Transport of Resistance – Inducing Sterols in Phloem Sap of Barley[§]

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After root application of [7α - ^3H]- 7β -hydroxysitosterol and [$3\alpha,6\beta$ - $^3\text{H}_2$]- 6α -hydroxylathosterol these sterols could be detected in the leaves and phloem sap feeding aphids. These results imply that the phloem sap is a sterol transport system in barley plants.

Introduction

Plants are affected by a multitude of fungal diseases, which often cause high yield losses in all kinds of crops. Hofferek (1980) reported that spraying of barley plants with a root extract of barley can induce resistance toward the fungal pathogen *Puccinia striiformis* West. The plasma membranes of barley roots have an exceptionally high content of free phytosterols (57 mol% of total lipids in comparison to 7% in the membranes of spinach leaves). Analysis of the sterol composition showed that sitosterol accounts for nearly two thirds of the total amount (Rochester *et al.*, 1987). Therefore it was not surprising that the resistance-inducing compounds of barley roots could be identified as 7α -hydroxysitosterol (**1**), 7β -hydroxysitosterol (**2**) and 7-oxositosterol (**3**) (Schabdach *et al.*, 1995). These oxysterols can only be found in the roots. Stem and leaves of untreated plants have a content below detection level of GC and GC-MS coupling. Their metabolism in barley roots has also been investigated (Vesper, 1998).

Fungal resistance was induced after spraying the leaves with 7-oxysitosterols. After leaf application of [$3\alpha,7\alpha$ - $^2\text{H}_2$]- 7β -hydroxysitosterol (**6**) and [3α - ^2H]-7-oxositosterol (**7**) these sterols could never be detected in the roots. After root application the two 7-oxysitosterols were found in the leaves (König and Seifert, 1998). The 7-oxysitosterols ap-

plied via the roots should be transported through the xylem into leaves. The question is, whether oxysterols occur also in the phloem? In this publication we report on the analysis of phloem sap of barley collected by means of aphids. For example, this method has been used before for the detection of the indolizidine alkaloid swainsonine in honeydew excreted by phloem selective pea aphids (Dreyer *et al.*, 1985). Instead of collecting the phloem exudate we have extracted and analysed the aphids. The aphid species used, *Sitobion avenae* Fabr., is a wide-spread herbivorous insect on grasses. It is a selective phloem feeder and commonly used for sampling pure phloem sap via the exuding stylet technique (Fisher and Frame, 1984). This practice of severing aphid stylets is often used to study phloem physiology (Barlow and McCully, 1972). In some cases other phloem-selective insects are also used, as for example leafhoppers and planthoppers on rice (Kawabe *et al.*, 1980).

Results and Discussion

After root application of 7-oxositosterol (**3**, 300 $\mu\text{g/ml}$) for 5 days the sterol could be detected by GC-MS in the roots and leaves of barley but not in the phloem sap feeding aphids. When the roots of barley plants were immersed for 2 days in a 1% aqu. ethanolic solution of [$3\alpha,7\alpha$ - $^2\text{H}_2$]- 7β -hydroxysitosterol (**6**) or [3α - ^2H]-7-oxositosterol (**7**) (100 $\mu\text{g/ml}$ per compound), 0.33 ± 0.04 $\mu\text{g/g}$ fr. wt of **6** or 0.67 ± 0.08 $\mu\text{g/g}$ fr. wt of **7** were found in the leaves (König and Seifert, 1998). In the experiment with the aphids, the concentration of 7-oxosi-

[§] Part 2 in the series “7-Oxysterol induced resistance towards rusts in barley”.

tosterol in the phloem sap was too low to detect **3** in the aphids. If the roots of barley plants were cut off and the leaves were immersed for 9 days in a 0.5% aqu. ethanolic solution of **3** (300 µg/ml), 7-oxositosterol could be identified by GC-MS in the leaves and in the aphids. Since the aphids used are selective phloem feeders, 7-oxositosterol is a phloem-mobile compound.

After application of [7α - ^3H]- 7β -hydroxysitosterol (**4**, 50 µg/ml) and [$3\alpha,6\beta$ - $^3\text{H}_2$]- 6α -hydroxylathosterol (**5**, 150 µg/ml) the radioactivity increased in the roots (**4**, 47.7 Bq/mg dry wt; **5**, 5432 Bq/mg dry wt), leaves (**4**, 4.6 Bq/mg dry wt; **5**, 267 Bq/mg dry wt) and aphids (**4**, 0.25 Bq/mg dry wt; **5**, 11.3 Bq/mg dry wt) (Table I). The concentration of [$3\alpha,7\alpha$ - $^2\text{H}_2$]- 7β -hydroxysitosterol (**6**) after exogenous application to the leaves was determined three days (0.39 ± 0.03 µg/g fr. wt) and for weeks (0.36 ± 0.04 µg/g fr. wt) after application (König and Seifert, 1998). The concentrations indicate the absence of any metabolization and deuterium-hydrogen exchange of **6** in the leaves. Therefore it is no reason to assume that the tritium of [7α - ^3H]- 7β -hydroxysitosterol (**4**) or [$3\alpha,6\beta$ - $^3\text{H}_2$]- 6α -hydroxylathosterol (**5**) was completely transferred to other compounds and the detection of tritium in the aphids is based on artifacts.

The application of radiolabelled sterols can prove the downstream transport of sterols together with photosynthates. The aphids on the barley leaf surface incorporated radioactivity by feeding for 9 days (**4**) or 7 days (**5**) of the phloem sap containing the tritiated sterols **4** or **5**. It is possible to calculate the amount of sterol which was absorbed by a single aphid. Aphids in the first group (50 µg/ml of **4**) contained a mean of 0.44 ± 0.02 ng of sterol and aphids of the second group (150 µg/ml of **5**) contained 1.02 ± 0.37 ng. The oxysterol concentration in the phloem sap could be estimated as follows: Unattended aphid species excrete between 20 and 40 nl honeydew per aphid and hour (Heimbach, 1986; Völkl *et al.*, 1999; Fischer *et al.*, 2000). A similar honeydew production can be assumed for *Sitobion avenae* resulting in 480 – 960 nl honeydew per aphid and day. Assuming a value of 720 nl per aphid and day, an aphid of the first group fed approximately 6480 nl (9 days) and of the second group 5040 nl (7 days). The concentrations of **4** and **5** in the phloem sap of barley seedlings should be about 68 ng/ml and 202 ng/ml.

The results are very consistent considering the different sterol concentrations. Our experiments demonstrate that small amounts of oxysterols are

Table I. Radioactivity of barley plants and aphids after root application of [7α - ^3H]- 7β -hydroxysitosterol (**4**) and [$3\alpha,6\beta$ - $^3\text{H}_2$]- 6α -hydroxylathosterol (**5**).

2 independent experiments; controls: experiments without **4** and **5**.

	Radioactivity [Bq/mg dry wt]	Concentration [µg/mg dry wt]	Number of Aphids
Compound		4	
Roots	47.7	0.71	300
Leaves	4.6	0.069	
Aphids	0.25	0.0037	
Roots control	0.005		200
Leaves control	0.002		
Aphids control	0.002		
Compound		5	
Roots	5432	4.28	40
Leaves	267	0.21	
Aphids	11.3	0.0089	
Roots control	0.13		40
Leaves control	0.01		
Aphids control	0.22		

1 mg dry wt of aphids = 8.38 aphids, **4**.

1 mg dry wt of aphids = 8.69 aphids, **5**.

Estimated concentration of **4** in the phloem: 68 ng/ml.

Estimated concentration of **5** in the phloem: 202 ng/ml.

Experimental

Seeds of barley (*Hordeum vulgare* L. cv "Mammut") were germinated on moist filter paper and after germination transferred onto perforated polystyrene plates. The seedlings were grown hydroponically in Münchner/2 solution (3 mM KNO₃, 2 mM Ca(NO₃)₂, 4 mM MgCl₂, 0.5 mM KH₂PO₄, 0.06 mM EDTA-Fe(III)-Na salt, 0.023 mM H₃BO₃, 0.005 mM MnCl₂, 0.4 µM ZnSO₄, 0.2 µM CuSO₄, 0.05 µM Na₂MoO₄) for two weeks.

Treatment with 7-oxositosterol (3)

The nutrient solution of barley plants consisted of Münchner/2 solution containing 0.25% of detergent (Palmolive®), 0.5% of EtOH, 300 µg/ml of **3** and was prepared as follows: 7-Oxositosterol (**3**) was dissolved in EtOH and diluted with the detergent containing Münchner/2 solution. The roots of the seedlings were placed in the Münchner/2 solution. After the nutrient solution was consumed (approximately 1 ml/day per plant), fresh solution was added. 40 adult aphids were put on each group of 15 plants at the same day as the sterol was applied. Plants were kept in a closed insect cage for 5 days. At the end, aphids were collected, frozen in liquid N₂ and dried at 60° for 2 days. The barley plants were separated in root- and shoot-parts and also dried at 60° for 2 days. Controls were grown in the same nutrient solution as described above but without **3**.

Barley plants, grown in potting soil for two weeks, were cut off under water just above the roots. The water contained 0.01% detergent to avoid air-bubbles which could plug the xylem vessels. The shoot parts were placed in the above described Münchner/2 nutrient solution which contained 300 µg/ml of **3**. The level of solution was maintained constant during the whole experiment. 30 adult aphids were put on each group of 15 plants at the same day as the sterol was applied. After 9 days the experiment was finished and the aphids and barley plants were treated as described above.

Aphids and plants were ground to a fine powder with mortar and pestle and were extracted four times with CHCl₃. The CHCl₃ extracts were evaporated and the residues purified by TLC (20 × 20 cm silica plates, cyclohexane/EtOAc 1:1 v/v). The bands with the R_f 0.45–0.50 were scraped off and

eluted with CHCl₃. The combined eluates were evaporated to dryness under reduced pressure. Samples were trimethylsilylated with N-methyl-N-trimethylsilyltrifluoroacetamide for 12 h at room temp. and analysed by GC and GC-MS. 3-O-trimethylsilyl-7-oxositosterol was identified by comparison of R_T and MS with the reference compound.

Capillary GC measurements were carried out under the following conditions: Fused silica-glass column DB1 (J&W Scientific, 29 m × 0.32 mm, 0.1 µm film thickness); injector temp. 280°; column temp. 3 min 80°, heat rate 3° min⁻¹ to 280°; FID temp. 290°; carrier gas H₂, flow rate 2 ml min⁻¹, split ratio 1:20. GC-MS measurements were performed on an instrument coupled to a GC fitted with a fused silica-glass column DB-5 (30 m × 0.32 mm, 0.1 µm film thickness); inj. temp. 280°; carrier gas H₂, flow rate 2 ml min⁻¹, splitless injection.

3-O-Trimethylsilyl-7-oxositosterol

R_T (GC, DB1) 28.93; MS *m/z* (rel. int.): 500 (M⁺, 65), 485 (12), 444 (6), 410 (18), 395 (26), 281 (34), 207 (100), 129 (15).

Treatment with [7α-³H]-7β-hydroxysitosterol (4) and [3α,6β-³H₂]-6α-hydroxylathosterol (5)

The ³H-labelled sterols (Schröder, 1995) were purified with prep. TLC (cyclohexane-EtOAc 1:1 v/v). A 50 µg/ml solution of **4** (specific radioactivity 67 kBq/mg) consisted of 0.5% EtOH and 0.1% detergent (Palmolive®) in Münchner/2 solution. A 150 µg/ml solution of **5** (specific radioactivity 1.27 MBq/mg) consisted of 0.5% EtOH and 0.25% detergent in Münchner/2 solution. Control plants were nourished with Münchner/2 solution containing 0.5% EtOH and 0.1% detergent. The roots of 2-week-old barley plants were placed in the appropriate solution, ensuring that the leaves had no contact with the solution. After the radioactive labelled sterol solution was completely consumed, fresh solution with the same unlabelled sterol in the same concentration was added (approximately 1 ml/day per plant). This was continued until the end of the experiment (**4**, 9 days; **5**, 7 days). Aphids and plants were harvested and dried as described above.

Dried aphids and plant parts were weighed and wrapped in ash-free filter-paper. The samples were oxidized in a sample oxidizer which collected the total combustion water. The tritium activity was counted in a liquid scintillation counter. A Packard Sample Oxidizer Model 307 was used according to the manual with a flow setting of 18 ml of monophase S and 2 ml of permafluor E. N₂ and

O₂ pressure were set at 5 bar and the carburization time was 45 sec. The yield of tritiated water was determined by combustion of known standards (on filter-paper as well). The tritium content was counted with a Packard Liquid Scintillation Analyzer 2500 TR. Counting time was 10 min, with samples kept in 30 ml HD-PE scintillation tubes.

- Barlow C. A. and McCully M. E. (1972), The ruby laser as an instrument for cutting the stylets of feeding aphids. *Canad. J. Zoology* **50**, 1497–1498.
- Behmer S. T. and Grebenok, R. J. (1998), Impact of dietary sterols on life-history traits of a caterpillar. *Physiol. Entomol.* **23**, 165–175.
- Brown D. J. and DuPont F. M. (1989), Lipid composition of plasma membranes and endomembranes prepared from roots of barley. *Plant Physiol.* **90**, 955–961.
- Carvajal M., Cooke D. T. and Clarkson D. T. (1996), Responses of wheat plants to nutrient deprivation may involve the regulation of water-channel function. *Planta* **199**, 372–381.
- Dreyer D. L., Jones K. C. and Molyneux R. J. (1985), Feeding deterrence of some pyrrolizidine, indolizidine, and quinolizidine alkaloids towards pea aphids (*Acyrtosiphon pisum*) and evidence for phloem transport of indolizidine alkaloid swainsonine. *J. Chem. Ecol.* **11**, 1045–1051.
- Fischer M., Völkl W. and Hoffmann K. H. (2000), Interspecific variations in aphid honeydew feeding on tansy: impact of the mutualism with ants. Poster, International Congress of Entomology, 20. – 26. August 2000, Iguacu, Brasilia.
- Fisher D. B. and Frame J. M. (1984), A guide to the use of the exuding-stylet technique in phloem physiology. *Planta* **161**, 385–393.
- Heimbach U. (1986), Freilanduntersuchungen zur Honigtauabgabe zweier Zierlausarten (Aphidina). *J. Appl. Entomol.* **101**, 396–413.
- Hofferek H. (1980), Oxidoreduktase-Aktivitäten und Metabolitenkonzentrationen in Gerste nach Infektion mit *Puccinia striiformis* West.. *Biochem. Physiol. Pflanz.* **175**, 499–515.
- Holfelder M. G. A. H., Steck M., Komor E. and Seifert K. (1998), Ricinine in phloem sap of *Ricinus communis*. *Phytochemistry* **47**, 1461–1463.
- Kawabe S., Fukumori T. and Chino M. (1980), Collection of rice phloem sap from stylets of homopterous insects severed by YAG laser. *Plant and Cell Physiol.* **21**, 1319–1327.
- Kieckhefer R. W. (1984), Cereal aphid (Homoptera: Aphididae) preference for and reproduction on warm-season grasses. *Environm. Entomol.* **13**, 888–891.
- Kieckhefer R. W. and Gellner J. L. (1988), Influence of plant growth stage on cereal aphid reproduction. *Crop Sci.* **28**, 688–690.
- Kitamura Y., Matuo N., Takashi T. and Miura H. (1991), The characteristics of atropine transport in seedlings and regenerated plantlets of *Duboisia myoporoides*. *J. Plant Physiol.* **137**, 613–618.
- Kitamura Y., Yamashita R., Miura H. and Watanabe M. (1993), Phloem transport of tropane and pyridine alkaloids in *Duboisia myoporoides*. *J. Plant Physiol.* **142**, 635–637.
- König M. (1997), Resistenzinduktion gegen Rostpilze bei Getreide durch oxidierte Sterole. Dissertation, University of Bayreuth, Germany.
- König M. and Seifert K. (1998), Uptake and translocation of exogenously applied 7-oxysterols in barley. *Phytochemistry* **49**, 1257–1263.
- Navari-Izzo F. and Izzo R. (1984), *In vivo* conversion of [4-¹⁴C]sitosterol and [22,23-³H]sitosterol to stigmasterol in barley seedlings. *Phytochemistry* **23**, 769–772.
- Orlich G. and Komor E. (1989), Methods in Enzymology, Transport in Plants, Phloem Transport. Academic Press, Inc., San Diego, New York, Berkeley, Boston, London, Sydney, Tokyo, Toronto, Vol. 174, 288–312.
- Rochester C. P., Kjellbom P., Andersson B. and Larsson C. (1987), Lipid composition of plasma membranes isolated from light-grown barley (*Hordeum vulgare*) leaves: Identification of cerebroside as a major component. *Arch. Biochem. Biophys.* **255**, 385–391.
- Rochester C. P., Kjellbom P. and Larsson C. (1987), Lipid composition of plasma membranes from barley leaves and roots, spinach leaves and cauliflower inflorescences. *Physiol. Plant.* **71**, 257–263.
- Schabdach H., John S., Steiner U. and Seifert K. (1995), Plant disease resistance inducing activity of 7-oxo- and 7-hydroxysterols. *Z. Naturforsch.* **50c**, 257–262.
- Schröder J. (1995), Synthese von Tritium-markierten Verbindungen des Steroidtyps. Diploma Thesis, University of Bayreuth, Germany.
- Vesper T. (1998), Metabolismus, Transport und Wirkungsweise Resistenz induzierender Sterole. Dissertation, University of Bayreuth, Germany.
- Völkl W., Woodring J., Fischer M., Lorenz M. W. and Hoffmann K. H. (1999), Ant-aphid mutualisms: the impact of honeydew production and honeydew sugar composition on ant preferences. *Oecologia* **118**, 483–491.
- Wink M. and Witte L. (1984), Turnover and transport of quinolizidine alkaloids. Diurnal fluctuations of lupanine in the phloem sap, leaves and fruits of *Lupinus albus* L. *Planta* **161**, 519.