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Stimulation of source-applied ^{14}C-sucrose export in *Vicia faba* plants by brassinosteroids, GA_3 and IAA

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SUMMARY

The effect of brassinosteroids (SSHB, 24-Epi), GA_3 and IAA on sucrose uptake and export from source leaf was studied in *Vicia faba* plants. Brassinosteroids, GA_3 and IAA applied exogenously to the source leaves were found to enhance the retention of ^{14}C-label and stimulate the uptake in discs of source leaves during 4-h treatment. The hormone-promoted sucrose uptake is probably due to modulation of the H^+-ATPase activity as indicated by increasing V_{max}-values for sucrose uptake. Enhanced transport of ^{14}C-compounds to the apical sink region was observed in SSHB- and GA_3-treated plants after 24 h. SSHB, GA_3 and IAA applied to the sink region of *Vicia* plants, promoted ^{14}C-radiolabel transport to the site of hormone-treated region. On the basis of this observation, we conclude that, like GA_3 and IAA, brassinosteroids affect ^{14}C-unloading processes.

Key-words: Brassinosteroids, ^{14}C-distribution, gibberellic acid, indole-3-acetic acid, phloem loading, phloem unloading, *Vicia faba.*

INTRODUCTION

One of the important determinants in plant productivity is the transport of photosynthates from source to sink and the utilization in sink tissue. Partitioning between the sinks is controlled by their relative strength. It has been suggested that phytohormones such as GA_3 and IAA may play a regulatory role in carbon partitioning and membrane transports of assimilates (Thomas 1985; Daie *et al.* 1986; Patrick 1987).

Brassinosteroids are novel plant growth regulators that may increase crop yield or improve crop quality (Yokota & Takahashi 1985; Mandava 1991). Interest in the physiological functions of natural steroids prompted us to investigate the influence of brassinosteroids on the assimilate partitioning. This paper presents the effects of exogenously applied natural SSHB, its synthetic analogue 24-Epi, GA_3 and IAA on the partitioning of ^{14}C-labelled photosynthates in *Vicia faba* plants as a result of altered phloem loading and unloading.

Abbreviations: EB: erythrosin B; FC: fusicoccin; GA_3: gibberellic acid; IAA: indole-3-acetic acid; SSHB: (22 S, 23 S)-homo-brassinolide; 24-Epi: 24-epibrassinolide; se-cc: sieve element/companion cell complex.

MATERIALS AND METHODS

Plant material

Broad bean plants (*Vicia faba* L. cv. Juno, Saat- und Pflanzgut Quedlinburg, Germany) were grown in a growth cabinet under controlled conditions as previously described (Petzold & Dahse 1988).

Application of phytohormones and ^{14}C-sucrose to intact leaves

For transport studies, 3-week-old *Vicia* plants (possessing four leaves and the cotyledons) were trimmed to retain the mature, fully expanded bifoliate leaf at the third node from the top (source) and the youngest, visible unfolded leaf including the plumula (sink region) 24 h before the start of the experiments. This procedure allowed the plants to establish a new source/sink balance as we can assume from preliminary studies using $NaH^{14}CO_3$.

Phytohormones (GA_3, IAA, 10^{-7}–10^{-5} mol l^{-1}) were dissolved in aqueous solutions containing 0·01% Tween 20 (Sigma). Three hours before the source leaf was exposed to ^{14}C-sucrose (5 mmol l^{-1}, 1 μCi ml^{-1}) 20 μl of these solutions were applied on the upper surface of the source leaf over an abraded area of 1 cm^2 (SiC powder, 1200 mesh, obtained from K. Schriewer, Hamburg, Germany) surrounded by lanolin paste.

In other experiments, the same phytohormones, except 24-Epi, were injected into the basal part of the sink region. Following a 4- or 24-h incubation period, plants were dissected into roots, stems (below and above the source leaves), source leaves and sink regions (apex). All experiments were conducted under illumination of 40 W 'white' fluorescent tubes (7·3 W m^{-2} at the apex level) at a temperature of 22°C and 70% r.h. Experiments were carried out in five-fold replicates and repeated at least three times. Each fraction of the plant was extracted in 10 ml 80% (v/v) ethanol at 80°C for 24 h. One ml of each ethanol-soluble extract was transferred to a scintillation vial containing 9 ml of scintillation fluid (7 ml toluene, 2 ml ethanol, 6 mg PPO, 2 mg POPOP) and radioassayed in a Packard Scintillation Counter at efficiencies of 70–80%.

Application of phytohormones and ^{14}C-sucrose to leaf discs

Experiments with discs from the detached fourth leaf were carried out the day before the whole plant experiment. Sixty min before the start of uptake, the midrib of the leaf was excised and the lower epidermis was stripped off to facilitate uniform uptake from the incubation medium. Discs (10 mm in diameter) were punched from the area between large veins, rinsed with distilled water and incubated for 30 min with the stripped side onto 10 ml of standard medium (250 mmol l^{-1} mannitol, 0·5 mmol l^{-1} $CaCl_2$, 0·25 mmol l^{-1} $MgCl_2$, buffered at 5·5 with 10 mmol l^{-1} MES (2[N-morpholino] ethanesulphonic acid)-NaOH with or without various concentrations of brassinosteroids, GA_3 or IAA. Discs were then transferred to identical solutions containing 5 mmol l^{-1} radiolabelled sucrose (5 ml, 5 discs, 0·5 μCi) and incubated in darkness at 25°C for 1 h. After having been rinsed with three chases of standard medium for 1 min each, the discs were rapidly blotted on filter paper and transferred to Tricarb-vials with 5 ml ethanol. After extraction and decolorization by adding 100 μl of 10% benzoylperoxide, the radioactivity was counted as described above.

Separation and identification of radioactive compounds in the ethanol-soluble fractions were performed by chromatography on cellulose-thinlayers (butanol/ethanol/water: 52/33/15 (v/v/v) and chloroform/acetic acid/water: 6/7/1 (v/v/v). Authentic ^{14}C-labelled sucrose fructose and glucose were used as markers.

Table 1. Effect of SSHB, GA_3 and IAA (100 μmol l^{-1} each) on the ^{14}C-activity in ethanol-soluble and -insoluble fractions of *Vicia faba* source leaves after 4 h incubation. Values in the presence of SSHB, GA_3 and IAA were significantly different from control ($P \leqslant 5$)

Hormone treatment	*Vicia faba* source leaves (dpm 10^3 g f wt.$^{-1}$)		% of total ^{14}C fixed
	Soluble	Insoluble	
Control	8420	935	11
SSHB	10 272	1180	11
GA_3	9683	1019	10
IAA	9262	1029	11

Activity of acid invertase was determined based on the method of Daie *et al.* (1986) using the Nelson assay procedure.

Chemicals

SSHB and 24-Epi were synthesized from stigmasterol as described before (Adam & Marquardt 1986) and were dissolved in a stock solution of methanol. The final methanol concentration never exceeded 1% and had no effect on the plant during the experimental period. FC was dissolved in absolute ethanol and added drop by drop to an appropriate volume of hot (70%) distilled water under continuous stirring to give a 1 mmol l^{-1} stock solution. The final ethanol concentration was 0·6% in the stock solution. EB was dissolved in distilled water to yield a 10 mmol l^{-1} stock solution.

Uniformly labelled ^{14}C-sucrose, D-^{14}C-glucose and D-^{14}C-fructose (specific activities 2·4, 9·0 and 9·6 GBq mmol, respectively) were obtained as aqueous solutions from the Radiochemical Centre Amersham (UK).

RESULTS

^{14}C-Distribution after treatment with SSHB, GA_3 and IAA of the source leaf

An analysis of the ^{14}C-activity in the source, stem and sink tissues following 4-h treatment of the source established that more than 90% was present in the ethanol-soluble fraction of both the control and hormone-treated plants (Table 1). Based on this observation, the ^{14}C-activity in this fraction is taken as a measure for ^{14}C-material.

In the short-term (4 h) an increased retention of ^{14}C-label was observed in the source leaf fraction in the presence of SSHB, GA_3 and IAA (Fig. 1a). The upward and downward movements towards apical sink and roots were significantly smaller than in the control. SSHB-treatment showed the strongest effect. This hormone-induced retention of ^{14}C-label in the source leaves may have reflected a higher metabolic conversion of ^{14}C-sucrose. Chromatographic separation of the ^{14}C-material in the source leaf tissue, however, showed that the percentage of unmetabolized sucrose was similar (*c.* 80% of the total activity on the chromatogram) for both control and SSHB- and GA_3-treated plants while glucose and fructose made up the bulk of the remaining ^{14}C-activity (Table 2). In contrast, IAA seemingly enhanced sucrose metabolism as indicated by the higher percentage of glucose and fructose in the ethanolic extract (Table 2).

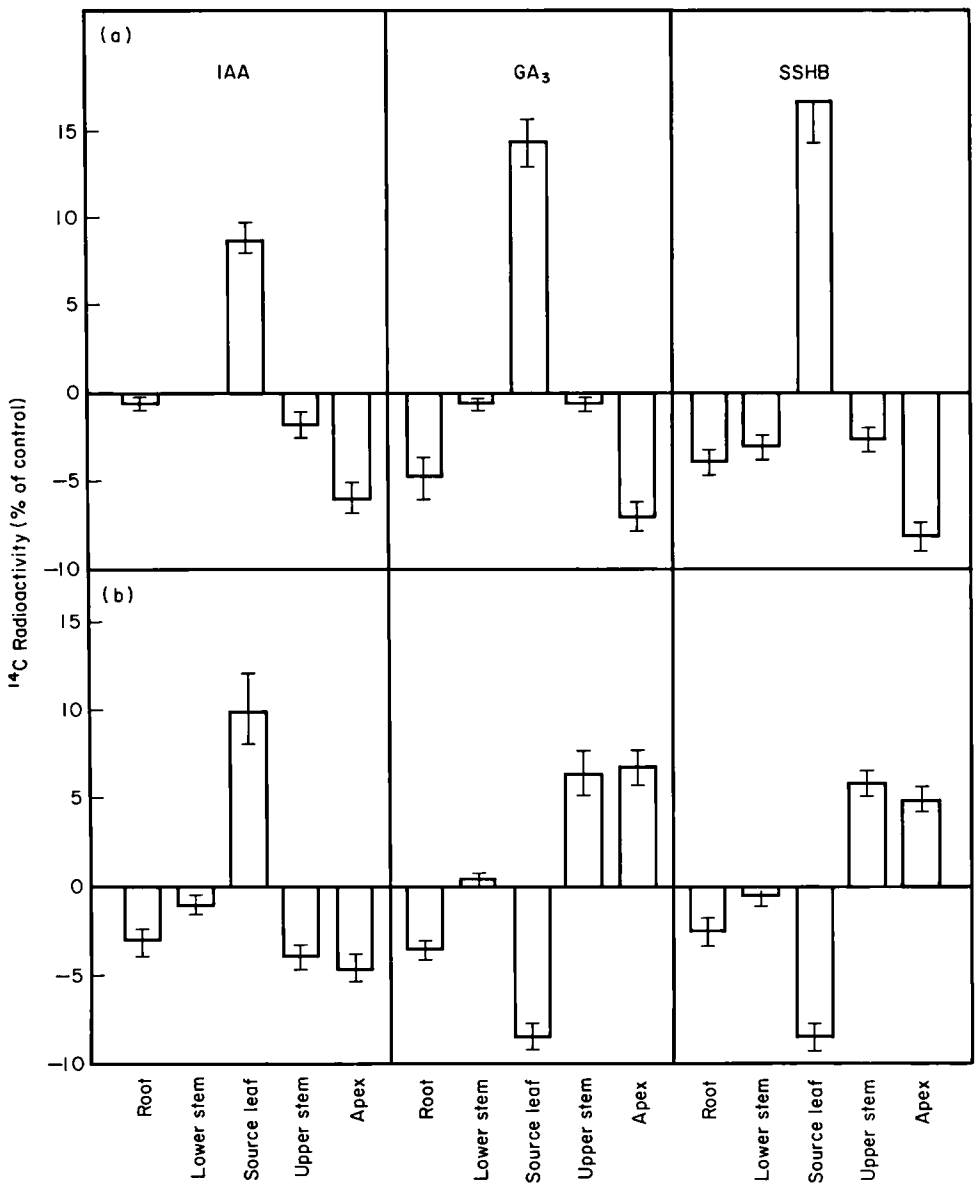

Fig. 1. Effect of SSHB, GA_3 and IAA (20 µl, 10 µmol l^{-1}) applied on the source leaf on the ^{14}C-distribution to the root and apex for 4 h (a) and 24 h (b). Treatment as described in materials and methods. The amount of ^{14}C-label was expressed as percentage of the total amount of label in different plant parts of control (without hormones). Values are means of four replicates. Vertical bars indicate ± SE.

The percentage of the ^{14}C-label in various parts of a *Vicia* plant after a 24-h experiment is summarized in Table 3. The pattern of ^{14}C-distribution was changed drastically under the influence of phytohormones. The upward movement toward the apical sink region was significantly enhanced in the SSHB- and GA_3-treated plants compared to the control (Fig. 1b), whereas the portion of the total ^{14}C recovered from the roots was lower after

Table 2. Chemical nature of ^{14}C-activity found in the ethanolic extracts of source tissues of *Vicia faba* plants 4 h after ^{14}C-sucrose application. Each value is the mean of three replicates. *Values statistically different from control ($P \leqslant 0{\cdot}05$).

Hormone treatment (10^{-6} mol l^{-1})	^{14}C-activity (10^5 dpm g f wt.$^{-1}$)	Radioactivity (% total ^{14}C-activity on chromatogram) co-chromatograming		
		Sucrose	Glucose	Fructose
Control	1·22	78·2	6·7	2·3
SSHB	1·41*	81·4	8·2	3·1
GA_3	1·38*	79·7	7·5	3·2
IAA	1·32*	75·4	10·2	5·2

Table 3. Percent of ^{14}C-label in different parts of *Vicia* plant 24 h after application of ^{14}C-sucrose to an abraded source leaf. Average of three experiments ± SE

Organ	% of label in plant
Labelled leaf (source)	70·0 ± 6·5
Upper stem segment	2·3 ± 0·3
Apex	14·4 ± 2·8
Lower stem segment	3·0 ± 1·5
Roots	9·8 ± 2·4

treatment with SSHB, GA_3 and IAA (Fig. 1b). In contrast to the results with SSHB and GA_3, radioactivity tended to remain in the source leaf in IAA-treated plants. As shown in Table 2, IAA may stimulate the metabolic breakdown of sucrose. Invertases, sucrose synthetase and sucrose phosphate synthase are involved in the immediate metabolism of sucrose. The activity of these enzymes appears to be a potential target for hormonal regulation (Cheikh *et al.* 1990; Estruch & Beltran 1991).

Earlier reports suggested that exogenous application of auxin and gibberellic acid stimulated invertase activity (Daie *et al.* 1986; Weil & Rausch 1990). This possibility was tested in hormone-treated source leaves. Cell wall acid invertase was present in control leaves, exhibiting a low degree of activity (around 0·8 μmol mg^{-1} protein h^{-1}). The activity of acid invertase increased significantly after 24 h in IAA-treated and to some extent in GA_3- and SSHB-treated leaves (Table 4).

The enhancing effect of SSHB and GA_3 on the movement towards the apical sink region after 24 h may be due to previous export of phytohormones from the source to the sink. GA_3 is reported to be translocated via the phloem to the sink region (Aloni *et al.* 1986; Patrick & Mulligan 1989), where it may promote sink activity such as growth (Aloni *et al.* 1986). Therefore, the effect of SSHB, GA_3 and IAA, when applied to the sink region, on the ^{14}C- export from the source was determined.

Table 4. The effect of SSHB, GA_3 and IAA on cell-wall acid invertase activity in *Vicia* source leaf tissues expressed as percentage of the activity in controls (0·8 μmol mg protein^{-1} h^{-1}). Determinations were made after 24 h of incubation. Values represent the mean of five samples. *Value is significantly different from control

Hormone treatment (10 μmol l^{-1})	Invertase activity (%)
Control	100
SSHB	137
GA_3	132
IAA	185*

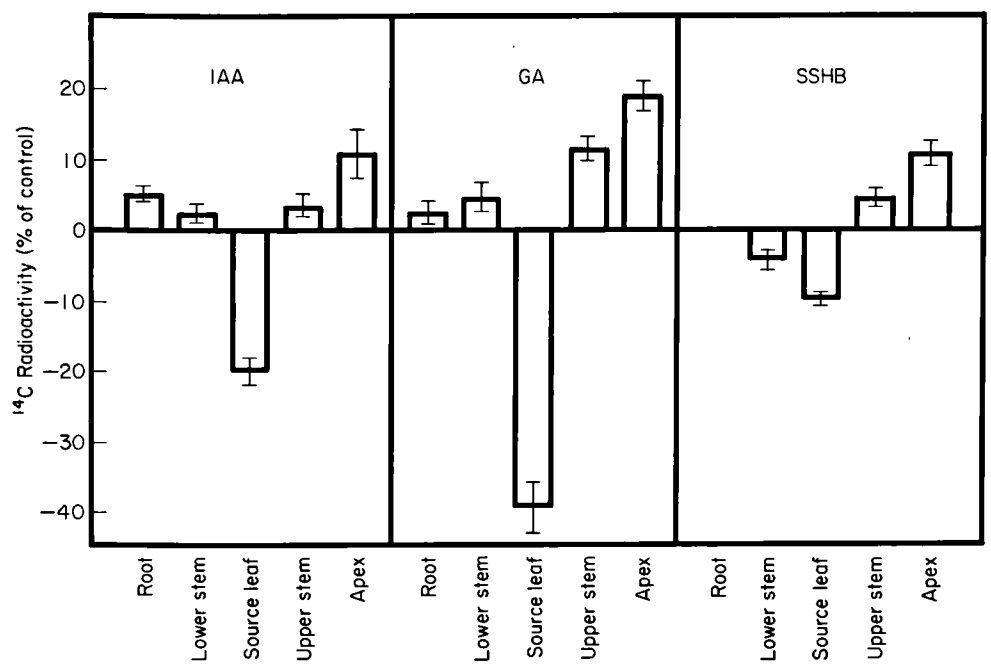

Fig. 2. Distribution of ^{14}C-label in the plant after ^{14}C-sucrose was applied on source leaf and SSHB, GA_3 and IAA were injected into a sink leaf for 24 h. The amount of ^{14}C-label was expressed as percentage of control. Values are means of four replicates. Vertical bars indicate ±SE.

Effect of sink-applied phytohormones on ^{14}C-distribution in Vicia *plants*

After injection of SSHB, GA_3 or IAA into the sink region, the export of ^{14}C-label from the source was stimulated, predominantly towards the apical sink (Fig. 2). Phytohormone treatment did not alter the net photosynthesis of source leaves (data not shown). Increased

Table 5. Stimulating effect of brassinosteroids (SSHB, 24-Epi), GA_3, IAA and FC on ^{14}C-sucrose uptake by source leaf discs of *Vicia faba* and its inhibition by EB. EB was present only during incubation wth labelled sucrose. Data expressed as the means of three replicates (10 discs per replicate) ±SE. The percentage of inhibition by EB is in parentheses. *Treatment values significantly different from control ($P \leq 0{\cdot}05$). n.d. = not determined

Hormone treatment	Sucrose uptake (μmol g f wt.$^{-1}$ h^{-1})	
	−EB	+EB (100 μmol l^{-1})
Control	3·06±0·03	2·08±0·05 (32)
FC (10 μmol l^{-1})	4·56±0·07	1·82±0·03 (60)
SSHB		
1 μmol l^{-1}	4·85±0·08	n.d.
10 μmol l^{-1}	5·08±0·06	2·18±0·04 (57)
24-Epi (10 μmol l^{-1})	4·66±0·03	n.d.
GA_3		
1 μmol l^{-1}	5·43±0·06	n.d.
10 μmol l^{-1}	4·73±0·04	2·45±0·02 (48)
IAA (10 μmol l^{-1})	3·71±0·09	2·04±0·06 (45)

amounts of ^{14}C-label were found in the roots under the influence of GA_3 and IAA, but not in the presence of SSHB. The ^{14}C-partitioning in intact plants (Figs 1 and 2) is the result of the effects of SSHB, GA_3 and IAA on the influx/efflux balance in source, path and sink. To obtain detailed insight into the nature of hormone-induced retention of ^{14}C-compounds in the source tissue, we used a technique with stripped leaf discs according to Van Bel & Ammerlaan (1981), Delrot (1981) and Lemoine *et al.* (1984).

Effects of SSHB, GA_3 and IAA on ^{14}C-sucrose uptake into the leaf discs of Vicia faba

Preliminary experiments showed that the sucrose uptake by leaf discs was linear during the first hour of incubation. A 1-h period was chosen for determination of the initial uptake velocity.

Brassinosteroids (SSHB, 24-Epi), GA_3 and to a lesser extent, IAA significantly increased sucrose uptake by leaf discs (Table 5). This hormone-promoted uptake may be due to stimulation of H^+-sucrose co-transport which has been characterized by Delrot & Bonnemain (1984) for *Vicia* leaves. Concentration-dependence of ^{14}C-sucrose uptake into the source leaf discs of *Vicia* is biphasic, as in many other systems (Giaquinta 1983; Wimmers & Turgeon 1991) (data not shown). The kinetic parameters of the high-affinity sucrose uptake in *Vicia* leaves (Table 6) are similar to those reported for *Beta vulgaris* (Giaquinta 1983), *Commelina benghalensis* (Van Bel & Koops 1985) and *Pisum sativum* (Wimmers & Turgeon 1991). The apparent kinetic parameters calculated from Lineweaver–Burk plots of the saturable component are $K_m = 2{\cdot}72$ mmol l^{-1} and $V_{max} = 4{\cdot}87$ μmol h^{-1} g^{-1} fw. and represent the high-affinity system for sucrose in mature leaves of *Vicia faba* (Delrot & Bonnemain 1984). In the next step we examined the effect of phytohormones on the high-affinity system for sucrose.

Table 6. Effects of phytohormones on the apparent kinetic parameters K_m and V_{max} of the high-affinity uptake system for sucrose by leaf discs of *Vicia faba* at up 5 mmol l^{-1} ^{14}C-sucrose in the medium. Conditions were as described in materials and methods. Average data of 10 discs ± SE, three replicates. *Differs significantly from the control ($P \leq 0{\cdot}05$)

Hormone treatment	V_{max} (μmol g f wt.$^{-1}$ h^{-1})	K_m (mmol l^{-1})
Control	4·87 ± 0·02	2·72 ± 0·04
FC (100 μmol l^{-1})	6·32 ± 0·04	2·70 ± 0·06
SSHB (10 μmol l^{-1})	6·49 ± 0·03	2·74 ± 0·04
GA_3 (10 μmol l^{-1})	6·42 ± 0·07	2·70 ± 0·03
IAA (10 μmol l^{-1})	5·87 ± 0·06	2·68 ± 0·05

Brassinosteroids, GA_3 and IAA increased the V_{max} significantly, whereas the K_m was not greatly affected (Table 6). FC and EB were used for comparison. FC is a well-known stimulator of the plasma membrane bound H^+-ATPase (De Michelis *et al.* 1991) and a promoter of sucrose uptake in leaf tissues of *Vicia faba* (Delrot 1981), *Phaseolus vulgaris* (Sturgis & Rubery 1982) and *Egeria densa* (Dahse *et al.* 1990). Erythrosin B has been used in plant tissues as an inhibitor of H^+-ATPase activity *in vitro* and proton extrusion *in vivo* (Cocucci & Marrè 1986; Beffagna & Romani 1988). The effect of FC was in the same order of that of the phytohormones; EB strongly inhibited the sucrose uptake (Table 5).

DISCUSSION

Exogenously applied phytohormones affect assimilate distribution in higher plants (discussed by Patrick 1987) but the underlying mechanisms remained unclear. The present results indicate that phytohormones (SSHB, GA_3, IAA) influence the source-sink transport of assimilates at several levels, some of which are identified here.

Sucrose uptake and phloem loading in leaf tissues are dependent on a plasma membrane proton pump which energizes the proton–sucrose symport (Delrot & Bonnemain 1984; Wimmers & Turgeon 1991). The rapid promotive effect of SSHB, GA_3 and IAA on sucrose uptake in leaf discs (during 60 min) suggests a phytohormone-induced alteration in the kinetic properties of the uptake system. Promoted sucrose uptake (Table 5) correlates with increased V_{max} (Table 6). SSHB, GA_3 and IAA probably increase the motive force for sucrose uptake. This agrees with the proposed interaction of FC, GA_3 and IAA with the plasma membrane proton pump in *Phaseolus* and *Vicia* leaf discs (Sturgis & Rubery 1982; Aloni *et al.* 1988) and in several other plant materials (Kazama & Yamaki 1976; Katsumi & Kazama 1978; Scherer 1984; Santoni *et al.* 1991). The promotion of sucrose uptake by SSHB, GA_3 and IAA is in the same range as the GA_3-stimulation of serine uptake by *Nicotiana tabacum* cells (Smith 1978) and of sucrose uptake by isolated vein networks of *Pisum sativum* leaves (Estruch *et al.* 1989) and by isolated vascular bundles of *Apium graveolens* (Daie *et al.* 1986). The EB-reduction of the FC- and phytohormone-enhanced sucrose uptake is a further argument for the proposed interaction of SSHB, GA_3 and IAA with the proton pumping ATPase. Moreover, electrophysiological data and pH measurements of the external medium indicated the stimulation of an electrogenic proton pump in *Egeria* leaf cells by brassinosteroids (Dahse *et al.* 1991). The additional proton-motive

force was large enough to stimulate the uptake of amino acids and sucrose by the mesophyll during a 1-h incubation period.

Microautoradiography showed that, after 4 h, ^{14}C-label was absorbed to a higher extent by the mesophyll than in the minor veins of *Vicia* source leaves (not shown). Under the same conditions but in the presence of hormones (GA_3, IAA) the mesophyll cells are more densely labelled than the mesophyll of control (U. Petzold, unpublished data). Apparently, the short-term hormone treatment promotes the ^{14}C-sucrose uptake by the mesophyll, whereas the ^{4}C-transfer from the mesophyll to the veins is reduced. Our results agree with observation of Lucas (1985) that all cell types in a leaf disc are capable of sucrose uptake which reflects a general retrieval mechanism by the mesophyll and the phloem.

The question arises why only the mesophyll would benefit from the extra proton-motive force generated by the action of SSHB, GA_3 and IAA. However, the strong effect of IAA on the ^{14}C-sucrose conversion (Table 2) indicates a modified metabolic ^{14}C-partitioning which may prevent sucrose efflux from the mesophyll. Stimulation of the proton extrusion by the phytohormones also promotes the activity of acid invertase (Table 4) which will enhance breakdown of apoplastic sucrose. As se-cc is often unable to absorb glucose and fructose (Giaquinta 1983), a high cell wall-invertase activity would prevent phloem loading and assimilate transport. This may explain the effects of phytohormones on the retention in the mesophyll (Tables 1, 2, 5 and 6) and the retardation of the export (Fig. 1a).

In longer (24 h) studies, SSHB and GA_3 have been shown to alter the distribution of ^{14}C-materials within the whole plant (Fig. 1b). The mechanism for the enhanced export towards the apical region is not clear. In *Vicia* plants, long-term (24 h) exposure to GA_3 of source leaf enhanced growth in the apical sink region (Aloni *et al.* 1986) where sucrose is rapidly utilized (utilization sink, Oparka 1990) after symplastic unloading (Patrick 1990). These experimental data correlated with the observation that ^{14}C-GA_3 was accumulated progressively from 2 to 24 h in young upper stem parts and the apex (Aloni *et al.* 1986). Similar results were obtained after application of ^{3}H-SSHB in whole *Vicia* plants (U. Petzold & G. Adam, unpublished data).

The switch between 4 and 24 h from export-hindrance to export-promotion by SSHB and GA_3 (Figs 1a and b) has not yet been assessed. Under these experimental conditions, SSHB and GA_3 may have moved via the phloem to the apical sink. The promotive effect of locally applied phytohormones (Fig. 2) demonstrates the positive effect of SSHB, GA_3 and IAA on the ^{14}C-transport to the sink. As the mechanism of phloem unloading has not been fully anlaysed, the exact involvement of phytohormones in this process is unclear. It can be stated, however, that the phytohormones induce enhancement of the sink strength with a commensurate effect on phloem unloading. The resulting increased turgor gradient between source and sink is expected to stimulate the phloem loading (Fig. 1b). The contradictory effect of IAA on phloem loading (Fig. 1b) and phloem unloading (Fig. 2) remains to be solved. Under influence of IAA ^{14}C-metabolites may have been sequestered in pools or compartments. Shortage of ^{14}C available for phloem loading may explain the absence of a promotive IAA-effect on ^{14}C-export after 24 h (Fig. 1b).

Mechanisms linking the above factors and processes for control of partitioning will be further investigated in our laboratory.

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