F2)</td>
<td>294</td>
<td>286*</td>
<td>87</td>
</tr>
<tr>
<td>MK1 × PM11 (F3)</td>
<td>62</td>
<td>67*</td>
<td>28</td>
</tr>
<tr>
<td>AN/AN</td>
<td>20</td>
<td>16*</td>
<td>2</td>
</tr>
<tr>
<td>PY/PY</td>
<td>15</td>
<td>16*</td>
<td>4</td>
</tr>
<tr>
<td>g11/g11</td>
<td>12</td>
<td>16*</td>
<td>3</td>
</tr>
<tr>
<td>cer2/cer2</td>
<td>14</td>
<td>16*</td>
<td>5</td>
</tr>
<tr>
<td>m1/m1</td>
<td>19</td>
<td>16*</td>
<td>12</td>
</tr>
</tbody>
</table>

* Expected segregation for a single recessive nuclear mutation.
  * Expected segregation for two nuclear recessive mutations assorting independently (9:3:3:1).
  * Significant difference (P < 0.05).
CHILLING SENSITIVE MUTANT OF ARABIDOPSIS

Figure 2. Effect of temperature on the relative growth rate of wild-type (WT) and mutant Arabidopsis.

elsewhere (3). Leaf lipids were quantified as previously described (4). The total lipid was also chromatographed on a Unisil column (Clarkson Chemical Co.) with elution solvents: hexane, hexane-chloroform (2:1), chloroform, and ethanol, respectively. Sterols were contained in the chloroform fraction and sterol-esters were contained in the hexane-chloroform fraction. Free sterol composition of leaves of wild-type and mutant plants was determined using capillary gas chromatography (30 m SPB-1 column at 255°C) with cholesterol as a quantitative standard.

14CO2 Labeling of Plants

Two week old plants were illuminated (150 μE m-2 s-1) in the presence of 14CO2 for 24 h at either 22°C or 13°C. In some cases the plants were pretreated at 13°C for 1 d prior to labelling. The general method for 14CO2 feeding in the light has been described previously in detail (27). A 12.5 cm pot with three mutant and three wild-type plants was enclosed in a gas-tight plexiglass chamber that was connected by Tygon tubing in a closed circuit to a gas-tight pump and a stoppered flask containing 8% phosphoric acid. At the beginning of the labeling period [14C]-NaHCO3 was injected into the acid to yield a final specific activity of 14CO2 in the system of 280 μCi mmol-1 and a CO2 concentration of 31.8 mL L-1. After 24 h the aerial portion of each plant was harvested and ground in chloroform:methanol (1:1). The homogenate was washed two times in 0.9% NaCl. Insoluble compounds were pelleted by centrifugation at 3000g for 5 min. The chloroform fraction containing nonpolar compounds was reserved. The aqueous fraction was applied to a Dowex-50(H+) column (0.5 × 3.5 cm) and neutral and acid compounds were eluted with water. Basic compounds were eluted with 8 mL of 2 N NH4OH. The neutral and acid components were then applied to a Dowex-1 (formate) column (0.5 × 3.5 cm) and the neutral components were eluted with 2.5 mL of H2O. The acid components were eluted with 6 mL of 6 M HCl. The percent distribution of 14C assimilate was determined for each fraction.

Chromatography of 14C-Labeled Nonpolar Compounds

Plants were labeled with 14CO2 for 24 h as described above. Plants labeled at 13°C were pretreated at 13°C for 24 h immediately prior to labeling. Plants were harvested and nonpolar compounds were extracted into chloroform (4). Lipids were separated by TLC on silica gel coated plates (Baker) as described by Kates (13) and on high-performance TLC silica gel plates (Whatman LHP-K). On each TLC an equal amount of lipid extract (in dpm) was analyzed for all samples. Polar lipids were separated by chromatography in chloroform:methanol:7 m ammonia (65:25:1 by volume). Neutral lipids were separated by chromatography in hexane:diethyl ether:formic acid (80:20:2 by volume). High performance TLC of neutral lipids was performed as described (29). The plate was first developed in benzene:diethyl ether:acetic acid (60:40:1:0.05 by volume). The solvent was allowed to migrate to 5.5 cm above the origin. The plate was dried and developed in hexane:diethyl ether (94:6 by volume) to a groove scraped 7 cm above the preadsorbent layer. Individual lipids were identified by comparing their Rf values with those of reference standards. The amount of radioactivity in regions of the chromatograms was quantified using a Bioscan System 200 Imaging scanner.

[14C]Mevalonic Acid Labeling of Free Sterols and Steryl-Esters

dl-[2-14C]Mevalonic acid (49.4 mCi mmol-1) was diluted to 27 μCi ml-1 with 0.025% Triton-X 100 and applied to both leaf surfaces of 2 week old plants. The plants were illuminated (150 μE m-2 s-1) at either 22°C or 13°C. The aerial portion of two plants were harvested at various times and non-polar compounds were extracted into chloroform (as described above).

Analysis of Steryl-Esters

Neutral lipids from the labeling experiments were separated using high performance TLC as described above. Cholesterol and cholesterol-linoleate were chromatographed in the first and last lane on each TLC plate and were visualized by staining with iodine vapor while sample lipids were protected from staining. Steryl-esters were extracted into hexane, dried under N2 and saponified with 5% KOH in 95% methanol at

<table>
<thead>
<tr>
<th>Table II. Effect of Temperature on Tissue Viability</th>
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<tbody>
<tr>
<td>Tissue</td>
</tr>
<tr>
<td>Wild type</td>
</tr>
<tr>
<td>Germination (%)</td>
</tr>
<tr>
<td>Leaf Chl content (mg/g fresh weight)</td>
</tr>
<tr>
<td>Root growth (mm)</td>
</tr>
<tr>
<td>Callus growth (fold increase)</td>
</tr>
</tbody>
</table>

* Significant difference (P < 0.05).
Figure 3. Relative Chl content of mutant Arabidopsis throughout a 6 d 13°C treatment regime. Control plants were maintained at 22°C for the duration of the experiment. Treated plants were chilled at 13°C for 1 to 6 d after which they were returned to 22°C. The amount of Chl on a fresh weight basis was measured for wild type and mutant plants on each day of the experiment. Values given represent the amount of Chl in the mutant relative to wild type for a given treatment.

80°C for 1 h (8). One mL of 0.95% NaCl was added to the mixture and free sterols were extracted into hexane and precipitated with a 1% digitonin solution as previously described (13). The fatty acid potassium salts were not extracted into hexane and remained in the aqueous phase. The 4-demethyl sterols and 4,4-dimethyl sterols were identified by chromatography with cholesterol and lanosterol respectively. Reference compounds were obtained from Sigma. The identity of 4-monomethyl sterol was inferred from its Rf (16).

RESULTS
Isolation and Genetic Analysis of a Chilling Sensitive Mutant

Wild-type Arabidopsis is a chilling-resistant species that survives prolonged exposure to temperatures as low as 4°C without any apparent injury. An ethyl methane sulfonate mutagenized (M<sub>2</sub>) population of Arabidopsis was screened for plants which exhibited cold-induced chlorosis. M<sub>2</sub> plants were maintained at 22°C for 2 weeks and then transferred to 13°C for several days. The mutant line PM11 was recovered as a plant which was normal in appearance at 22°C but became chlorotic approximately 3 d after being shifted to 13°C (Fig. 1). The mutant was rescued after 3 d of chilling by returning it to 22°C.

To determine the mode of inheritance of chilling sensitivity in PM11, the mutant line was crossed to wild type and the chilling phenotype of the progeny was examined. F<sub>1</sub> plants from this cross did not become chlorotic when exposed to chilling temperatures (Table I). However, the F<sub>2</sub> progeny segregated 1:3 (chilling sensitive:resistant) (Table I). Together, these results indicate that the chilling-sensitive phenotype of PM11 is due to a single recessive nuclear mutation at a locus which we have designated chsl.

The chsl locus was assigned to a chromosome by scoring the F<sub>2</sub> progeny from a cross between MK1 and PM11 for chilling-sensitivity and for each of five recessive mutations carried by MK1 (Table I). In order to assess chilling-sensitivity, two week old F<sub>2</sub> plants were chilled at 13°C for 3 days. This treatment was sufficient for unambiguous scoring of the chilling phenotype, yet allowed complete recovery of the injured plants. Four of the five MK1 markers, py, gll, cer2, and msl, segregated independently from the chsl locus. Only an, the mutation carried by chromosome 1, showed linkage to chsl (Table I). The distance between an and chsl was estimated to be 28.6 cM by the method of Suiter et al. (28). Since an is located at the extreme end of chromosome 1, chsl appears to be located in the region of the map between ga4 and thl (18).

Growth of Mutant and Wild-Type Arabidopsis

To establish the threshold temperature for chilling sensitivity in PM11, the growth rate of mutant and wild type plants was measured over a range of temperatures from 9°C to 32°C. The two lines had similar growth rates above 22°C, whereas the growth rate of the mutant was reduced at 20°C and was essentially zero below about 18°C (Fig. 2). All subsequent studies on PM11 were conducted at 13°C to ensure full expression of the chilling-sensitive phenotype.

The effect of low temperature on germination, leaf Chl content, root elongation, and callus growth was measured by
transferring tissues from wild-type and mutant plants from 22°C to 13°C for 6 to 10 d. There was no apparent effect on germination, root growth or callus growth (Table II). This observation suggests that the chsI mutation specifically affects an essential component of the leaf.

Leaf Chlorosis

Chlorosis is a common symptom of chilling injury in chilling-sensitive species such as cucumber, sorghum, and corn (10, 23, 26). This measure of chilling injury was used to assess the chilling sensitivity of PM11 by measuring the Ch1 content of plants grown at 22°C and of plants shifted to 13°C for one to 6 d (Fig. 3). No reduction in Ch1 content was observed in plants grown at either 22°C or 13°C for 1 d. There was also no reduction in Ch1 in wild-type plants treated for 6 d at 13°C (results not presented). However, when PM11 was treated at 13°C for 2 d, then returned to 22°C, it exhibited a sharp reduction in Ch1 content that became apparent on the third day of the experiment. These results indicate that the cellular events responsible for chlorosis preceded the loss of Ch1 by at least 24 h. This suggests that in PM11, as in many naturally occurring chilling-sensitive species, chlorosis is a symptom of chilling injury and does not directly reflect the primary cause. Recovery of Ch1 content following transfer to non-chilling temperature was observed in plants treated at 13°C for up to three days but not after longer treatments. Thus, chilling injury becomes irreversible after the third day of chilling treatment.

To explore the possibility that the chilling-induced chlorosis in PM11 was due to photooxidative damage to Ch1, mutant and wild type plants were illuminated with 5 or 150 μE m⁻² s⁻¹ of continuous illumination at 13°C for 6 d. Similar levels of leaf chlorosis were observed following both treatments (data not shown). Since there was no apparent correlation with light intensity and the degree of chlorosis, these results indicate that the mechanism of chilling injury in PM11 does not primarily involve photooxidation of Ch1.

Electrolyte Leakage

A characteristic physiological response associated with chilling damage in chilling-sensitive species is cold-induced electrolyte leakage (24). This is thought to be due to changes in the physical state of membranes at chilling temperatures. As a second criterion of the “chilling-sensitive” nature of PM11, the effect of chilling on electrolyte leakage was studied in wild type and PM11 plants. From the results in Figure 4 it can be seen that the mutant exhibits rapid loss of ions following transfer from low temperature back to normal growth temperatures. In this respect it mimics the effects observed in naturally occurring chilling sensitive plants both with respect to ion leakage, and that it exhibits the effects following transfer to elevated temperature. These results suggest that whatever the basis of the chilling-sensitive phenotype of the mutant, the syndrome of effects is comparable to that observed in chilling-sensitive species.

Metabolite Analysis

To identify the possible biochemical defect in PM11, a broad analysis of metabolites was performed by labeling plants for 24 h at 22°C or 13°C with ¹³C and then fractionating and analyzing the major products by a combination of col-

Table III. Distribution of ¹⁴CO₂-Labeled Metabolites following a 48 h Chilling Treatment at 13°C

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Percent Incorporation</th>
</tr>
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<tbody>
<tr>
<td>Basics</td>
<td>Wild type 5.7 11.1</td>
</tr>
<tr>
<td>Neutral</td>
<td>PM11 47.7</td>
</tr>
<tr>
<td>Acid</td>
<td>Wild type 12.5</td>
</tr>
<tr>
<td>Nonpolar</td>
<td>PM11 19.7</td>
</tr>
<tr>
<td>Insoluble</td>
<td>Wild type 6.9</td>
</tr>
<tr>
<td>Recovery</td>
<td>PM11 92.5</td>
</tr>
</tbody>
</table>

Figure 5. TLC of ¹³CO₂-labeled polar lipids isolated from wild type and mutant Arabidopsis. Lipids were isolated from wild type (A) and mutant (B) plants labeled at 22°C, and from wild-type (C) and mutant (D) plants labeled at 13°C. Although neutral lipids and unknown lipids are not well-resolved in this system, a broadening of the uppermost band due to accumulation of the unknown lipid is apparent in lane D. TLC was performed in chloroform:methanol:Ammonia (65:25:1 by vol.). NL, neutral lipids; S, free sterols; MGD, monogalactosyldiacylglycerol; PA, phosphatidic acid; PG, phosphatidyl glycerol; PE, phosphatidyl ethanolamine; DGD, digalactosyldiacylglycerol; PC, phosphatidyl choline; UL, unknown lipid.
leaf of each plant, and fractionating the lipids by TLC. A total of 57 F2 plants were analyzed, of which 18 were both chilling sensitive and positive for the cold-induced lipid. The other 39 plants did not display either of these phenotypes. Therefore, the gene controlling the accumulation of radioactivity in the cold-induced lipid is tightly linked to the chsl mutation.

**Characterization of the Cold-Induced Lipid**

A variety of techniques for chromatographic fractionation of lipid extracts from chilled plants revealed one consistently reproducible difference between mutant and wild type. As shown in Figure 6A, chilled mutant plants accumulated label in a lipid that comigrated with authentic steryl-ester. No differences were observed between wild type and mutant plants labeled at 22°C (data not shown). In order to establish that the neutral lipid observed in the polar lipid separation (Fig. 5) was the putative steryl-ester, neutral lipids isolated from a polar lipid separation were rechromatographed in a neutral lipid solvent system. The putative steryl-ester was only observed in the neutral lipid fraction of chilled PM11 plants (Fig. 6B). In total, the chill-induced lipid cochromatographed with authentic steryl-ester in three neutral lipid solvent systems suggesting that this lipid is a steryl-ester.

Quantitation of the amount of radioactivity in the putative steryl-ester indicated there was an approximately 10-fold increase in the proportion of radioactivity in this lipid in chilled PM11 plants compared with wild type. By contrast, the amount of label in free sterols and total neutral lipids in the mutant was not significantly different from wild type (Table IV). Therefore, the increase in the putative steryl-ester is specific and not due to a general effect on lipid metabolism.

The possibility of an effect on glycerolipid composition was also explored by preparing lipids from plants which had been grown at 22°C for 2 weeks then shifted to 13°C for up to 4 d. The major leaf lipids were separated and quantified, then individually analyzed for acyl chain composition by gas chromatography of fatty acid methyl esters. No changes in the abundance of specific lipid classes or the acyl composition of the individual lipids were observed (data not shown). These results indicate that the chilling sensitivity of PM11 is not due to a defect in glycerolipid metabolism.

**Analysis of the Putative Steryl-Ester**

Steryl-esters are composed of a long chain fatty acid esterified to the 3'-OH of a sterol (25). Saponification of the cold-

**Table IV. Proportion of Radioactivity from 14CO2 Incorporation in Some Lipids following a 48 h Chilling Treatment at 13°C**

<table>
<thead>
<tr>
<th>Assay</th>
<th>Percent Radioactivity in Total Lipids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild type</td>
</tr>
<tr>
<td>Steryl-ester</td>
<td>0.7 ± 0.3*</td>
</tr>
<tr>
<td>Free sterols</td>
<td>5.8 ± 0.4</td>
</tr>
<tr>
<td>Neutral lipids</td>
<td>17.7 ± 1.5</td>
</tr>
</tbody>
</table>

* Values represent means ± se (n = 3).
Arabidopsis and steryl-ester the prechosis and chlorosis in plants accumulate the sterols. In order to determine the sterol composition of the steryl-esters which accumulate in the mutant at low temperature, plants were labeled with $^{14}$C-mevalonic acid for 72 h. No differences were observed in the sterol composition of the steryl-esters from wild type and mutant plants labeled at 22°C. However, when plants were labeled at 13°C, the proportion of label in 4,4-dimethylsteryl-ester and 4-monomethylsteryl-ester was much greater in the mutant than in the wild type (Fig. 10). These results indicate that the chsl mutation preferentially stimulates the accumulation of label in steryl-esters derived from the earliest intermediates of the sterol biosynthetic pathway.

A preliminary analysis of the effect of chilling temperatures on wild-type and mutant plants indicated that the content of free sterols in wild type and mutant plants were not quantitatively different for plants grown at 22°C or chilled at 13°C for 2 d (results not presented). Leaf tissue from wild type or mutant plants contained approximately 160 μg free sterol per g fresh weight when grown at either 13°C or 22°C. The major free sterols observed were sitosterol, campesterol, stigmasterol, and cholesterol. Steryl-esters, which represented less than 5% of the total sterols in all samples, were not accurately quantitated. However, there did not appear to be major differences in the amount of steryl-esters in the leaves of chilled mutant and wild type plants. Additional studies will be necessary in order to reconcile the results of the labeling studies with measurements of the steryl-ester content.

**DISCUSSION**

**The chsl Locus in Arabidopsis**

Following several days of low temperature treatment, the chsl mutant displayed leaf chlorosis and electrolyte leakage, two symptoms typically associated with chilling damage in naturally occurring chilling sensitive species. In these and related respects, the phenotype of the mutant mimics naturally occurring chilling sensitivity. In an attempt to determine the function of the chsl locus, the mutant line PM11 was subjected to physiological and biochemical analysis. In plants induced steryl-ester followed by solvent extraction and TLC revealed the identity of the sterol constituents as 4,4-dimethylsterol, 4-monomethylsterol, 4-demethylsterol, and an unidentified sterol (Fig. 7). These four compounds were also precipitated by digitonin which specifically precipitates free sterols but not sterol conjugates or other lipids (data not shown).

In order to obtain more rapid and specific labeling of the sterol pool, plants were labeled by applying $^{14}$C-mevalonic acid to wild type and mutant plants for 3 to 72 hours at chilling and non-chilling temperatures. In all cases, squalene was the first compound labeled, followed by the 4,4-dimethylsterols, 4-monomethylsterols, and steryl-esters (see sterol pathway, Fig. 8). At the end of the experiment most of the label was present in the 4-demethylsterols and steryl-esters. However, in chilled mutant plants the proportion of label in the steryl-ester fraction was significantly increased (Fig. 9). These results support the conclusion that steryl-esters specifically accumulate during chilling in PM11. Further, since the increase in steryl-esters is apparent 24 h after chilling, it precedes chlorosis and electrolyte leakage by approximately 48 h. Thus, the accumulation of steryl-esters is the earliest identifiable phenotype associated with chilling injury in PM11.

**Figure 7.** TLC of $^{14}$CO$_2$ labeled neutral lipids isolated from mutant Arabidopsis and steryl-ester hydrolysis products. Lipids were isolated from mutant plants labeled at 13°C (A) and chromatographed using high performance TLC system. Lipids that cochromatographed with cholesterol-linoleate (i.e. steryl-ester) were isolated, saponified, and then rechromatographed (B). SE, steryl-ester; FAME, fatty acid methyl ester; TAG, triacylglycerol; S, free sterol; 1, 4,4-dimethyl sterol; 2, 4-monomethyl sterol; 3, 4-demethyl sterol; 4, unknown sterol.

![TLC of $^{14}$CO$_2$ labeled neutral lipids isolated from mutant Arabidopsis and steryl-ester hydrolysis products.](image)

**Figure 8.** Steryl-ester metabolic pathway. Asterisk (*) indicates steps involving two or more discrete chemical reactions; a, steryl-ester biosynthetic enzyme(s); b, steryl-ester hydrolase(s).
homzygous for the chsl mutatation, leaf tissue became highly susceptible to chilling injury while roots, seeds, and callus were not adversely affected by exposure to low temperature. However, the chilling sensitivity of leaves did not appear to be due to a defect in photosynthesis or respiration, as exposure to nonpermissive temperature for two days had no effect on either of these processes (results not presented). The earliest chilling-induced difference detected between mutant and wild type was a 10-fold increase in the amount of radioactivity accumulating in the steryl-esters of the mutant. No other changes in neutral or polar lipids were observed, indicating that the altered steryl-ester levels was due to a specific effect on this class of compounds and not a general effect on lipid metabolism.

There are several possible explanations for the cold-induced accumulation of label in the steryl-esters in PM11. One possibility is that the wild type chsl gene product encodes an enzyme that functions in steryl-ester metabolism which is altered or defective in the mutant. According to this hypothesis, the accumulation of steryl-esters would be directly responsible for chilling injury. A potential candidate for the defective enzyme is a steryl-ester hydrolase (12). In accordance with the pathway illustrated in Figure 8, the predicted effect of a defective steryl-ester hydrolase would be increased levels of steryl-esters. If the chsl gene encodes a steryl-ester hydrolase, the temperature dependent increase in steryl-esters could be accounted for in two ways: either the mutation results in a cold sensitive form of a constitutively expressed enzyme, or the mutation results in a completely inactive form of a low temperature-induced enzyme. Detailed characterization of the mechanisms which regulate steryl-ester hydrolase activities in Arabidopsis will be necessary to test these and related possibilities.

An alternate explanation for the accumulation of radioactivity in the steryl-esters in PM11 is that it is a secondary effect of the chsl mutation and, consequently, a symptom of chilling injury rather than a cause. For example, the defective chsl gene product may alter a membrane component which exerts a secondary effect on steryl-ester metabolism resulting in increased steryl-ester levels. In this case, the accumulation of steryl-esters may be a useful diagnostic tool for studying the initial stages of chilling injury. The possibility that low temperature treatment results in altered steryl-ester levels in chilling sensitive species has not previously been examined.

The role of steryl-esters in plants is not known (9, 25). A variety of suggestions have been made pertaining to their function, although none have been experimentally verified. Steryl-esters occur in leaves, roots, tubers, and seeds where they are reportedly present in the nuclear, chloroplast, mitochondria, and microsomal membrane fractions (7, 15, 25). Generally they represent from 1 to 15% of the total sterols, although in wheat endosperm they reportedly account for up to 82% of the total sterols. The 4-demethyl steryl-esters have been suggested to function in sterol transport, analogous to their role in mammalian systems (14). However, translocation of cholesterol-palmitate could not be demonstrated in higher plants (1) and no information is available concerning the intra- or intercellular transport of steryl-esters. The esters of 4,4-dimethyl and 4-monomethyl sterols may be involved in the regulation of sterol biosynthesis in animals and a similar,

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**Figure 9.** Incorporation of $[^{14}C]$mevalonic acid into steryl-esters. Wild-type (WT) and mutant Arabidopsis were labeled with $[^{14}C]$ mevalonic acid at $22^\circ C$ and $13^\circ C$ for 3 to 72 h. Values represent the percent of total radioactivity incorporated into the steryl-ester fraction ± SE ($n = 6$).

**Figure 10.** TLC of $[^{14}C]$mevalonic acid-labeled steryl-ester fraction. Steryl-esters isolated from wild-type (A) and mutant (B) plants labeled at $22^\circ C$ for 72 h and from wild-type (C) and mutant (D) plants labeled at $13^\circ C$ for 72 h were saponified and then separated by chromatography using high performance TLC. The same amount of radioactivity was applied to each lane. 1, 4,4-Dimethyl sterol; 2, 4-monomethyl sterol; 3, 4-demethyl sterol; 4, unknown sterol.
though unexplored, role has been suggested in plants (2, 9). Given the current understanding of the function of steryl esters, it is not possible to predict the cause or effect of increased levels of radioactivity in this class of compounds in Arabidopsis at low temperatures.

In spite of the uncertainties regarding the function of steryl esters, the lipophilic character of these compounds suggests that the accumulation of steryl esters in the mutant at low temperatures reflects an effect of the chs1 mutation on membranes. This is generally consistent with the large body of suggestive evidence that membranes are the primary site of injury in chilling sensitive plants (19–21). Significantly, electrolyte leakage was also a symptom of chilling injury in PM11, suggesting membrane damage. Although free sterols have been shown to influence membrane permeability and fluidity in animal cells (30), studies of the effect of cholesterol-palmitate on membrane permeability (8) have led to the conclusion that steryl esters do not act as membrane stabilizers. However, it is possible that the accumulation of steryl-esters observed in the mutant following chilling may reflect alterations in the pool of sterols functioning in membrane stabilization.

Chilling Sensitive Mutants

Although we have demonstrated that it is possible to identify mutants which cannot be distinguished from naturally chilling sensitive species by any useful criteria, the mutant approach to study chilling tolerance is not without limitations. Theoretically, two types of mutations could give rise to chilling sensitivity; those which inactivate a gene specifically required for survival at low temperature and those which result in a cold sensitive form of a gene product. The essential problem is that there is no good criterion to determine which class of mutations is responsible for chilling sensitivity in the chs1 or any other mutant. While the former class of "chilling resistance" genes may have an important role in chilling tolerance, the significance of cold sensitive mutations in natural chilling phenomena is questionable. It is formally possible to create a cold sensitive mutation in any constitutively expressed essential gene product. However, the degree to which low temperature inactivation of such enzymes contributes to chilling sensitivity in nature is not known. Without an understanding of the molecular basis of chilling tolerance, the role of cold sensitive proteins cannot be resolved. Therefore, some but not all 'chilling resistance' genes identified by the mutant approach may be relevant to chilling tolerance. This leaves open to question the significance of the chs1 locus in naturally occurring chilling sensitive species.

A second problem with the use of mutants is that, even though a single gene can be shown to be involved, it can nevertheless be very difficult to determine the biochemical basis of chilling sensitivity. Indeed, the identity of the mutant gene product responsible for chilling sensitivity in PM11 has not been established. This problem can be studied in one of two ways. The approach used here relies on the identification of biochemical differences between the mutant and wild type lines. In principle, a specific hypothesis can then be formulated and tested to determine whether the biochemical difference is directly due to the presence of a mutant gene product and thereby responsible for chilling sensitivity. Another approach would be to directly isolate the 'chilling resistance' gene and attempt to elucidate its function. While methods are currently being developed for cloning genes in Arabidopsis based solely on the mutant phenotype and genetic map position (5), it is frequently not possible to determine the function of isolated genes. Since both approaches are quite laborious, the question arises as to how many other ways the chilling-sensitive phenotype can arise. If there are only a few loci involved, it may be feasible to characterize all of them at both the biochemical and molecular level. On the other hand, if mutations at many different loci can result in the same phenotype, the genetic approach would probably not be useful.

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LITERATURE CITED