7-OXOPENTADECAN-2-OL ESTERS - A NEW EPICUTICULAR WAX LIPID CLASS

by

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Keywords: 2-hydroxy-7-pentadecanone esters, barley, *eceriferum* mutants, β -ketoacyl elongase

The alkan-1-ol and alkan-2-ol esters (primarily, C₃₅-C₄₈ and C₃₃-C₃₅, respectively) of barley epicuticular waxes are known to arise via two different biosynthetic pathways. Herein we identify a third type of ester (approx. 0. 2% by weight of spike wax), hitherto unknown in these plant lipids. The esters were isolated via preparative TLC together with the longer chain primary alcohols. From the effects of chemical treatments analyzed with the aid of TLC and GC, the presence of an oxo group in the alcohol moiety was deduced. GC-MS of the esters and their alcohol moieties before and after NaBH₄ treatment plus of the fatty acid moieties resulted in the identification of at least three 7-oxopentadecan-2-ol esters (C₃₃, ₃₅ and ₃₇). 2,7-Dihydroxypentadecane was synthesized and its mass spectrum found to be identical to that of the reduced ester alcohol moiety. A determination of the relative amounts of these esters in ten different genotypes supports the contention that biosynthetically the pentadecan-2-ol esters are precursors of those containing an oxo group on carbon 7.

I. INTRODUCTION

The synthesis and deposition of barley epicuticular waxes is determined by the *eceriferum* genes. To identify those *cer* genes which can serve as tools in unravelling the biosynthetic pathways of these very long chain molecules, the

composition of all the lipid classes from spike mutants excepting the unidentified trace components is being determined. During the past year when primary alcohol fractions from mutants of the *cer-cqu* locus were analyzed with gas chromatography, a series of unexpected peaks

Abbreviations: BSA = bis-trimethylsilylacetamide; BSTFA = N,N-bis-trimethylsilyltrifluoroacetamide; GC = gas chromatography; MS = mass spectrometry; TLC = thin layer chromatography; TMCS = trimethylchlorosilan; TMS = trimethylsilyl.

often appeared in the positions characteristic for C₃₂, ₃₄ and ₃₆ primary alcohols. These peaks were not present when acetate derivatives of the alcohols were prepared and analyzed. In the present communication we present evidence which attributes these peaks to a homologous series of 7-oxopentadecan-2-ol esters. This name is preferred to 2-hydroxy-7-pentadecanone esters as it indicates the biosynthetic origin of these lipids which is also discussed herein.

2. MATERIALS AND METHODS

Seeds of barley (*Hordeum vulgare* L.) cv. Bonus as well as of the *eceriferum* mutants *cer-c*³, *-c*²⁹, *-c*⁴⁴, *-c*⁶¹, *-c*⁶³, *-c*⁷³, *-c*⁸⁹, *-c*⁹⁵, *-q*³⁵, *-q*¹⁴⁵⁹, *-u*⁴⁷², *-u*^{602a} and *-u*^{602b} (4,5) were planted and grown in the Phytotron (14) at the Swedish University of Agricultural Science, Stockholm between 1980 and 1983 using the most recently specified conditions while *cer-q*⁴² and *-u*⁶⁹ (4) were grown in 1978 using the procedures standard at that time (6). In 1967 the mutants 602a and 602b were isolated as two out of five homozygous *eceriferum* M₂ plants originating from a single spike of an M₁ plant. Allele tests carried out with mutant 602a revealed that it was allelic to *cer-u*. Such tests were not carried out on mutant 602b as it was assumed to be identical to 602a (U. LUNDQVIST, personal communication). Both mutants have identical phenotypes. Recent chemical analyses of the spike waxes have shown that whereas mutant 602b has the composition expected for a *cer-u* mutant (19), 602a does not (P. VON WETTSTEIN-KNOWLES, unpublished). Thus although 602b is very likely an allele of *cer-u*, it will not be so designated until the appropriate crosses have been carried out.

Epicuticular waxes were collected from heading spikes (21), and subjected to thin layer chromatography (TLC) on 20×20 cm plates. Benzene proved to be a more suitable developing solvent than amylene stabilized chloroform (Merck) and was used unless specified. Silica gel H (Merck) plates were used for all waxes lacking β-diketone lipids, whereas copper acetate-silica gel H "hybrid" TLC plates (21) were used for those having β-diketone lipids;

namely, Bonus and the mutants 3, 63, 69, 472, 602b and 1459. From TLC plates of the available waxes of the six *cer-c* mutants, 29, 44, 61, 73, 89 and 95, the 0.6 cm of gel directly above the primary alcohols was pooled and extracted with chloroform to yield the extract that was used for chemical analyses.

To quantitate the relative amounts of the primary alcohols and 7-oxopentadecan-2-ol esters, the silica gel containing the primary alcohols together with the 0.6 cm directly above was extracted with chloroform. To ensure that all of both lipid classes had been recovered, the extract of the one cm of gel on either edge of the just described band was analyzed by GC.

The procedures used for preparation of alcohol acetates, reduction using NaBH₄, oxidation using K₂Cr₂O₇, transmethylation using BF₃ in CH₃OH and making of trimethylsilyl (TMS) derivatives using bistrimethylsilylacetamide (BSA) have been published (7, 8, 15, 19, respectively). Alternatively TMS derivatives were prepared by adding to the three times acetone dried samples, 100 μl pyridine, 100 μl of N,N-bis-trimethylsilyltrifluoroacetamide (BSTFA), and 8 μl trimethylchlorosilane (TMCS). The capped vial was held at 60 °C for 20 min, whereupon the solution was concentrated using dried N₂ and a suitable volume of hexane added.

To ascertain whether 7-hydroxypentadecan-2-ol esters were present in a spike wax, the gel between the primary alcohols and the free fatty acids at the origin was extracted with chloroform since chemical analyses (section 3.1.) indicated that this was the position at which they would be located. An aliquot was subjected to GC. Thereafter, one half of the sample was treated with K₂Cr₂O₇ and the other half with BSTFA, whereafter both were subjected to GC.

The procedure used to synthesize 2,7-dihydroxypentadecane is outlined in Figure 1. It is one (IV) of the five possible products (I - V) that can arise via this series of reactions. TMS derivatives of the reaction products were made by treating with BSTFA, TMCS and pyridine in hexane solution at 50 °C for a few min.

Quantitative GC analyses were carried out using 3% SP-2100 columns and the instruments described previously (6, 21). Qualitative GC

Figure 1. The procedure used for synthesizing 2,7-dihydroxypentadecane (IV) potentially gives rise to four additional products: I, 2-hydroxyhexane; II, 2,7-dihydroxyoctane; III, 5-hydroxytridecane; and V, 8, 13-dihydroxydocosane. The Grignard reagent was prepared from 42.4 mmol of 1,4-dibromobutane (Fluka) and 84.4 mmol of magnesium in tetrahydrofuran 50 ml (THF). To the stirred solution, initially at room temperature, 34 mmol of nonanal was added dropwise over 5 min. After cooling in an ice bath, 60.5 mmol of ethanal in 5 ml THF was added. Thereafter, the reaction mixture was treated with 50 ml water, acidified with 30 ml 4 M-HCl and extracted with 50 ml ethyl ether. The ethereal phase was dried over Na_2SO_4 . Removal of the solvents on the rotary evaporator gave a semi-solid product mixture (6.0 g). Reaction of the double Grignard reagent with one equivalent of an aldehyde gives rise to products I and III and with two equivalents of aldehydes to products II, IV and V.

analyses were carried out using a Packard 437 gas chromatograph with a single 2 mm (i. d.) $\times$ 1 m glass column containing 10% SE-30 on Chromosorb W, HP, 80-100 mesh. The operating conditions used for the latter instrument while very similar to those described for the former (6)

were modified where necessary to give as identical separations as possible. Gas chromatography-mass spectrometry (GC-MS) analyses were performed on a VG 7070F instrument equipped with a VG 2035 Data System and a Pye Unicam Series 204 gas chromatograph using a 2 mm (i. d.)

Figure 2. Effect of BSTFA (upper) and $K_2Cr_2O_7$ (middle) treatments on the components of the original TLC extract (lower) as revealed by SE-30 GC. Peaks 1, 3, 5 and 7 represent the TMS derivatives of C_{26} , C_{28} , C_{30} and C_{32} primary alcohols which are represented by peaks 2, 4, 6 and part of 8, respectively. The non- C_{32} primary alcohol part of peak 8 plus peaks 9 and 11 are due to the C_{33} , C_{35} and C_{37} 7-oxopentadecan-2-ol esters, respectively. Peak 10 is an unknown.

$\times 1.5$ m glass column packed with 5% OV-101 on Chromosorb W, HP, 80-100 mesh; carrier gas (He), approx. $25 \text{ ml} \cdot \text{min}^{-1}$; ion source temp., 220°C ; ionization energy, 70 eV.

3. RESULTS AND DISCUSSION

3.1. Identification of 7-oxopentadecan-2-ol esters

Subjection of an aliquot of the original extract to TLC suggested the presence of two partially overlapping lipid classes. Injection of another aliquot into the gas chromatograph gave the series of peaks shown in the lower trace of Figure 2. Peaks 2, 4 and 6 as well as part of peak 8 were ascribed to C_{26} , C_{28} , C_{30} and C_{32} primary alcohols, respectively, on the basis of the following observations. i) Treatment of the extract with $K_2Cr_2O_7$ resulted in their disappearance (middle curve, Figure 2) since they had been converted to free fatty acids. The latter were recoverable at the

origin of TLC plates. ii) Treatment with acetyl chloride converted them into acetate derivatives that co-chromatographed on TLC and GC with standards. iii) Treatment with BSTFA produced TMS derivatives (upper curve, Figure 2), which eluted from the SE-30 column (peaks 1, 3, 5 and 7) at the same times that standards did. iv) Treatment with $NaBH_4$ or BF_3 in CH_3OH had no effect on their relative mobilities as assayed by TLC and GC. If, in the initial TLC isolation amylene stabilized chloroform replaced benzene, a much larger proportion of the original extract was primary alcohols.

As illustrated in Figure 2, treatment with $K_2Cr_2O_7$ or BSTFA had no effect on the elution times of part of peak 8 or peaks 9 and 11 suggesting that they represented the lipid class overlapping the primary alcohols upon TLC. Removal of the latter with $K_2Cr_2O_7$ permitted firstly the establishment of an $R_f = 0.26$ for the unknown lipid class vs 0.19 for the primary alcohols from *cer-c* spike waxes (ave. 39% C_{26} , 18% C_{28} , 16% C_{30} and 14% C_{32}). Secondly, a weight % distribution of 20, 69 and 11 between the remaining part of peak 8 plus peaks 9 and 11 was determined using SP-2100 columns. Treatment of the original extract with $NaBH_4$ reduced the R_f of the unknown to 0.07 . After preparative TLC separation of the reduced unknowns from the primary alcohols, an aliquot was injected into the gas chromatograph. No peaks appeared between 270 – 342°C . Subsequent to treatment with BSA, however, three peaks in the proportions expected were observed less than 2°C higher than the respective peaks in the chromatogram of the starting unknown homologues (peaks 8, 9 and 11, Figure 2).

Subjecting the original extract to BF_3 in CH_3OH treatment followed by TLC revealed a loss of the unknown lipid class and appearance of two other spots, one at the origin and the other with an $R_f = 0.64$. Both were recovered by preparative TLC. The latter was found to behave in TLC and GC as C_{16} , C_{18} , C_{20} and C_{22} fatty acid methyl ester standards. A mol % distribution of 1, 16, 65 and 18, respectively, was calculated after SP-2100 GC. Integrator units divided by the effective carbon number (number of carbons minus 1.25) = mol (9). A minor peak suggesting trace amounts of a C_{24} homologue was also

present. GC of the material at the origin gave no peaks although subsequent to treatment with BSA a single peak eluting at 199 °C was present. The lack of a visible peak before BSA treatment as in the previous paragraph is presumed due to a combination of peak broadening and/or adsorption on the column and the small amount of sample. When treatment with NaBH₄ preceded that with BF₃ in CH₃OH, identical results were obtained except that GC of the material at the origin gave a peak appearing at 198 °C that after treatment with BSA appeared at 208 °C.

On the basis of the above observations, the unknown lipid class was identified as an ester. At least three homologues occur having acid moieties of 18, 20 and 22 carbons esterified to an unknown alcohol moiety containing an oxo group. The structure of this alcohol was determined with the aid of GC-MS and synthesis of the derived diol.

The TMS derivative of the reduced unknown alcohol, obtained as described above and assumed to be a diol, gave the easily interpreted spectrum shown in Figure 3A. Assuming the intense peaks at m/z 117, 215 and 275 represent TMS oxonium ions, the diol could be 2,7-dihydropentadecane. Undoubtedly two diastereomers occur, although they can not be resolved by the packed GC column used. The chirality at carbon 2 is unknown. The molecular ion peak is not visible. The highest mass peak is at m/z 373 ($M - 15$). The mass spectrum of the TMS derivative of the ester alcohol itself (Figure 3B) shows a molecular ion peak at m/z 314 and an intense TMS oxonium ion peak at m/z 117 which again demonstrates the presence of a 2-hydroxy group. Peaks are found revealing the position of the presumed oxo function on carbon 7, namely acylium ion peaks at m/z 141 and 201 and a McLafferty rearrangement ion peak at m/z 216, but they are of relatively low intensity.

To our knowledge neither 2-hydroxy-7-pentadecanone nor the reduction product 2,7-dihydropentadecane has been reported in the literature. The diol synthesized as described in Figure 1 was not isolated in a pure state. An aliquot of the product mixture was converted to TMS derivatives and analyzed by GC-MS. The derivatives of the products II - V (Figure 1) were present in approximately equal amounts. If

product I was synthesized, which is likely since product III was, its derivative would have been lost together with the other volatile compounds in the solvent peak which was dumped in the GC-MS separator. The mass spectrum of product IV which must be a mixture of stereoisomers was identical to that obtained for the TMS derivative of the reduced alcohol from the presently studied esters (Figure 3A). We think that this result strongly supports our identification of the alcohol moiety as 2-hydroxy-7-pentadecanone.

The three major new esters and their NaBH₄ reduction products were also analyzed by GC-MS. The highest mass peaks in the spectra of the latter (illustrated for eicosanoate in Figure 3C) arise from TMS oxonium ions formed by loss of a C₈ alkyl radical from the molecular ions. These peaks as well as the more intense peak at m/z 215 which is due to the other possible TMS oxonium ion indicate the position of the oxy function. Other peaks in the higher mass part of the spectra include an intense peak at m/z 185 which is probably formed by loss of fatty acid residues from the highest mass TMS oxonium ions via McLafferty rearrangement processes and a number of peaks derived from the fatty acid parts of the esters.

The highest mass peaks in the spectra of the three new esters (Figure 3D) correspond to ions formed by loss of C₇H₁₄ from the molecular ions in McLafferty rearrangement processes. These indicate the position of the oxo function on carbon 7 and are consistent with the presence of an acylium ion peak at m/z 141. Peaks characteristic for the fatty acid residues are also found. As in Figure 3C this is illustrated for the eicosanoate containing ester (Figure 3D). Neither in the spectra of the esters themselves nor of their reduction products are peaks present which could reveal the position of the hydroxy group in the alcohol moiety. This absence led to the isolation and structural characterization of this part of the esters as described above.

Finally the identity of the C₁₆, 18, 20 and 22 fatty acid methyl esters obtained by transesterification of the esters with BF₃ in CH₃OH was confirmed by MS. We thus conclude that in addition to the three major new esters – 7-oxopentadecan-2-ol stearate, 7-oxopentadecan-2-ol

Figure 3. Mass spectra of the TMS derivatives of the compounds used in the identification of the 7-oxopentadecan-2-ol esters. A. 2,7-dihydroxypentadecane. B. 2-hydroxy-7-pentadecanone. C. 7-hydroxypentadecan-2-ol eicosanoate (6-hydroxy-1-methyltetradecyl eicosanoate). D. 7-oxopentadecan-2-ol eicosanoate (6-oxo-1-methyltetradecyl eicosanoate).

Figure 4. Proposed biosynthetic origin of the 7-oxopentadecan-2-ol esters in barley from alkan-2-ol esters. Brackets enclose a hypothetical intermediate. The arrows indicate direction of synthesis of the carbon chains of the acid and alcohol moieties of the alkan-2-ol esters. Note, carbon-7 with the oxo group corresponds to carbon-9 synthetically which was a methyl carbon of an acetyl-coenzyme A donor unit.

eicosanoate and 7-oxopentadecan-2-ol docosanoate – a trace amount of 7-oxopentadecan-2-ol palmitate is present in the barley epicuticular waxes studied.

3. 2. Biosynthetic origin of 7-oxopentadecan-2-ol esters

Two types of esters are known in barley, those with alkan-1-ol and those with alkan-2-ol moieties. They have been most extensively studied in *cer-u*⁶⁹ spike waxes (20), where the latter type account for 35% by weight (= 62 mol %) of this lipid class. Since these esters amount to 11% by weight (21) of this wax and there is approximately 1 mg wax per spike at the age when the wax is collected (21), roughly 70 and 40 µg of the alkan-1-ol and alkan-2-ol esters are present on each spike. Of the latter 13 and 20 µg, respectively, are accounted for by the C₃₃ and C₃₅ homo-

logues, each of which consists of two isomers, one having a C₁₃ and the other a C₁₅ alkan-2-ol moiety (see Figure 4). The C₁₅ alkan-2-ol esters total 24 µg per spike. While *cer-c*³ and wild type Bonus spikes have similar proportions of the two ester types, a considerable range in composition occurs as illustrated in the second column in Table I. Four genotypes totally lack the alkan-2-ol esters and three have increased proportions. In *cer-c*⁶³ and -^{602b} the elevation is small, but in -*u*^{602a} a doubling of the wild type amount occurs.

If, as suggested in Figure 4, the alkan-2-ol esters are biosynthetic precursors of the newly identified esters, then one might expect a correlation between the presence and absence of these two types of lipids in spike waxes. That in general this deduction holds is shown by a comparison of the data in the second and third columns of Table I. To determine whether the relative amounts of the new esters varies in parallel to

Table I.

Amounts of C₃₃ and C₃₅ alkan-2-ol esters and 7-oxopentadecan-2-ol esters in spike waxes of Bonus and nine *cer* mutants.

Genotype	%	C ₃₃ + C ₃₅	Presence of 7-oxo-C ₁₅ - 2-ol esters ^{b)}	C ₂₆ OH
		Total esters ^{a)}		C ₃₅ 7-oxo-C ₁₅ - 2-ol ester ^{c)}
Bonus		29	+	21.5
<i>cer-u</i> ⁶⁹		30	+	18.1
- <i>c</i> ³		26	+	21.4
- <i>c</i> ⁶³		39	+	4.3
- <i>u</i> ^{602b}		36	+	5.4
- <i>u</i> ^{602a}		56	+	0.9
- <i>u</i> ⁴⁷²		0	-	
- <i>q</i> ³⁵		0	-	
- <i>q</i> ⁴²		0	-	
- <i>q</i> ¹⁴⁵⁹		0	-	

^{a)} Alkan-1-ol and -2-ol esters are present in a single band on TLC plates. The wt % of the two major 2-ol esters (C₃₃ and ₃₅) in this chloroform extractable band has been determined via GC using SP-2100 columns. See text.

^{b)} Based upon SP-2100 GC analyses of simultaneously isolated primary alcohols and 7-oxopentadecan-2-ol esters. The - = absence and the + = presence designations are based upon a minimum of C₂₆ primary alcohol integrator units = 3.1×10^6 in the former group and a range of $0.9\text{--}3.8 \times 10^6$ in the latter group.

^{c)} Mol ratio; calculated using the appropriate effective carbon numbers (9), which for C₂₆ primary alcohol (C₂₆ OH) = 25.4 and for C₃₅ 7-oxopentadecan-2-ol ester = 32.75. The 26 carbon homologue is always the dominant primary alcohol ranging from 37–58% (by wt as acetates) with a mean value of 46% for the six genotypes concerned. The 35 carbon homologue is always the dominant ester, representing 65, 68 and 56% (by wt) in *cer-c*³, -*c*⁶³ and -*u*^{602a}. No significant difference can be detected in the homologue distributions of the two individual *cer-c* mutants and that for the pooled *cer-c* mutants given in the text.

those of the proposed precursor esters, the amount of the former relative to another lipid class was determined. The primary alcohols were chosen as they are known to be synthesized via a different elongation system from that leading to the esterified alkan-2-ols (18) which is not influenced by the studied *cer-cqu* mutations (21). Furthermore, the primary alcohols and the 7-oxopentadecan-2-ol esters can be simultaneously separated from almost all the other wax lipids by TLC, and the relative amounts of the major homologue of each lipid class determined by GC (see footnote c, Table I). Results from such analyses are presented in column 4 of Table I. For each mol of C₃₅ 7-oxopentadecan-2-ol ester in Bonus, *cer-u*⁶⁹ and -*c*³ roughly 20 mol of C₂₆ primary alcohol are present. The ratio is decreased to 5 mol in *cer-c*⁶³ and -*u*^{602b}, while in -*u*^{602a} the two specified lipids occur in approximately the same frequency. We thus conclude

that not only is there a correlation between the presence and absence of the suggested precursor alkan-2-ol esters and the new esters, but that when present the relative amounts of these two lipid classes vary in a parallel fashion.

By weight the ratio of the C₂₆ primary alcohol to the C₃₅ 7-oxopentadecan-2-ol ester isolated from *cer-u*⁶⁹ is 93:7. Since the primary alcohols are 3% by weight of the spike wax in this genotype (21) of which the 26 carbon homologue accounts for 58% (Table I, footnote c), each spike has roughly 17 µg of this compound and 1 µg of the C₃₅ ester. The latter lipid class amounts to approximately 2 µg per spike which is 0.2% by weight of the epicuticular wax.

A comparison of the upper and lower lines in Figure 4 reveals that whereas the same four acids serve as moieties for both types of esters the same is not true for the alcohol moieties. Of the two minor (C₁₁ and ₁₇) and two major (C₁₃ and ₁₅)

alkan-2-ols present in the precursor esters only the C₁₅ was present in their 7-oxo derivatives. This conclusion which is based upon GC-MS studies is in accord with the following observation. For *cer-u*⁶⁹ a calculation of the mol % distribution of the fatty acids esterified to pentadecan-2-ol gives C₁₆ = 0.6, C₁₈ = 18.5, C₂₀ = 69.5 and C₂₂ = 11.5, whereas that for those esterified to tridecan-2-ol gives C₁₆ = 4, C₁₈ = 61 and C₂₀ = 35 (20). The former distribution is remarkably similar to that given in section 3. 1. for the fatty acid moieties of the newly analyzed esters which were isolated from the mixture of *cer-c* mutants.

In plant and insect epicuticular waxes the hydroxyl group of long chain secondary alcohols is believed to be inserted into a preformed carbon chain (1, 2, 3). The hydroxyl group is then in some cases oxidized giving rise to long chain ketones (2, 3). A similar insertion process was invoked to explain the origin of the hydroxyl group in β -diketones (12, 16, 17), and thereafter extended to include the newly identified oxo- β -diketones (13). Given that this is true one would predict that 7-hydroxypentadecan-2-ol esters are potential intermediates in the biosynthesis of 7-oxopentadecan-2-ol esters (Figure 4). Although examination of *cer-u*^{602a} and *-u*⁶⁹ waxes did not reveal the presence of the hypothetical hydroxy intermediates, we are unable to rule out their existence. This situation is not unique as illustrated by the recent analyses of oxo and hydroxy derivatives of hentriacontan-14,16-dione in the Triticeae (10, 11, 13). Out of 53 species with β -diketones 48 also have hydroxy- β -diketones. Fifteen of the latter contain oxo- β -diketones in addition. Among the mono and disubstituted β -diketones studied, hydroxy groups were identified on carbons 4, 8, 9, 25 and 26 and oxo groups on carbons 10 and 25. The data lead firstly to the conclusion that a number of different hydroxylation systems exist, and secondly to the deduction that whereas the oxo group on carbon 25 presumably arises via a hydroxy intermediate the same need not be true for the oxo group on carbon 10. Interestingly, an oxo group on carbon 10 is three carbons away from the closest oxo of the β -dione group which is one carbon less than the distance between the oxo on carbon 7 and the methyl plus oxygen attached to carbon 12 of the 7-oxopentadecan-2-

ol esters. From this aspect it should be noted that an oxo group is not inserted on carbons 5 or 7 of the presumably available tridecan-2-ol esters. The above discussion leads us to deduce that the enzyme system in barley giving rise to the oxo group in the pentadecan-2-ol moieties of the esters is almost certainly distinct from that inserting the hydroxy group into the β -diketones although both lipid classes are derivatives of the same elongation system (18).

ACKNOWLEDGEMENTS

We are indebted to M. PETERSEN for outstanding technical assistance; to A. S. STEINHOLZ and N. RASMUSSEN for preparing the figures; and to the personnel of the Phytotron in Stockholm for growing the plants.

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