Light Stimulation of Cold Acclimation: Production of a Translocatable Promoter[1, 2]

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Summary. The light stimulation of cold acclimation of *Hedera helix* L. var. Thorndale has been shown to result in the production of translocatable promoters of hardiness. Movement of the promoters from an illuminated donor portion to a darkened receptor portion was demonstrated. The majority of transport was acropetal through the phloem and to a much lesser degree basipetal through the xylem. In the early stages of hardening, transport was strictly acropetal. It is suggested that acropetal transport is under the influence of a mobilizing center located in the apex of the plant. Mobilization of the promoters of hardiness was induced by applications of ^{6}N-benzyladenine. Attempts to characterize the light-generated promoters through fixation of $^{14}CO_2$ and subsequent translocation of ^{14}C-labeled compounds from the illuminated donor to the darkened receptor indicated that the translocatable promoting material was either some component of the Dowex 1 fraction or sucrose. Furthermore, the hardiness of leaves was significantly increased by sucrose solutions but not by equi-molar solutions of glucose, galactose or mannitol.

The development of cold hardiness in woody plants involves a number of complex changes influenced by many environmental factors, most notably low temperature and light. Previously, light has been found to greatly enhance the rate and degree of cold acclimation of *Hedera helix* as a photosynthetic rather than a photoperiodic stimulus (5). It was desirable to determine if the light stimulation resulted in the production of some translocatable substance or if it was merely a localized response as in anthocyanin formation.

Materials and Methods

Method of Cold Acclimation. Unless otherwise stated, the standardized procedure for artificial cold acclimation of *Hedera helix* L. var. Thorndale (English Ivy) was used as previously described (6). Intact plants were placed at 5° for a period of 6 weeks. During this time an 8-hour photoperiod was provided by fluorescent and incandescent lamps having a combined intensity of 600 ft-c at plant level. Irrigation was supplied as necessary, usually at weekly intervals.

Method of Freezing and Thawing. Artificial freezing was accomplished by wrapping the samples in aluminum foil and placing them in insulated boxes, which were then placed in a freezer at −6°. After the temperature inside the boxes reached −6°, 1 box then remained at this temperature for 2 hours, and the remaining boxes were transferred to the next freezer set at −12°. The process continued with freezers set at 6° intervals. The rate of temperature drop resulting from this procedure was approximately 3.5° per hour. Following a 2-hour hold period at the desired temperature, the insulated boxes were removed to a 5° cooler and allowed to thaw. The following day, survival was determined.

Method of Testing Survival. The viability of tissue samples was determined by the refined triphenyl tetrazolium chloride (TTC) test (7). All killing points are means of a minimum of 3 replications and are separated by Duncan's new multiple range test. Means not followed by the same letter are significantly different at the 5% level.

Translocation of Hardiness Promoters. To determine if the light stimulation of cold acclimation resulted in the production of a translocatable substance, grafting of hardy plants to unhardy plants and reciprocal grafts were attempted. Attempts to secure a graft union were unsuccessful, since conditions required to maintain the plants in a hardened state were not conducive to formation of a graft

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union. Alternatively, conditions conducive to formation of a graft union caused rapid dehardening of the plants.

An alternative approach took advantage of the differential hardening response in the light and in the dark (5). Different portions of intact plants were covered with aluminum foil to exclude light; the illuminated portion was considered the donor and the darkened portion the receptor. If the light stimulation of cold acclimation resulted in the production of a translocatable substance, the dark receptor should have exhibited a significant increase in hardiness as compared to a totally darkened plant.

Incorporation and Translocation of $^{14}CO_2$. In an attempt to characterize the light-generated promoter, fixation of $^{14}CO_2$ and subsequent translocation of the ^{14}C-labeled compounds from the illuminated basal portion to the darkened receptor were studied. Upper portions of plants were covered with aluminum foil, and the plants placed under standard hardening conditions for 2 weeks before the plants were exposed to $^{14}CO_2$. Upon termination of the feeding period (12 hrs at 5°), 3 plants were immediately frozen and retained for chemical analyses. The remaining plants were returned to the 5° chamber for continuation of the hardening process, and further samples were taken after an additional 1, 2, or 3 weeks of hardening.

Samples were separated into leaves and stems from both the light and dark portions of individual plants, lyophilized and ground to 40 mesh in a Wiley mill. Samples of 100 mg were extracted for 6 hours with 80 % (v/v) ethanol in a micro-Soxhlet apparatus. The extract was then fractionated 6 times with petroleum ether (density 0.67–0.69), and the petroleum ether fraction was dried under an air stream, and retained for radioactivity determinations. The ethanol fraction was concentrated under reduced pressure and further fractionated on Dowex 50 W (H^+) and then Dowex 1 (OH^-) columns. The extract was passed through the columns at a rate of 3 ml per minute, and followed by two 15 ml volumes of 80 % ethanol. The Dowex 50 W column was eluted by successive additions of 40 ml 0.4 N NH_4OH in 80 % (v/v) ethanol, 15 ml 80 % (v/v) ethanol, 15 ml deionized H_2O, 15 ml 4.0 N NH_4OH and 30 ml deionized water. The Dowex 1 column was eluted with successive additions of 40 ml 0.4 N HCl in 80 % (v/v) ethanol, 15 ml 80 % (v/v) ethanol, 15 ml deionized water, 15 ml 4.0 N HCl, and 30 ml deionized H_2O. Eluates of the respective columns were collected and then taken to dryness under reduced pressure, and the residue was taken up in 2 ml of 10 % isopropanol and retained for radioactivity determinations.

The neutral effluent, comprised mainly of sugars, was further purified by thin layer chromatography. Silica gel G plates, prepared with 0.1 N boric acid in place of water aided considerably in separation of the sugars. Plates were developed in methyl ethyl ketone:acetic acid:water (6:2:2) (v/v) and sprayed with aniline diphenylamine phosphate (1 g diphenylamine + 1 ml aniline/100 ml acetone + 10 ml phosphoric acid).

Radioactivity determinations were made in a Tricarb liquid scintillation spectrometer. The silica gel portions of the plates containing the individual sugars were scraped into scintillation vials, and 2 ml of absolute alcohol was added to elute the sugars from the silica gel. Scintillation fluid, BBOT [2,5-*bis*-2-(5-tert-butylbenzoxazoly)-thiophene] in toluene (4 g/liter) was added, and the vials were vigorously mixed. Within 30 minutes, the silica gel settled to the bottom of the vial. Counting efficiency was about 70 %.

Concentrations of individual sugars were determined by the anthrone method (11). Three ml of anthrone solution [0.2 g anthrone/100 ml of 75 % (v/v) H_2SO_4] were placed in centrifuge tubes in an ice bath, and 1 ml of distilled water was layered over the anthrone solution. Areas of the silica gel G plates containing the individual sugars were then scraped into the centrifuge tubes and thoroughly mixed. Samples were placed in a hot water bath at 100° for 10 minutes; after cooling and centrifuging, the A_{630} was determined. Standard solutions of individual sugars were assayed each time and standard curves generated for the individual sugars.

Results

Translocation of Hardiness Promoters. Initially, alternate leaves of individual plants were covered with aluminum foil. In addition, control plants with all or none of the leaves covered were also included. After 6 weeks, hardiness of leaves, either illuminated or darkened, on the same plant was essentially the same and was not significantly different from hardiness of leaves from plants entirely exposed to light. Leaves from all 3 treatments were significantly hardier than leaves from plants entirely covered (table I). Stem tissue from plants with alternate leaves covered was significantly less hardy than samples from the illuminated control plants. This probably would be a reflection of reduced photosynthetic area which was previously shown to influence stem hardiness (5).

Table I. *Effect of Covering Alternate Leaves on Cold Acclimation of Ivy*

Means followed by different letters are significantly different at the 5 % level by Duncan's new multiple range test.

Treatment	Killing point (°) Leaves	Stems
Light control	—15.5 a	—19.9 a
Light/dark–light	—15.1 a	—17.4 b
Light/dark–dark	—13.9 a	—16.9 b
Dark control	—10.8 b	—10.2 c

Table II. *Effect of Covering Various Plant Portions on Cold Acclimation of Ivy Plants After 6 and 12 Weeks Exposure to Cold Hardening Conditions*

Means followed by different letters are significantly different at the 5 % level by Duncan's new multiple range test.

Treatment	Killing point (°) 6 Weeks of hardening Leaves	Stems	12 Weeks of Hardening Leaves	Stems
Light control	—20.0 a	—20.1 a	—22.2 a	—22.5 a
Light–donor	—15.8 b	—12.3 b	—21.6 a	—22.2 a
Dark–receptor	— 9.9 c	—12.9 b	—18.9 b	—19.0 b
Dark–receptor	—15.5 b	—17.1 a	—19.3 b	—20.1 b
Light–donor	—20.7 a	—18.6 a	—19.7 b	—22.6 a
Dark control	— 9.9 c	—11.7 b	—10.7 c	—12.8 c

A modification of the above experiment was to exclude light from a larger continuous number of leaves. Plants were covered with aluminum foil so that either the entire upper half or entire lower half of the plant was illuminated, with the remainder of the plant being covered. After 6 and 12 weeks of exposure to standard acclimation conditions, hardiness of the different portions was determined.

After 6 weeks, only darkened receptor portions acropetal to the illuminated donors exhibited an increase in hardiness (table II). Leaves in the darkened portion located acropetally to the illuminated donor were hardy to −15.5°, while leaves from the entirely darkened plants were only hardy to −9.9°. If the darkened receptor was basipetal to the illuminated donor, hardiness of leaves (−9.9°) in this portion was the same as hardiness of leaves from the entirely darkened control (−9.9°).

Stems exhibited a somewhat anomolous behavior in that stem tissue from a plant with the upper portion exposed to light was not significantly different in hardiness from the dark control (table II). However, stem tissue in any portion of a plant with the base exposed to light was significantly hardier than the dark control and was equivalent to the light control.

After 12 weeks, the preferential translocation pattern was no longer evident, and the darkened receptor benefited from the light donor regardless of its orientation, acropetal or basipetal (table II).

The experiment was repeated but modified by including 2 controls, one with 10 leaves and the other with 5 leaves, for both the light and dark treatments. In addition, both upper and lower portions of the 10-leaf controls were sampled for individual hardiness determinations after 6 weeks under the standard hardening conditions. These added precautions were taken to eliminate any possible differences in hardiness due to sampling different areas on a plant or due to different amounts of leaf area. There was no significant difference in hardiness of the dark controls with respect to the upper or lower portion of the 10-leaf control or any sections compared with the 5-leaf control (table III). This was true for either stem or leaf tissue. In similarly sampled light controls, leaves from the lower half of the 10-leaf control were only slightly less hardy than leaves from the upper half or from the 5-leaf control. Hardiness of stem samples was identical from both regions of the 10-leaf control and slightly higher than the 5-leaf control.

The results confirmed reception of the hardiness promoters by darkened receptors and that the preferential acropetal translocation exhibited after 6 weeks was real and repeatable. Also confirmed was the poor hardening response of illuminated stems if they were located in the upper half of the plant.

Table III. *Effects of Covering Various Plant Portions on Cold Acclimation of Ivy After 6 Weeks Exposure to Hardening Conditions*

Means followed by different letters are significantly different at the 5 % level by Duncan's new multiple range test.

Treatment	Killing point (°) Leaves	Stems
Light Control (5 leaves)	—17.1 ab	—17.5 cd
Light control (10 leaves)	—19.0 a	—20.6 ab
Light control (10 leaves)	—15.6 bc	—23.3 a
Light–donor	—15.8 bc	—14.4 e
Dark-receptor	—10.1 d	—14.7 e
Dark–receptor	—14.0 c	—15.4 de
Light–donor	—16.4 bc	—19.6 bc
Dark control (10 leaves)	—10.5 d	— 9.1 f
Dark control (10 leaves)	—10.4 d	—10.3 f
Dark control (5 leaves)	— 9.0 d	—10.0 f

Influence of the Apex on Cold Acclimation. Aside from the demonstration of preferential acropetal translocation of the hardiness promoters, these experiments stimulated interest in the nature of the apparent mobilizing influence of acropetal dark sections. To further investigate if the apex was responsible for this phenomenon, the effect of apex removal on cold acclimation was studied. Plants with the apex and small unexpanded leaves removed were hardened under standard conditions along with intact plants. Hardiness of stems and leaves from decapitated plants was not significantly different from the controls.

Table IV. *Effect of ^{6}N-Benzyladenine Applications on Alternate Leaves on Cold Acclimation of Ivy After 6 Weeks Exposure to Cold Hardening Conditions*

Means followed by different letters are significantly different at the 5 % level by Duncan's new multiple range test.

Treatment	Killing point (°) Leaves	Stems
Control plants	—13.7 a	—16.9 a
Treated plants: leaves + ^{6}N-benzyladenine	—15.7 b	—15.4 b
Treated plants: leaves —^{6}N-benzyladenine	—13.5 a	—15.3 b

Effect of ^{6}N-benzyladenine on Cold Acclimation. Since kinetin and various kinin derivatives have been shown to act as mobilizing agents (1), the influence of ^{6}N-benzyladenine on distribution of the hardiness promoters was studied. Alternate leaves of plants were treated with ^{6}N-benzyladenine (100 mg/liter + wetting agent) and placed under standard hardening conditions with control plants, the alternate leaves of which were treated only with wetting agent (0.05 % v/x Ortho X-77). Hardiness of leaves treated with ^{6}N-benzyladenine was significantly increased over the untreated leaves from the same plant and over leaves from the control (table IV). Internodes below treated or untreated leaves were individually sampled for hardiness determinations. Samples from below either a treated or untreated leaf were identical in hardiness, and both were significantly less hardy than stem tissue from control plants.

Effect of Girdling and Defoliation on Translocation of Hardiness Promoters. To further characterize movement of the hardiness promoters, the influence of girdling on their movement was investigated. Plants were girdled midway down the stem by removing a band of tissue 1 cm wide down to the xylem. Five leaves were left on both sides of the girdle. Plants were placed under standard hardening conditions, which also suppressed regeneration of any phloem connections. Hardiness determinations revealed that there was little difference in hardiness of leaf or stem tissue above or below the girdle.

Elaboration of the experiment employed defoliation on either side of the girdle. Hardiness of the remaining leaves was the same regardless of their position with respect to the girdle (table V). Hardiness of stem tissue in any area retaining leaves was equal to the control, and was significantly greater than the defoliated regions of stems. However, if the defoliated stem section was acropetal to the girdle it was significantly less hardy than the defoliated stem section basipetal to the girdle.

Table V. *Effect of Girdling and Defoliation on Cold Acclimation of Ivy Leaves and Stems after 6 Weeks Exposure to Cold Hardening Conditions*

Means followed by different letters are significantly different at the 5 % level by Duncan's new multiple range test.

Treatment	Killing point Leaves	Stems
Control	—19.1 a	—19.7 a
Girdled	—17.7 a	—20.2 a
	—18.5 a	—20.7 a
Girdled	defoliated	—11.6 c
	—18.0 a	—21.6 a
Girdled	—18.2 a	—21.2 a
	defoliated	—16.4 b

Characterization of the Light-Generated Promoters. In an attempt to characterize the light-generated promoters, fixation of $^{14}CO_2$ and subsequent translocation of the ^{14}C-labeled compounds from the illuminated basal donor to the darkened receptor were studied. Immediately after the feeding period, illuminated leaves (donor portion) were labeled in all of the fractions (table VI). The majority of the ^{14}C resided in the Dowex 1 fraction (organic acids and phosphorylated compounds) and in the effluent of the ion-exchange resins (sugars). Furthermore, in the sugar fraction of illuminated leaves ^{14}C was found in all of the isolated sugars. However, in leaves of the darkened receptor portion or in stems of either donor or receptor portions (table VII), ^{14}C-labeled compounds were present only in the Dowex 1 fraction and sucrose of the sugar fraction. After 1 or 2 weeks following feeding, the amount of label present in the sucrose fraction of illuminated leaves decreased rapidly while the amount of label in the Dowex 1 fraction decreased less rapidly. Not until 3 weeks after feeding did any of the label appear in the petroleum ether or Dowex 50 W fractions or in sugars other than sucrose in the darkened receptor leaves. Appearance of the ^{14}C in these fractions in darkened receptor stems was earlier but the amounts were very insignificant.

Effect of Exogenous Sugars on Cold Acclimation. Since the data indicate that the translocatable promoter might be a sugar, attempts were made to

Table VI. *Distribution of ^{14}C in the 80% Ethanol Extract of Leaves of Illuminated and Darkened Portions of Ivy Plants After Various Intervals at 5° Following Exposure to $^{14}CO_2$*

$^{14}CO_2$ was generated from 2.5 mg of $Ba^{14}CO_3$ (0.322 mc) by the addition of lactic acid. Plants were exposed to the $^{14}CO_2$ for 12 hours at 5°. The light intensity was 600 ft-c.

Fraction	CPM/mg dry wt Weeks after feeding $^{14}CO_2$ 0 Light	0 Dark	1 Light	1 Dark	2 Light	2 Dark	3 Light	3 Dark
Petroleum ether	55	0	95	0	125	0	110	10
Dowex 50 W	35	0	20	0	25	0	20	5
Dowex 1	2840	405	1910	285	1935	220	1400	410
Effluent	3315	290	1900	235	990	160	800	295
Raffinose	35	0	20	0	20	0	20	25
Unknown	145	0	65	0	70	0	50	0
Sucrose	3310	260	1280	175	620	120	430	205
Fructose	80	0	70	0	40	0	30	10
Glucose	125	15	175	10	95	15	190	5

Table VII. *Distribution of ^{14}C in the 80% Ethanol Extract of Stems of Illuminated and Darkened Portions of Ivy Plants after Various Intervals Following Exposure to $^{14}CO_2$.*

See table VI for conditions during exposure to $^{14}CO_2$.

Fraction	CPM/mg dry wt Weeks after feeding $^{14}CO_2$ 0 Light	0 Dark	1 Light	1 Dark	2 Light	2 Dark	3 Light	3 Dark
Petroleum ether	0	0	25	10	25	5	105	45
Dowex 50 W	0	0	5	0	0	0	10	10
Dowex 1	150	60	700	265	435	210	565	490
Effluent	175	50	870	410	485	185	600	425
Raffinose	0	0	25	10	15	5	30	15
Unknown	0	0	10	5	5	0	15	5
Sucrose	175	40	835	385	380	170	420	385
Fructose	0	0	5	5	25	0	5	0
Glucose	0	0	10	0	20	0	70	20

Table VIII. *Killing Points of Ivy Leaves Floated on 0.25 and 0.50 M Solutions of Galactose, Glucose, Mannitol and Sucrose for 6 Weeks at 5° in the Dark*

Killing point of leaves before treatment was —9.2°. Means followed by different letters are significantly different at the 5% level by Duncan's new multiple range test.

Treatment		Killing point (°)
H_2O		—13.5 a
Galactose	0.25 M	—14.9 a
	0.50 M	—14.4 a
Glucose	0.25 M	—14.4 a
	0.50 M	—13.5 a
Mannitol	0.25 M	—14.9 a
	0.50 M	—13.4 a
Sucrose	0.25 M	—17.4 b
	0.50 M	—17.3 b

induce hardiness with exogenous sugars. Leaves were floated on various sugar solutions at 5° in the dark, and hardiness determinations were made after 6 weeks.

Only leaves floated on sucrose exhibited an increase in hardiness that was significantly different from the water control (table VIII). Glucose or galactose failed to significantly increase the hardiness, while equimolar concentrations of mannitol were also ineffective.

Discussion

Translocation of Hardiness Promoters. Results presented in tables I to III indicate that the light enhancement of hardiness resulted in the production of a translocatable entity, but in the early stages of cold acclimation, the hardiness promoters

were only translocated in an acropetal direction. Thus, darkened receptor portions benefited from the illuminated donors only when the receptor was acropetal to the donor, and would indicate that there was some mobilizing influence of the apex.

The poor hardening response of illuminated acropetal stems may also be an indication that translocation of the hardiness promoters is dependent on some mobilization center located in the apex. That is, if the illuminated donor is basipetal to the dark receptor, there is mobilization to the acropetal portion which introduces the hardiness promoters into the stems. However, if there is no mobilizing center below the light donor (which there does not appear to be as evidenced by the lack of increased leaf hardiness), there will be little or no introduction of the hardiness promoters into the stems.

After 12 weeks, the mobilizing influence of the acropetal portion of the plant was not evident, and hardiness of darkened receptors regardless of orientation approached that of illuminated donors and was true for both leaf and stem tissues. Thus, the flow of hardiness promoters initially proceeds acropetally, while later during the cold acclimation process the flow is less restrictive. Repetition of the experiment confirmed the preferential acropetal translocation and also demonstrated that differences manifested were not the result of differential hardening in the upper or lower portions.

The increased hardiness of leaves treated with ^{6}N-benzyladenine indicates that the promoters are responsive to a mobilizing center: either the apex, or one simulated by ^{6}N-benzyladenine applications. The increase in hardiness of the leaves apparently came at the expense of stem hardiness, since untreated leaves were as hardy as the control leaves. Internodes below treated and untreated leaves were identical in hardiness, but were significantly less hardy than stem tissue from control plants. Apparently promoters present in the stems are more easily diverted to the simulated mobilization center.

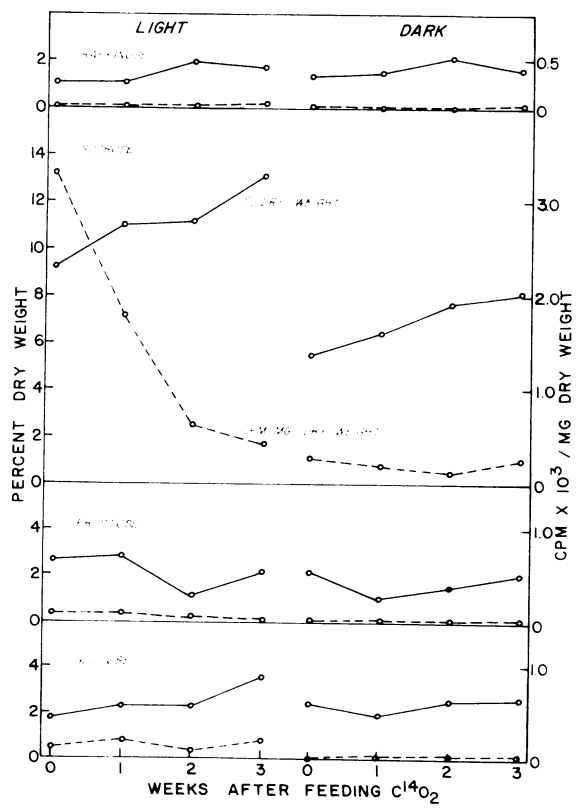

FIG. 1. Sugar content and distribution of ^{14}C in leaves after feeding $^{14}CO_2$. See table VI for conditions during exposure to $^{14}CO_2$.

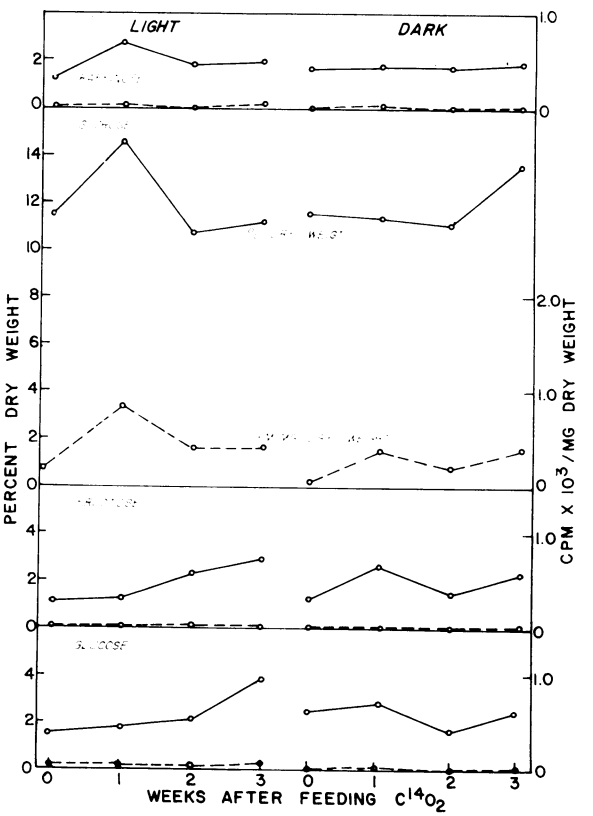

FIG. 2. Sugar content and distribution of ^{14}C in stems after feeding $^{14}CO_2$. See table VI for conditions during exposure to $^{14}CO_2$.

The girdling experiments indicated that the roots do not influence hardiness of aerial portions of the plants. While girdling alone does not eliminate the possible influence of roots, it has been previously reported that cuttings harden equally as well as intact plants (6). Combination of defoliation with girdling indicated that acropetal transport of the hardiness promoters was largely through the phloem and hence impeded by girdling. However, the hardiness promoters were also translocated basipetally to a much lesser degree through the xylem, which was not inhibited by girdling.

Characterization of the Light-Generated Promoters. Since ^{14}C-labeled compounds were found in the Dowex 50 W and petroleum ether fractions only in illuminated leaves initially after feeding, and since there was little redistribution of the label into these fractions in the dark receptor, metabolites associated with these fractions do not appear to be representative of the light-generated promoters. It appears that the light generated promoting material to cold hardiness consists of either some component of the Dowex 1 fraction or sucrose. Lack of redistribution of the label into sugars other than sucrose in the dark receptor de-emphasizes their role in cold acclimation.

Analyses of sugar content (figs 1, 2) further support the conclusion that sucrose is more important than the other sugars in cold acclimation. While glucose, sucrose and raffinose increased in concentration in illuminated leaves, only sucrose increased in concentration in the dark receptor. Also the concentrations of glucose, fructose and raffinose were similar in leaves in both the donor and receptor portions of the plants, but only the sucrose concentration was higher in the donor leaves than in the receptor leaves. This difference in concentration would be compatible with the donor and receptor concept and would also account for the higher degree of hardiness in the donor leaves over receptor leaves. Concentrations of sugars and distribution of ^{14}C in sucrose were equal in both donor and receptor portions of stems.

This rapid equilibration of sucrose concentration and ^{14}C-labeled compounds could be responsible for the similarity of hardiness of donor and receptor portions of stems. Thus, in stems where hardiness of the donor and receptor portions are similar, sucrose concentrations and ^{14}C distribution are similar. However, in leaves hardiness was greater in donor portions, which also had sucrose and ^{14}C contents higher than the receptor portions.

While there have been previous reports of sugar solutions increasing hardiness (3, 4, 8, 9, 10), there are also examples of other solutes having similar effects (2, 4). These latter reports are the basis for the theory that the protective influence of solutes is not unique to sugars, but is dependent on their ability to penetrate the cells and increase the osmotic potential (2, 4). On the contrary, Trunova (8) has presented evidence that even though some sugars, such as rhamnose, penetrate into the cells and accumulate in significant quantities, they do not increase hardiness. Cold hardiness was only increased by sugars, such as sucrose, that were readily metabolized. In *Hedera helix,* the indirect evidence suggests that of the sugars accumulated and translocated, sucrose is the most important in relation to cold hardiness. This is further substantiated by the fact that of the exogenous sugars added, only sucrose increased cold hardiness, while equi-molar concentrations of galactose, glucose, or mannitol were ineffective.

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