

Andrew Richardson · Rochus Franke · Gerhard Kerstiens
Mike Jarvis · Lukas Schreiber · Wieland Fricke

Cuticular wax deposition in growing barley (*Hordeum vulgare*) leaves commences in relation to the point of emergence of epidermal cells from the sheaths of older leaves

Received: 11 February 2005 / Accepted: 15 March 2005 / Published online: 7 June 2005
© Springer-Verlag 2005

Abstract In grasses, leaf cells divide and expand within the sheaths of older leaves, where the micro-environment differs from the open atmosphere. By the time epidermal cells are displaced into the atmosphere, they must have a functional cuticle to minimize uncontrolled water loss. In the present study, gas chromatography and scanning electron microscopy were used to follow cuticular wax deposition along the growing leaf three of barley (*Hordeum vulgare* L.). 1-Hexacosanol (C₂₆ alcohol) comprised more than 75% of extractable cuticular wax and was used as a marker for wax deposition. There was no detectable wax along the first 20 mm from the point of leaf insertion. Deposition started within the distal portion of the elongation zone (23–45 mm) and continued beyond the point of leaf emergence from the sheath of leaf two. The region where wax deposition commenced shifted towards more proximal (basal) positions when the point of leaf emergence was lowered by stripping back part of the sheath. When relative humidity in the shoot environment was elevated from 70% (standard growth conditions) to 92–96% for up to 4 days prior to analysis, wax deposition did not change

significantly. The results show that cuticular waxes are deposited along the growing grass leaf independent of cell age or developmental stage. Instead, the reference point for wax deposition appears to be the point of emergence of cells into the atmosphere. The possibility of changes in relative humidity between enclosed and emerged leaf regions triggering wax deposition is discussed.

Keywords Cell development · Cuticle · *Hordeum* · Leaf growth · Relative humidity · Wax

Abbreviations FID: Flame ionization detector · GC: Gas chromatography · LEV: Leaf elongation velocity · MS: Mass spectrometry · POE: Point of emergence · REGR: Relative elemental growth rate · RH: Relative humidity · SEM: Scanning electron microscopy

Introduction

The aerial surfaces of land plants are protected by an outer barrier of lipid compounds known as the cuticle. The cuticle is primarily composed of cutin, a polyester matrix of hydroxy and hydroxy epoxy C₁₆ and C₁₈ fatty acids (Kolattukudy 1996), which is overlaid and embedded with long chain hydrocarbon waxes such as primary and secondary alcohols, aldehydes, alkanes, ketones and fatty acids (Kunst and Samuels 2003). The functions of these surface waxes include: (1) to prevent uncontrolled water loss (or uptake) through the epidermis (e.g. Kerstiens 1996a; Riederer and Schreiber 2001; Ristic and Jenks 2002; Oliveira et al. 2003), (2) to protect against ultraviolet radiation (Barnes et al. 1996; Long et al. 2003), (3) to defend against attack by pathogens (e.g. Jenks et al. 1994; Kerstiens 1996b; Mariani and Wolters-Arts 2000) and insects (e.g. Eigenbrode 1996), and (4) to reduce water retention on the plant surfaces by controlling surface wettability (Barnes et al. 1996; Barthlott and Neinhuis 1997; Beattie and Marcell 2002).

A. Richardson (✉) · W. Fricke
Division of Biological Sciences,
University of Paisley, Paisley, PA1 2BE, UK
E-mail: rich-se0@wpmail.paisley.ac.uk
Tel.: +44-141-8483502
Fax: +44-141-8483663

R. Franke · L. Schreiber
Department of Ecophysiology, IZMB,
University of Bonn, 53115 Bonn, Germany

G. Kerstiens
Lancaster Environment Centre,
Department of Biological Sciences,
Lancaster University,
Lancaster, LA1 4YQ, UK

M. Jarvis
Department of Chemistry,
University of Glasgow, Glasgow, G12 8QQ, UK

Mutants in putative wax biosynthetic enzymes showed that surface waxes maybe also have a role in the control of plant organ development (Yephremov et al. 1999; Bird and Gray 2003) and pollen-stigma interactions (Bianchi et al. 1990; Preuss et al. 1993).

Monocotyledonous plants, such as grasses, differ fundamentally from dicotyledonous plants in the way their leaves expand and grow. This has implications for cuticle development. In dicot plants, leaves are enclosed in buds or are folded or rolled up at their earliest developmental stages. Increase in leaf surface area is achieved through significant expansion of cells in both longitudinal and lateral directions, giving dicot leaves their typical broad-shaped appearance. As leaf area expansion occurs in the open atmosphere, a (comparatively) dry environment, cuticular waxes must be deposited continually and in parallel to cell expansion.

In contrast, in monocotyledonous plants, increase in leaf area is largely due to longitudinal expansion of cells within a 'sheltered' environment created by sheaths of older leaves (Fig. 1a). Cells are produced by an intercalary meristem close to the point of leaf insertion (base of leaf). The newly produced cells elongate within a zone that stretches to about 20–60 mm from the point of leaf insertion, depending on species and leaf number (Palmer et al. 1996; reviewed in Fricke 2002). As cells exit the elongation zone, they continue some lateral expansion before emerging from encircling sheaths of older leaves into the comparatively dry atmosphere. At that stage, cuticular waxes must be sufficiently synthesized and deposited to fulfill their function(s).

The process of cuticular wax deposition and leaf development has been widely studied in dicots (see recent reviews by Bird and Gray 2003; Kunst and Samuels 2003). However, little information is available for the monocots with only one study of leek (*Allium porrum*), having been conducted (Rhee et al. 1998), and none for grasses. It is not known (1) when during grass leaf epidermal cell development waxes are deposited; (2) whether wax deposition is completed by the time cells emerge into the dry atmosphere or whether it continues post-emergence; (3) whether cuticular wax deposition in grass leaves commences in a cell-age or position dependent manner or in response to any particular environmental signal.

In the present study, we have addressed the above questions and provided for the first time information on cuticular wax deposition in grass leaves. Gas chromatography was used, together with scanning electron microscopy (SEM) analyses, to follow the deposition of cuticular wax along the growing leaf three of barley. The hypothesis was tested to prove that a gradient in relative humidity, RH, between emerged (low RH) and enclosed leaf portion (RH near 100%) acts as a signal for the commencement of cuticular wax deposition. By peeling back sheaths of older leaves, it was tested whether wax deposition in the developing leaf three of barley is related to cell age or position (e.g. set distance from leaf base) or to the point where cells emerge into the dry atmosphere (point of emergence, POE).

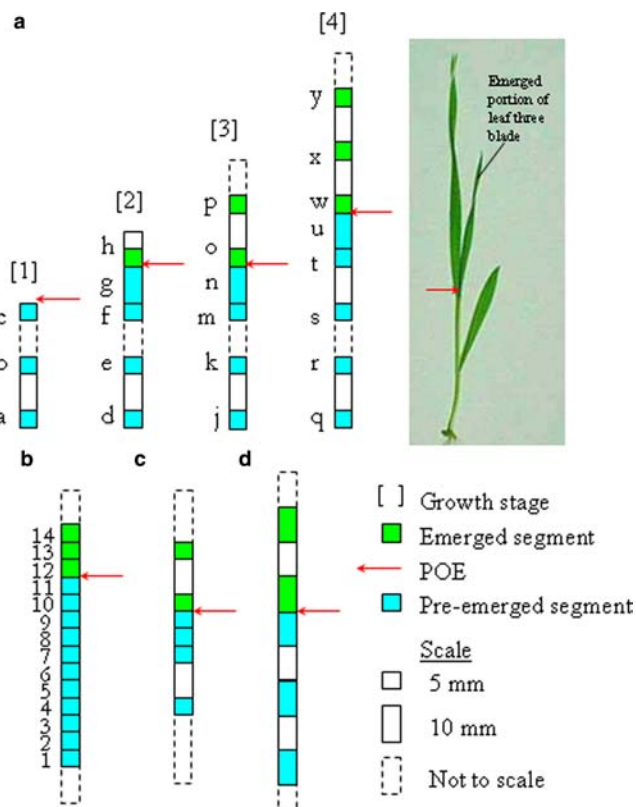


Fig. 1 Location of leaf segments analysed in experiments 1–3. **a** Experiment 1: 5 or 10 mm segments collected from barley leaf three at four growth stages (pre-emerged (stage 1), 1 cm (stage 2), 5 cm (stage 3), and 10 cm (stage 4) emerged). Segments *a*, *d*, *j*, and *q* were taken 2–5 mm from the leaf base, while *b*, *e*, *k* and *r* were taken 25–30 mm from the leaf base. All other segments were taken using the point of emergence (POE) of leaf three from the sheath of leaf two as the reference point for measurement. Schematic of barley plant at growth stage 4. **b** Experiment 2: 3 mm segments collected from barley leaf three at growth stage 3. Segments were taken using the POE as the reference point for measurement. **c** Experiment 3: 5 mm segments collected from barley leaf three at growth stage 3. Segments were taken using the POE as the reference point for measurement. **d** Experiment 3: 10 mm segments collected from barley leaf three at growth stage 3. Segments were taken using the POE as the reference point for measurement. All segments collected in triplicate, each comprising samples from three individual plants except **b** (comprising samples from five individual plants)

Materials and methods

Plant growth

Seeds of barley (*Hordeum vulgare* L. cv. Golf; Svalöf Weibull AB, Svalöv, Sweden) were imbibed overnight in water, germinated in the dark over 0.5 mM CaSO₄ for 3 days and exposed to light for a further 3 days while remaining on CaSO₄ solution. Seedlings were transferred on modified, aerated Hoagland solution (macronutrients: 0.5 mM MgSO₄, 2 mM Ca(NO₃)₂, 2 mM KNO₃, 0.5 mM NH₄H₂PO₄, 0.5 mM (NH₄)₂HPO₄ and 0.5 mM NaCl; micronutrients: 6.25 μM H₃BO₃, 0.125 μM CuSO₄, 0.5 μM MnSO₄, 0.4 μM ZnCl₂,

0.19 μM Na_2MoO_4 and 18 μM FeNaEDTA), which was contained in 1 l glass beakers wrapped in tin foil (four plants per beaker). Plants were grown in a growth chamber (Snijders Scientific Microclima 1000) at 70% relative humidity (RH) and 350–400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation at third-leaf level, during a 16 h, 21°C day–8 h, 15°C night cycle. Plants were analyzed at four different stages of development of the growing leaf three. Leaf three was analyzed either just before emergence from the sheath of leaf two (growth stage 1) or following the emergence (growth stages 2–4) (for details, see Fig. 1a).

Plant harvest and experimental design

The aim of experiments was to pin-point at millimeter-resolution the gradient of cuticular wax formation along the elongating leaf three. To avoid interference of differences between plant batches in interpretation of data, each type of experiment was conducted on a separate batch of plants. Typically, 50–150 plants were grown in parallel, and only those plants with a leaf three length within ± 5 mm of the desired growth stage were selected for analyses. Leaf three was separated from the surrounding sheath tissues of leaf one and two and from the younger leaf four, which developed enclosed by leaf three. The desired segments of leaf three were cut at various positions along the blade using razor blades which were mounted at fixed distances. In experiments 1, 3 and 4, corresponding segments from three plants were pooled to give one sample, and three replicate samples were prepared (i.e. 3×3 plants). In experiment 2, segments from five plants were pooled, and three replicate samples were prepared (i.e. 3×5 plants). In experiment 3, for each batch, segments from four plants were pooled, and three replicate samples were prepared (i.e. 3×4 plants). Prior to pooling segments, the exact length of each segment was measured using a micrometer to enable the calculation of leaf surface area. All samples were dried and stored over silica gel in micro-centrifuge tubes which had the center of the cap removed and replaced with fine nylon mesh.

Experiment 1 A combination of 5 and 10 mm long segments were cut from plants at growth stages 1–4 and labeled a–y (see Fig. 1a). Segments a, d, j, and q were taken 2–5 mm from the leaf base, while b, e, k and r were taken 25–30 mm from the leaf base. All other segments were taken using the point of emergence (POE) of leaf three from the sheath of leaf two as the reference point for measurement.

Experiment 2 A series of 3 mm long segments were taken from plants at growth stage 3 and labeled 1–14 (see Fig. 1b). Eleven segments were taken below the POE of leaf three from the sheath of leaf two and three segments were taken from above the POE.

Experiment 3 A series of segments were taken from three batches of plants at growth stage 3 or 4 that had

been grown at elevated relative humidity for 2–4 days prior to sampling. Elevated humidity was achieved through covering shoots with a perforated polyethylene bag. For the harvest, plants were removed individually from bags and quickly sectioned. For each batch an identical series of segments were taken from control, non-bagged plants. For batch (i), plants were analysed at growth stage 3, 3 days post-covering, and four 5 mm-long segments were taken along leaf three below the POE from the sheath of leaf 2, and two 5 mm long segments were taken from above the POE (see Fig. 1c). Control plants were grown at the chamber relative humidity setting (70%). For batch (ii), the growth chamber fan speed was reduced by 75% from that used for batch (i) to reduce potential RH removing airflow through the polythene bags. Plants were analysed at growth stage 3, 4 days post-covering, and three 10 mm-long segments were taken along leaf three below the POE from the sheath of leaf 2 and two 10 mm long segments were taken from above the POE (see Fig. 1d). Control plants were grown at the chamber relative humidity setting (70%). For batch (iii), plants were grown outside the growth chamber, at ambient laboratory temperature and at reduced PAR (250–300 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and further-reduced air flow. Plants were analysed at growth stage 4, 2 days post covering, and three 10 mm long segments were taken along leaf three below the POE from the sheath of leaf 2, and two 10 mm-long segments were taken from above the POE (see Fig. 1d). Control plants were grown at ambient laboratory RH of 55%.

Experiment 4 Plants at growth stage 4 were experimentally manipulated by lowering the POE through stripping back the sheath of leaf two by 30 mm (see Fig. 2). One 5 mm segment was collected above the new POE at 3.5 or 7 hours post-stripping. In addition, a control sample (time zero) was taken from separate plants immediately after stripping.

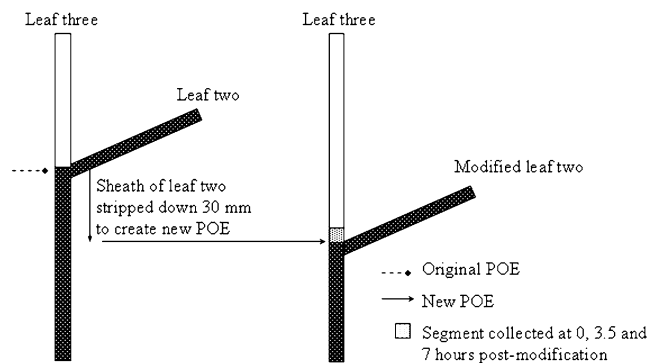


Fig. 2 Location of leaf segments collected for experiment 4. 5 mm segments were collected from barley leaf three at growth stage 3. Segments were taken using the new POE as the reference point for measurement at 0, 3.5 and 7 h post-modification of barley leaf two sheath. Segments were collected in triplicate, each comprising samples from three individual plants

Determination of relative humidity

Standard relative humidity (70% RH) during growth was controlled by the growth chamber. Relative humidity within bags during RH-experiments was measured using a Digitron 2020R Thermohygrometer (Sifam Instruments, Torquay, UK). Briefly, a small slit was made in the bag and the probe (diameter ca 2 cm) was gently introduced. The slit was then held around the probe to create a seal while the RH reading was taken (approx 30 seconds duration).

Determination of segment surface area

To relate wax amounts to leaf surface area, the length of segments and the cross-sectional width of upper and lower leaf surfaces of segments had to be known. Multiple hand-cut cross-sections were taken from within each of the segments a–y, from plants at growth stages 1 to 4 (Fig. 1a). Six plants were sectioned at each growth stage. Cut sections were placed in a droplet of water on a microscope slide, covered with a cover slip and photographed using a Zeiss optical microscope and MC63 photo micrographic camera against a 1 mm-scale. The resulting images were scanned, converted to TIFF files and the upper and lower cross-sectional widths determined using software package Image 4.0.2 for Windows, downloaded as freeware from <http://www.scioncorp.com> (O'Neal et al. 2002). For each section the sum of the upper and lower cross-sectional widths was calculated. Corresponding values were averaged to obtain the mean cross-sectional leaf width at that particular segment location.

Experiment 1 formed the basis of all other experiments; leaf surface area was determined directly for each respective segment and growth stage via length multiplied by total width. For experiments 2, 3 and 4, in which growth stages 3 and 4 were analyzed, the cross-sectional width of segments that were located at positions not analyzed in experiment 1 was obtained by extrapolation from plots of average total cross-sectional width against distance from POE (data not shown).

Leaf elongation and relative elemental growth rates (REGR)

Elongation velocity of the growing leaf three was calculated by the increase in leaf length, measured with a ruler over a 24 h period. Velocities at growth stage 1 could not be determined as the leaf had not yet emerged. The location and distribution of REGR and the length of the elongation zone of leaf three was determined at growth stages 3 and 4 by the pin-pricking method (Schneider et al. 1987; Fricke and Peters 2002). Pricking reduced elongation velocity of leaf three by 45 and 52%, respectively, and this reduction was corrected for when calculating REGR profiles.

Cuticular wax extraction and analysis

Leaf segments were individually immersed in 1,000 μ l chloroform (CHCl_3) for 10 s at room temperature. During preliminary studies, segments were dipped sequentially for 1 s, then 5 s, then 10 s. Extraction for 10 s proved to be the optimal dipping duration to ensure that the majority of surface cuticular waxes were extracted without co-extraction of contaminating internal lipid compounds from the cut edges of the segments. (Schreiber and Schönherr 1993). The resulting extract of cuticular waxes was spiked with 5 μ g of tetracosane as an internal standard for quantifying individual wax components. After evaporation of the chloroform at 55°C under a stream of nitrogen, compounds containing free hydroxyl and carboxyl groups were derivatized to their TMSi ethers and esters with 20 μ l *N*-*O*-bis-trimethylsilyltrifluoroacetamide(e) (BSTFA) (Machery-Nagel, Germany) and 20 μ l dry pyridine for 40 min at 70°C. Prior to analysis, the reaction mixture was diluted with 100 μ l CHCl_3 .

Cuticular wax compounds were quantified after capillary gas chromatography using an Agilent 8690 N gas chromatograph (GC) equipped with an on-column injector and flame ionization detector (FID) (Agilent Technologies, Böblingen, Germany). DB-1 columns (30 m \times 0.32 mm \times 0.1 μ m) (JW Scientific, Agilent Technologies) and hydrogen as a carrier gas (2 ml min⁻¹) were used. A sample volume of 1 μ l was injected per run and wax compounds were separated with the following temperature parameters: injection at 50°C, 2 min at 50°C, 40°C min⁻¹ up to 200°C, 2 min at 200°C, 3°C min⁻¹ up to 310°C, then 30 min at 310°C.

Cuticular wax compounds were identified by combined GC and mass spectrometry (MS) using an identical gas chromatograph equipped with an Agilent 5973 N quadropole mass spectrometer (Agilent Technologies, Böblingen, Germany). The column and temperature parameters were as per the GC-FID. The carrier gas was helium at a rate of 2 ml min⁻¹. Compounds were identified from their EI-MS spectra (70 eV, *m/z* 50–700) using a mass spectral library created in-house.

Scanning electron microscopy (SEM)

A series of 3 mm long segments were taken that corresponded to positions 1, 4, 7, 11, 12 and 14 of experiment 2 (plant growth stage 3) (see Fig. 1b). Segments were cut longitudinally into two halves at the midrib, mounted on SEM stubs with double-sided carbon tape (one half with adaxial and the other with abaxial surface uppermost) and dried over silica gel. Dried sections were gold-coated for 1 min using an M-scape gold sputter coater. Surface images were collected at 1, 3 or 25 K magnification using a Hitachi s-4100 field emission SEM coupled with an Oxford Instruments Gem detector at a working distance of 10 mm and 7 keV accelerating voltage.

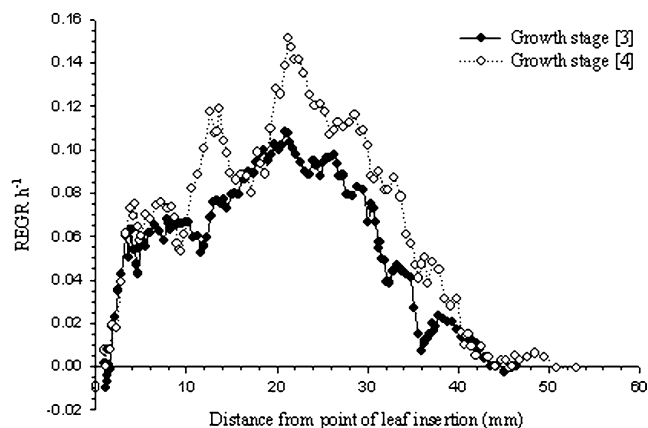


Fig. 3 Distribution of growth along the elongation zone of the barley leaf three at two growth stages. Relative elemental growth rates (REGR) plotted against distance from point of leaf insertion (base of leaf). REGR was corrected for decrease in leaf elongation due to pricking. Moving averages are shown (of five successive points). Plants analyzed at growth stages 3 and 4. Fourteen and ten plants were analysed for growth stages 3 and 4 resulting in a total number of 140 and 100 individual data points, respectively

Statistics

All data presented are means \pm standard deviation. Significance of differences between treatments was tested by t-test (Excel). For calculation of C_{26} deposition rates and wax doubling time, standard deviations of propagated errors were calculated using Gauss' Law.

Results

Leaf growth and development

The developing leaf three was analyzed at four different growth stages (1–4) (Fig. 1a). At each growth stage, leaf elongation was mainly due to elongation of the blade (rather than sheath), and all data apply to this tissue. At growth stage 1, leaf three had not emerged yet from the encircling sheath of leaf two; at growth stage 2, leaf three had emerged 1 cm. To see (1) how far the elongation zone reached from the point of leaf insertion and (2) where cells elongated at highest or lowest relative rates (expressed in percentage per hour), spatial profiles of relative elemental growth rate (REGR) were determined. Profiles were determined only for growth stages 3 and 4; at growth stages 1 and 2, leaf three was too thin at the base and the pricking approach caused too high a reduction in leaf elongation velocity (LEV). At growth stages 3 and 4 leaf three had emerged by 5 and 10 cm, respectively, and elongated at velocities of $1.37 \pm 0.21 \text{ mm h}^{-1}$ and $2.01 \pm 0.12 \text{ mm h}^{-1}$, respectively, over a 16 h/8 h day/night period. The length of the elongation zone and the profile of REGR at these two growth stages were similar: cells elongated up to 45 mm from the point of leaf insertion, and REGRs

were the highest halfway along the elongation zone (Fig. 3). Some lateral expansion of cells continued past the point where cell elongation ceased, as evidenced by a continued increase in cross-sectional width (not shown).

Analysis of epicuticular wax deposition

Initial GC-FID analysis indicated that the barley CHCl_3 -extractable cuticular wax was composed principally ($79.1 \pm 8.1\%$; means \pm SD, $n=17$) of one compound (amount of compound expressed as percentage of total extractable wax). GC-MS analysis identified this principal compound to be 1-hexacosanol (C_{26} alcohol) from the molecular mass of the TMS-derivative at m/z 454 and the diagnostic fragment ions m/z 439 ($\text{M}^+ - \text{CH}_3$) (with a relative abundance of 100); m/z 103 ($\text{CH}_2\text{OSi}(\text{CH}_3)_3$) (relative abundance 8); and m/z 75 ($\text{OSi}(\text{CH}_3)_2$) (relative abundance 17). Other compounds including *n*-alkanols, *n*-alkanes, *n*-alkanoic acids, *n*-alkanals and *n*-alkyl esters were also present (Table 1). For all experiments GC-FID was used to quantify all of the identified compounds. However, due to its predominance and near constant ratio to other wax compounds, 1-hexacosanol was chosen as an indicator for wax deposition.

Experiment 1 GC-FID showed that cuticular waxes were absent in the most basal segments at all growth stages (Fig. 4a–d). Wax deposition commenced between 29 mm to 45 mm below the POE of leaf three from the sheath of leaf two, at growth stages 2 to 4. At growth stage 1, significant wax deposition commenced somewhere between 57 mm to 34 mm below the POE.

Once wax deposition commenced, it increased linearly with distance from POE (Fig. 4). Distal to the elongation zone, the velocity of displacement of cells equaled LEV. Based on the LEV, the time taken for a cell to be displaced from the segment where wax was first detected to the segment furthest from the leaf base was determined. This made it possible to calculate the rate of wax deposition along the blade between these segments. Deposition rates for growth stages 2–4 between these segments were 0.17 ± 0.03 , 0.15 ± 0.04 and $0.20 \pm 0.02 \mu\text{g cm}^{-2} \text{ h}^{-1}$, respectively (means \pm SD) (Table 2). Wax was deposited at these rates before and

Table 1 Compounds in cuticular wax extracted from the emerged portion of leaf three of barley. Plants were analysed at growth stage [4], at segment y. The percentage content of compounds (amount of compound expressed as percentage of total extractable wax) is given in parentheses (means \pm SD; $n=3$)

Compound	Chain length
<i>n</i> -Alkanols	C_{22} (0.46 ± 0.01), C_{24} (2.15 ± 0.14), C_{26} (82.25 ± 1.04), C_{28} (1.19 ± 0.04)
<i>n</i> -Alkanes	C_{33} (0.67 ± 0.07)
<i>n</i> -Alkyl esters	C_{42} (1.54 ± 0.11), C_{44} (2.26 ± 0.11), C_{46} (3.95 ± 0.07), C_{48} (4.23 ± 0.32), C_{50} (1.02 ± 0.05)

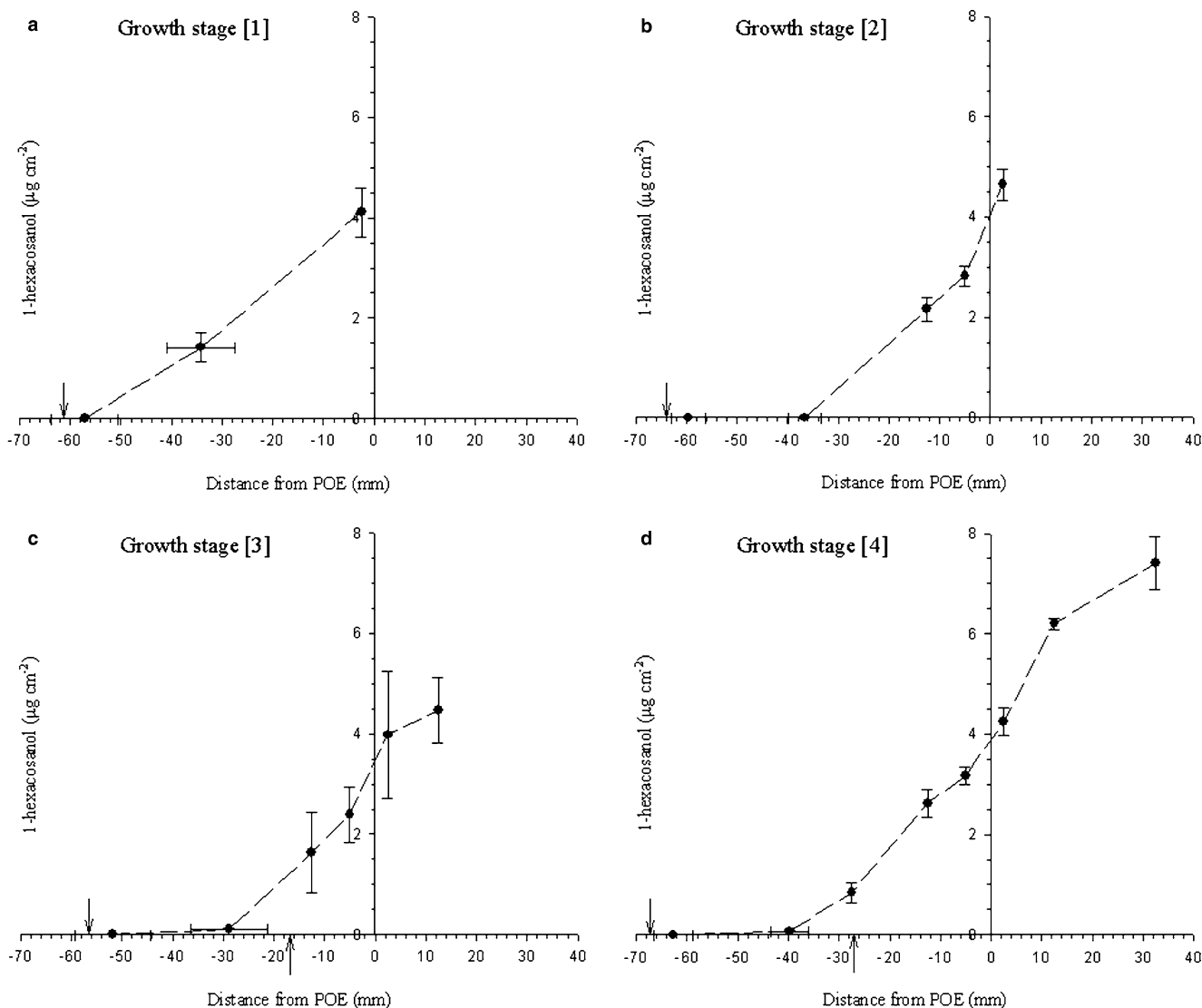


Fig. 4 Abundance of 1-hexacosanol ($\mu\text{g cm}^{-2}$ leaf surface) along the growing barley leaf at growth stage 1–4 (experiment 1). Means and SD from three replicates (each comprising three segments) were calculated from integrated GC-FID peaks. Data plotted at midpoint of segment from POE. The average distance of the leaf base from the POE is marked (*down arrow*). The point at which cell

elongation ceases is marked (*up arrow*). **a** Growth stage 1, total leaf length = 61.67 ± 6.63 mm, emerged blade = 0 mm. **b** Growth stage 2, total leaf length = 74.11 ± 3.33 mm, emerged blade = 10 ± 0 mm. **c** Growth stage 3, total leaf length = 109.33 ± 6.02 mm, emerged blade = 53.00 ± 5.29 mm. **d** Growth stage 4, total leaf length = 163.44 ± 8.76 mm, emerged blade = 96.22 ± 7.28 mm

past the POE. It was also possible to calculate deposition rates in absolute terms ($\mu\text{g wax h}^{-1}$). For growth stages 2–4 these rates were 0.13 ± 0.02 , 0.20 ± 0.06 and 0.40 ± 0.04 $\mu\text{g h}^{-1}$, respectively (means \pm SD) (Table 2). Therefore, the absolute quantity of total extractable cuticular wax present on growth stage 3 segment *k* and growth stage 4 segment *r* doubled every 62 ± 19 and 20 ± 5 min, respectively.

Density of the wax coverage immediately above the POE was similar for growth stages 2–4, averaging 4.60 ± 0.31 , 3.98 ± 1.26 and 4.25 ± 0.28 $\mu\text{g cm}^{-2}$, respectively (means \pm SD; $n=3$). Similarly, at growth stage 1 immediately below the POE the density of wax coverage was 4.1 ± 0.49 $\mu\text{g cm}^{-2}$ (means \pm SD; $n=3$). Comparison of wax coverage of growth stage 4 segment *y*

($7.4 \mu\text{g cm}^{-2}$) with a segment taken from a similar distance from the tip of a mature blade ($9.7 \mu\text{g cm}^{-2}$; two measurements, not shown) suggests that wax deposition at a particular leaf region continues throughout leaf development.

Experiment 2 followed wax deposition at growth stage 3 at higher spatial resolution. Wax deposition commenced 27 mm below the POE (Fig. 5). This was within the region pin-pointed by experiment 1 (28.8 ± 7.5 mm). The rate of 1-hexacosanol deposition and the absolute wax content was comparable to experiment 1, growth stage 3 (Table 2), as was the percentage composition of wax (not shown). The density of coverage by 1-hexacosanol immediately above the POE was reduced at 2.55 ± 0.09 $\mu\text{g cm}^{-2}$ as

Table 2 Wax deposition rates in growing barley leaves at three developmental stages (experiments 1 and 2). Deposition rates were calculated from differences in abundance of 1-hexacosanol between segment below the point of emergence (POE) where wax was first detected, to the segment furthest above the POE. Data which were used to calculate deposition rates or to convert rates from $\mu\text{g cm}^{-2} \text{h}^{-1}$ to $\mu\text{g h}^{-1}$ are also shown. Doubling time refers to the time it took for wax quantity (μg) in the segment below the POE to double

	Segment: below POE/above POE	Segment area (cm^2)	Leaf elongation Velocity (mm h^{-1})	Displacement time between segments (h)	Abundance of 1-hexacosanol		Deposition rate ($\mu\text{g h}^{-1}$)	Doubling time (min)
					(μg)	($\mu\text{g cm}^{-2}$)		
Experiment 1	Growth stage 2	e	1.35 ± 0.03	27.15 ± 3.77	0.03 ± 0.02	0.02 ± 0.04	0.13 ± 0.02	13 ± 9
		h	0.78 ± 0.04	30.14 ± 7.14	3.62 ± 0.30	4.65 ± 0.31	0.20 ± 0.03	62 ± 19
	Growth stage 3	k	1.91 ± 0.05	35.92 ± 2.36	0.21 ± 0.03	0.11 ± 0.10	0.40 ± 0.04	20 ± 5
		p	1.37 ± 0.01		6.10 ± 0.91	4.47 ± 0.66		
Experiment 2	Growth stage 4	r	1.97 ± 0.20		0.14 ± 0.03	0.07 ± 0.02	0.17 ± 0.03	48 ± 31
		y	1.98 ± 0.04		14.66 ± 1.00	7.41 ± 0.53	0.14 ± 0.03	
	Growth stage 3	3	1.85 ± 0.06	24.09 ± 3.51	0.14 ± 0.09	0.08 ± 0.05	0.17 ± 0.03	
		14	1.34 ± 0.02		4.57 ± 0.52	3.40 ± 0.42		

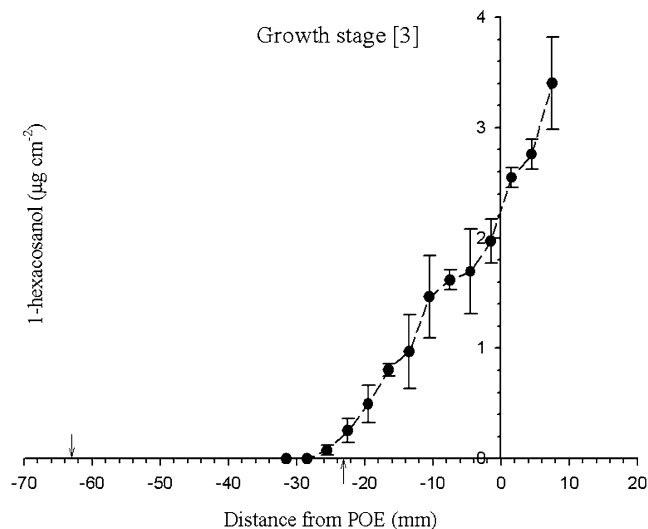
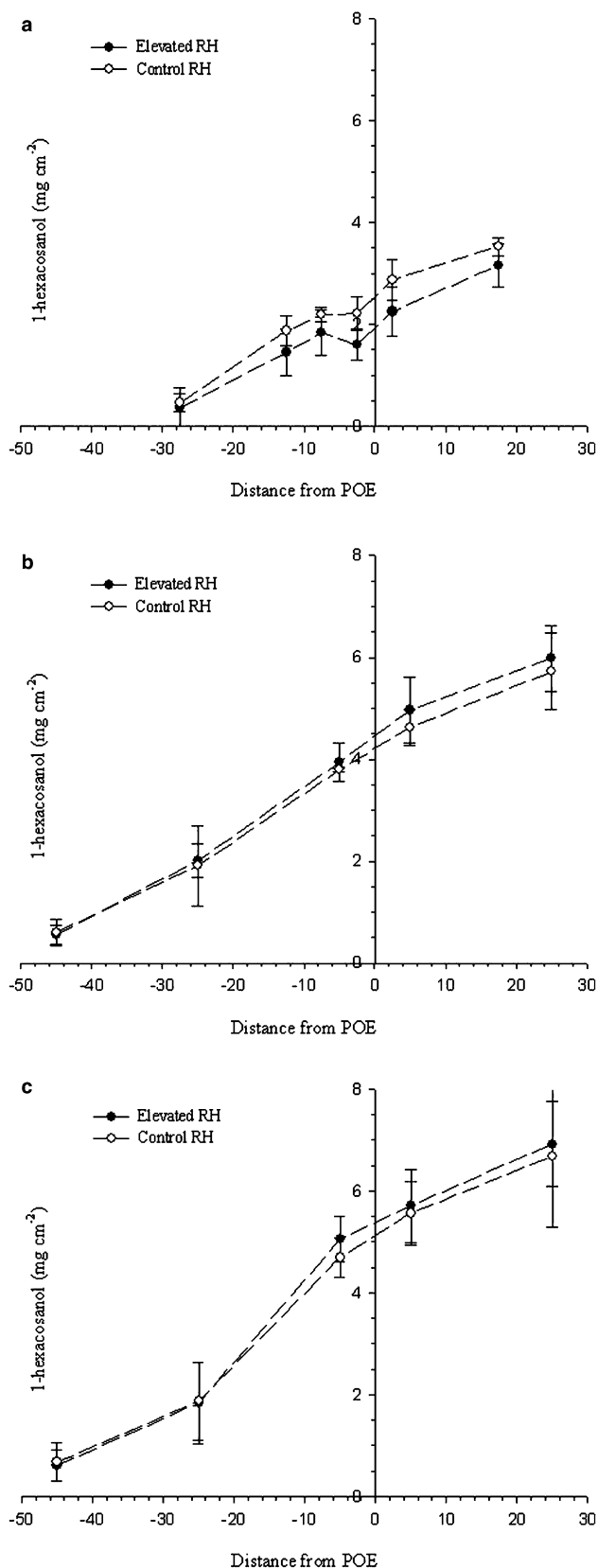


Fig. 5 High spatial-resolution analysis of 1-hexacosanol abundance ($\mu\text{g cm}^{-2}$ leaf surface) along the growing barley leaf at growth stage 3 (experiment 2). Means and SD from three replicates (each comprising five segments) were calculated from integrated GC-FID peaks. Data plotted at midpoint of segment from POE. The average distance of the leaf base from the POE is marked (down arrow). The point at which cell elongation ceases is marked (up arrow). Total leaf length = 113.73 ± 4.15 mm, emerged blade = 50.20 ± 0.41 mm

opposed to $4.25 \pm 0.09 \mu\text{g cm}^{-2}$ in Experiment 1. This might have been due to differences between experiments in the length of leaf three and sheath of leaf 2 (109.33 ± 6.02 and 56.33 ± 7.5 mm compared to 113.73 ± 4.15 and 63.53 ± 4.12 mm in Experiment 1, respectively), leaving less time for wax deposition in Experiment 2.

Experiment 3 This experiment was designed to test the effect of RH on cuticular wax deposition. The RH present within the bags differed for each batch. Batch (i) achieved an RH of ca 92%. Batch (ii) with reduced growth chamber fan speed, achieved 95% RH. Batch (iii) under ambient laboratory conditions, achieved an RH of 96%. Growing plants within bags at elevated RH had no visible effect on plant development, no measurable effect on LEV and resulted in no significant alteration to atmospheric CO_2 (data not shown). All three batches of plants failed to show a significant effect of elevated RH on 1-hexacosanol deposition along the elongating leaf three (Fig. 6a–c). Averaged over all leaf positions and batches of plants analysed, wax density in the enclosed and emerged portion of the blade of plants grown at elevated RH was $91 \pm 13\%$ ($n=10$) and $98 \pm 11\%$ ($n=6$) respectively, of values in control plants (means \pm SD). The percentage content of 1-hexacosanol within the total extractable wax was also unaffected by elevated RH; it was $78 \pm 6\%$ and $78 \pm 4\%$ in plants grown under elevated RH and control conditions, respectively (means \pm SD of 16 values, obtained from batches (i)–(iii)).

Experiment 4 The POE of leaf three from the sheath of leaf two was experimentally lowered by 30 mm



through stripping down carefully the sheath of leaf two (Fig. 2). At 3.5 h post-stripping, 1-hexacosanol concentration at the new POE had not changed. However,

Fig. 6 Effect of increase in atmospheric relative humidity on abundance of 1-hexacosanol along the growing barley leaf (experiment 3). Means and SD from three replicates (each comprising three or four segments) were calculated from integrated GC-FID peaks. Data plotted at midpoint of segment from POE. **a** Batch (i) Growth stage 3: high humidity; 92% RH, total leaf length = 96.89 ± 4.73 mm, emerged blade = 53.33 ± 2.29 mm. Control; 70% RH, total leaf length = 97.56 ± 4.82 mm, emerged blade = 53.22 ± 2.17 mm. **b** Batch (ii) Growth stage 3: high humidity; 95% RH, total leaf length = 107.88 ± 9.69 mm, emerged blade = 58.94 ± 4.14 mm. Control; 70% RH, total leaf length = 105.06 ± 5.84 mm, emerged blade = 58.9 ± 3.11 mm. **c** Batch (iii) Growth stage 4: high humidity; 96% RH, total leaf length = 156.58 ± 12.97 mm, emerged blade = 99.00 ± 7.17 mm. Control; 55% RH, total leaf length = 155.33 ± 8.80 mm, emerged blade = 101.92 ± 7.87 mm

between 3.5 h and 7 h post-stripping, the concentration of 1-hexacosanol increased by 90% (Table 3). Stripping back the sheath of leaf two reduced the LEV of leaf three by 17% ($P < 0.01$) at 3.5 h and by 8% at 7 h compared to velocities of non-disturbed plants.

Scanning electron microscopy (SEM)

SEM analysis detected only epicuticular waxes, in contrast to GC-FID which might also have detected intracuticular waxes removed during chloroform extraction. SEM supported the findings of the GC-FID with respect to the spatial profiles of wax deposition (Fig. 7). For segment 1, where GC-FID (Experiment 2) indicated that no waxes were present, SEM revealed an absence of epicuticular wax crystals on the epidermal surface (Fig. 7a). Segment 4, where GC-FID indicated that low quantities of wax were present, was the only segment for which SEM gave variable results between plants, showing regions with (Fig. 7b) or without (Fig. 7c) epicuticular wax crystals on the epidermal surface. For segments 7, 11, 12 and 14 where GC-FID showed a steady increase in the quantity of cuticular wax, SEM too revealed an increase in the abundance and density of epicuticular wax crystals on the epidermal surface (Fig. 7d–g).

Wax crystals were primarily linear and plate-shaped, oriented perpendicular to the cell surface and organized either singularly or joined together in groups. Individual

Table 3 Abundance of 1-hexacosanol in the segment above the new point of emergence of leaf three at different times following peeling back the sheath of leaf two (experiment 4). The means and standard deviations from three replicates (each comprising three segments) were calculated from integrated GC-FID peaks

Segment	Abundance of 1-hexacosanol ($\mu\text{g cm}^{-2}$)
Time zero, unstripped control	0.84 ± 0.20
3.5 hours post modification	0.80 ± 0.11
7 hours post modification	1.52 ± 0.32

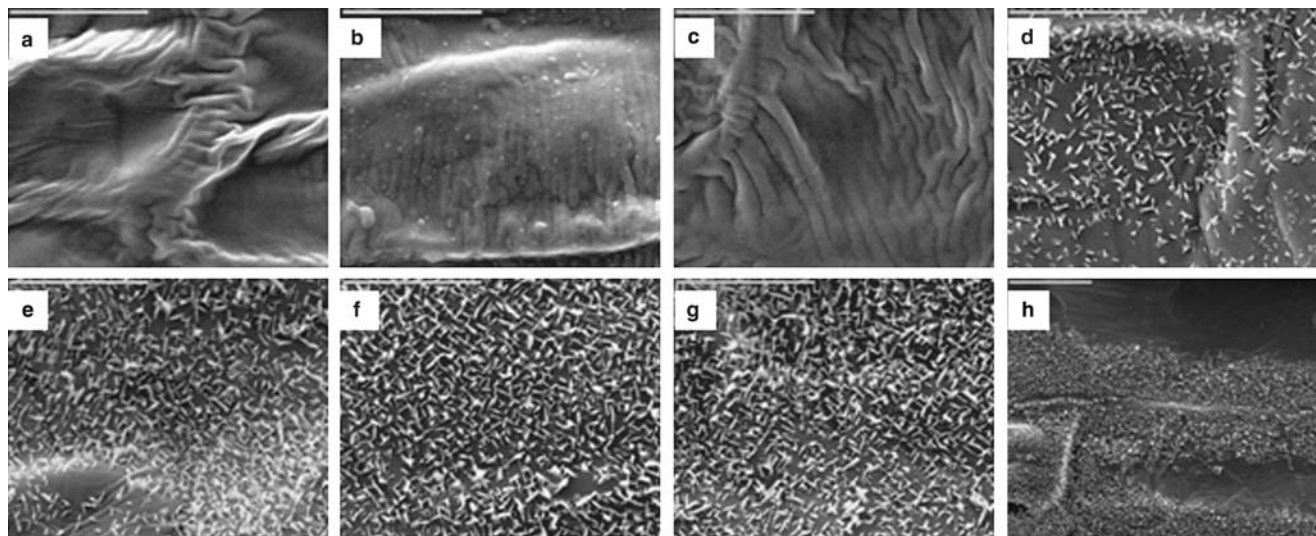


Fig. 7 SEM images of wax crystal development along the barley leaf three. Segments were taken from locations 1, 4, 7, 11, 12, 14 as per Fig. 1b. **a** Segment 1. **b** Segment 4. **c** Segment 4. **d** Segment 7. **e** Segment 11. **f** Segment 12. **g** Segment 14. **h** Segment 11. Scale bar; **a–g** = 5 μm , **h** = 10 μm

crystals attained a maximum size of 340 nm near the commencement of deposition (segment 7), rising to a maximum of 550 nm when fully developed (segment 14). This suggests that increases in both crystal density and crystal size contributed to increased wax concentrations along the developing leaf. No differences were noted in the abundance or form of crystals between adaxial and abaxial surfaces (data not shown). However, within each epidermal surface, the deposition of wax crystals was not uniform, with crystals often being most abundant in the region around guard cells. Differences were also noted within individual cells, with wax crystals often being concentrated near cell boundaries (Fig. 7h).

Discussion

The present study provides for the first time data on cuticle development in grass leaves. To the best of our knowledge, only one other study on cuticular wax deposition in monocots exists, and this study was carried out on leek (*Allium porrum*; Rhee et al. 1998), a member of the Lily family. Cuticular waxes are thought to be essential for achieving a low permeability to water for leaf surfaces (Schreiber and Schönherr 1993; Riederer and Schreiber 2001), and as such it can be expected that they must be sufficiently developed before the growing grass leaf emerges from sheaths of older leaves. The present study, which focused on cuticular waxes of the growing barley leaf, shows that deposition of cuticular waxes commences before emergence from sheaths and continues post-emergence. The data allow several conclusions (1–4).

1. Wax composition along the developing barley leaf does not change post emergence. 1-hexacosanol was the main wax component at all leaf and epidermal-

cell developmental stages analyzed, at least under the present growth conditions. No changes in the composition or percentage content of the waxes post emergence were noted. Post emergence modification of waxes has been reported for barley straw, where alcohol compounds are converted to aldehyde compounds (Wiśniewska et al. 2003).

Scanning electron microscopy revealed that the shape (plates) and orientation relative to the leaf surface (perpendicular) of wax crystals did not vary between leaf segments. As crystal shape and size is determined by the chemical composition of the wax (Koch et al. 2004), this observation provides additional support for uniformity in wax composition along the developing barley leaf.

2. Deposition of cuticular wax along the growing barley leaf commences but is not completed before emergence from sheaths of older leaves. Deposition of wax commenced about half-way within the portion of the growing blade enclosed by sheaths of older leaves and from here increased linearly with position along the blade. Since cells were displaced at steady velocities throughout this leaf region, the results imply that rates of wax deposition were steady too and differed little between enclosed and emerged leaf portions. In experiment 1, several growth stages were analyzed in plants that were derived from the same batch. The wax content at the point of emergence was consistent between growth stages, at about $4 \mu\text{g cm}^{-2}$. In other experiments, the wax content upon emergence was lower. For all experiments, plants were grown under controlled environmental conditions (light, temperature, relative humidity, day/night cycle) in the same growth chamber and they grew uniformly (about 80–90% of 14 days old plants were ready for analyses on the same day). The only possible differences between plant batches were the quality of air running through

the growth chamber (difference between air pollution levels during summer [low] and winter [high pollution] months), the age of seeds, and the bubbling rate during germination. Therefore, the results suggest that there is a set-point for the amount of wax at the POE, which is dependent on processes before the seedling stage or on air quality.

The cuticular wax profiles and SEM data clearly negate the hypothesis that wax deposition must be completed prior to emergence of the growing grass leaf into the dry air. In addition, during experiment 4, leaves were exposed to the dry atmosphere at regions that contained little wax, yet elongated at near-control velocities. Either (1) cuticular waxes are not as important for water retention in grasses as generally thought to be (Schreiber and Schönherr 1993; Riederer and Schreiber 2001); (2) intracuticular waxes constitute the primary permeation barrier to water (Vogg et al. 2004) and their deposition is completed before emergence (unlike the deposition of epicuticular waxes which is not completed, however GC-FID did not distinguish between intra- and epicuticular waxes) or is sufficiently completed to protect from water loss; or (3) the growing grass leaf can lose significant quantities of water through the cuticle without significant impairment of growth. The growing barley leaf consumes only about 1% of the quantity of water lost through transpiration for growth (Fricke 2002). Increased cuticular water loss in 'stripped' leaves could be compensated for by reduced stomatal transpiration or increased root hydraulic conductivity.

3. Cuticular wax deposition can occur in regions of cell elongation. The two processes are not incompatible. Overlaying the graphs of wax deposition at growth stages 3 and 4 with the zone of cell elongation (compare Fig. 6c, d with Fig. 3) at these growth stages demonstrates that wax deposition can commence prior to cessation of cell elongation. The size of the overlap is 10–26 mm at growth stage 3 and 17–25 mm, at growth stage 4. An overlap is also likely to occur at growth stages 1 and 2 but cannot be determined due to technical difficulties in obtaining REGR-profiles at these stages. Since wax deposition could not be measured in the same leaves used for determination for REGR profiles, the variability in overlap makes it impossible to say for definite that waxes were deposited in regions of cell elongation. However, the results strongly suggest that wax deposition is compatible with cell elongation. If so, this finding contrasts with the conclusion by Rhee et al. (1998) that wax deposition in the monocot leek (*Allium porrum*) always commences beyond the cell elongation zone. Therefore, grasses behave more like dicots where cuticle formation parallels cell expansion. However, unlike dicots, the deposition of cuticular waxes does not commence immediately following cell division, but, with some delay, in the distal portion of the elongation zone.

4. Wax deposition along the growing grass blade does not commence in relation to cell age or development; instead, POE is the reference point. The results suggest that the most important region of the barley third leaf, with regards to the commencement of cuticular wax deposition, is the POE from the enclosing sheath of the second leaf. This is evidenced by the consistency of the distance below the POE at which wax deposition commenced in Experiment 1, growth stages 2–4 (29–45 mm) and Experiment 2 (27 mm; growth stage 3). For Experiment 1, growth stage 1, it is not possible to give an exact measurement for distance since no points were analysed between –57 mm (no wax) and –34 mm (wax present). Furthermore, by peeling back the sheath of leaf two in Experiment 4 it was possible to create an artificial POE and induce wax deposition at that point. Such a response would not be expected if wax deposition occurred at a fixed (1) distance from the point of leaf insertion, or at a fixed (2) chronological or (3) developmental age of cells. Instead, the present results suggest that a signal with close relation to the POE triggers commencement of wax deposition in the growing barley leaf. The barley leaf system described here provides an excellent experimental setup to study the regulation of wax deposition at the molecular level. It remains to be shown to which degree the data obtained on barley are applicable to grasses (or cereals) in general.

Signals for wax deposition

What triggers commencement of wax deposition? Four potential signals come to mind when looking at the transition from an enclosed to an emerged leaf portion: relative humidity (Neinhuis et al. 2003; Hetherington and Woodward 2003; Talbott et al. 2003), light (Bird and Gray 2003); carbon dioxide (Bird and Gray 2003; Hetherington and Woodward 2003; Talbott et al. 2003) and ethylene (Broun et al. 2004). In the present study, relative humidity, RH, was tested, by reducing the gradient in RH between enclosed (close to 100% RH) and emerged leaf portion (ambient RH) through increase of ambient RH close to 100%. However, no significant effect of a reduced RH gradient on deposition or percentage content (of total wax) of 1-hexacosanol, was observed. The maximum-attainable ambient RH was 96%, and it cannot be ruled out that plants grown at higher ambient RH may show reduced wax deposition.

It could be argued that signals from the emerged portion of the growing leaf had moved already into enclosed leaf tissue and triggered wax synthesis which could not be altered by application of high RH. However, this possibility can be ruled out: during two of the three RH experiments, the tip of leaf three had not emerged at the time RH was increased.

Of the three other potential signals, carbon dioxide, ethylene and light, the latter appears to be the most likely

candidate to affect wax deposition along the growing barley leaf. The precise difference in photosynthetically active radiation between enclosed and emerged leaf portions is not known, but light could affect wax deposition through changes in photon flux density. In addition, the sheaths of older leaves are green, and the quality of light reaching an enclosed leaf region will differ from that experienced by emerged leaf regions. In particular, the red:far-red ratio of light reaching enclosed leaf tissue is bound to be higher and could affect cuticle synthesis or deposition through phytochrome.

Wax deposition at the cell level

Analysis of barley leaf segments by SEM revealed no apparent differences in the abundance or pattern of deposition of wax between the abaxial and adaxial surfaces. This contrasts with findings on leek (Rhee et al. 1998). At the cellular level, differences in the pattern of wax crystal deposition could be seen. Close to the region where wax deposition commenced, crystal density was often higher in the vicinity of guard cells (see also Rhee et al. 1998). Guard cells are thought to respond to humidity and CO₂ (Talbot et al. 2003) and it may be here that these potential signals for cuticle formation are perceived first and lead to initiation of wax synthesis. In non-stomatal cells and at early stages of wax deposition, wax appeared often first near cell boundaries, where walls of adjacent cells meet. Next to nothing is known about the transport paths of wax components between protoplast and epidermal surface. The present observations suggest that some components reach the surface via the middle lamella or radial cell walls.

Wax deposition rates

Rates of wax deposition along the growing barley leaf were comparable between developmental stages. Do these rates represent maximum rates and are they, therefore, potentially limiting the velocity of leaf elongation, emergence and photosynthetic yield (because insufficient wax deposition may cause excessive and detrimental water loss from emerged cells)? When the point of emergence of leaf three was lowered by peeling back the sheath of leaf two, wax deposition did not commence within the first 3.5 h, but between 3.5 h and 7 h. It cannot be said whether deposition commenced just after 3.5 h or before 7 h, but the average rate of wax deposition between 3.5–7 h and was similar to rates observed in non-disturbed plants. Therefore, it is possible that wax deposition in non-disturbed plants proceeds near its limit.

conclusion

The present study provides for the first time data on wax deposition along a growing grass leaf. The data

show that wax deposition continues past emergence of the growing leaf from sheaths of older leaves. The point of emergence appears to be the reference point for wax deposition. Onset of wax deposition neither occurs at a particular cell age nor at a cell-developmental stage. If changes in RH between enclosed and emerged leaf regions trigger wax deposition, then RH within the enclosed leaf portion must exceed 96%. There exist other potential signals triggering wax deposition, in particular light. The aim of future experiments to test these potential signals must be twofold: (1) to manipulate the micro-environment along the growing grass leaf; and (2) to obtain quantitative data about the gradient in potential signals (RH, ethylene, carbon dioxide, light) between emerged and enclosed leaf regions. The latter will present a particular technical challenge.

Acknowledgements This research was supported by the Biotechnology and Biological Sciences Research Council (BBSRC), UK, Grant 61/P18283. We would like to thank Eva Simanova and Tobias Wojciechowski from the University of Bonn for their help and advice concerning the GC analysis, Margaret Corrigan and Frank Placido from the University of Paisley for their help and tuition with the SEM and the referees for their helpful comments.

References

- Barnes J, Percy K, Paul N, Jones P, McLaughlin C, Mullineaux P, Creissen G, Wellburn A (1996) The influence of UV-B radiation on the physiochemical nature of tobacco (*Nicotiana tabacum* L.) leaf surface. *J Exp Bot* 47:99–109
- Barthlott W, Neinhuis C (1997) Purity of the sacred lotus, or escape from the contamination in biological science. *Planta* 202:1–8
- Beattie GA, Marcell LM (2002) Effect of alterations in cuticular wax biosynthesis on the physiochemical properties and topography of maize leaf surfaces. *Plant Cell Environ* 25:1–16
- Bianchi G, Murelli C, Ottaviano E (1990) Maize pollen lipids. *Phytochemistry* 29:739–744
- Bird SM, Gray JE (2003) Signals from the cuticle affect epidermal cell differentiation. *New Phytol* 157:9–23
- Broun P, Poindexter P, Osbourne E, Jiang Cz, Riechmann JL (2004) Win 1 a transcriptional activator of epidermal wax accumulation in *Arabidopsis*. *Proc Natl Acad Sci USA* 101:4706–4711
- Eigenbrode SD (1996) Plant surface waxes and insect behaviour. In: Kerstiens G (ed) *Plant cuticles—an integrated functional approach*. BIOS Scientific Publishers Limited, Oxford, pp 201–222
- Fricke W (2002) Biophysical limitation of cell elongation in cereal leaves. *Ann Bot* 90:157–167
- Fricke W, Peters PS (2002) The biophysics of leaf growth in salt-stressed barley: a study at the cell level. *Plant Physiol* 129:374–388
- Hetherington AM, Woodward FI (2003) The role of stomata in sensing and driving environmental change. *Nature* 424:901–908
- Jenks MA, Joly RJ, Peters PJ, Rich PJ, Axtell JD, Ashworth EN (1994) Chemically induced cuticle mutation affecting epidermal conductance to water vapour and disease susceptibility in *Sorghum bicolor* (L.) Moench. *Plant Physiol* 105:1239–1245
- Kerstiens G (1996a) Cuticular water permeability and its physiological significance. *J Exp Bot* 47:1813–1832
- Kerstiens G (1996b) Signaling across the divide: a wider perspective of cuticular structure-function relationships. *Trends Plant Sci* 1:125–129

- Koch K, Neinhuis C, Ensikat HJ, Barthlott W (2004) Self assembly of epicuticular waxes on living plant surfaces imaged by atomic force microscopy (AFM). *J Exp Bot* 55:711–718
- Kolattukudy PE (1996) Biosynthetic pathways of cutin and waxes, and their sensitivity to environmental stresses. In: Kerstiens G (ed) *Plant cuticles—an integrated functional approach*. BIOS Scientific Publishers Limited, Oxford, pp 83–108
- Kunst L, Samuels AL (2003) Biosynthesis and secretion of plant cuticular wax. *Prog Lipid Res* 42:51–80
- Long LM, Patel HP, Cory WC, Stapleton AE (2003) The maize epicuticular wax layer provides UV protection. *Funct Plant Biol* 30:75–81
- Marinari C, Wolters-Arts M (2000) Complex waxes. *Plant Cell* 12:1795–1798
- Neinhuis C, Koch K, Barthlott W (2001) Movement and regeneration of epicuticular waxes through plant cuticles. *Planta* 213:427–434
- Oliveira AFM, Meirelles ST, Salatino A (2003) Epicuticular waxes from caatinga and cerrado species and their efficiency against water loss. *An Acad Bras Cienc* 74:431–439
- O'Neal M, Landis DA, Isaacs R (2002) An inexpensive, accurate method for measuring leaf area and defoliation through digital analysis. *J Econ Entomol* 95:1190–1194
- Palmer SJ, Davies WJ (1996) An analysis of relative elemental growth rate, epidermal cell size and xyloglucan endotransglycosylase activity through the growing zone of ageing maize leaves. *J Exp Bot* 47:339–347
- Preuss D, Lemieux B, Yen G, Davis RW (1993) A conditional sterile mutation eliminates surface components from Arabidopsis pollen and disrupts cell signalling during fertilization. *Genes Dev* 7:974–985
- Reynhardt EC, Riederer M (1994) Structures and molecular dynamics of plant waxes. II. Cuticular waxes from leaves of *Fagus sylvatica* L. and *Hordeum vulgare* L. *Eur Biophys J* 23:59–70
- Rhee Y, Hlousek-Radojic A, Ponsamuel J, Liu D, Post-Beitenmiller D (1998) Epicuticular wax accumulation and fatty acid elongation activities are induced during leaf development in leeks. *Plant Physiol* 116:901–911
- Riederer M, Schreiber L (2001) Protecting against water loss: Analysis of the barrier properties of plant cuticles. *J Exp Bot* 52:2023–2032
- Ristic Z, Jenks MA (2002) Leaf cuticle and water loss in maize lines differing in dehydration avoidance. *J Plant Physiol* 159:654–651
- Schneider H, Nelson CJ, Coutts JH (1987) Assessment of spatial distribution of growth in the elongation zone of grass leaf blades. *Plant Physiol* 85:290–293
- Schreiber L, Schönherr J (1993) Mobilities of organic compounds in reconstituted cuticular wax of barley leaves: determination of diffusion coefficients. *Pestic Sci* 38:353–361
- Talbott LD, Rahvej E, Zeiger E (2003) Relative humidity is a key factor in the acclimatisation of the stomatal response to CO₂. *J Exp Bot* 54:2141–2147
- Vogg G, Fisher S, Leide J, Emmanuel E, Jetter R, Levy A, Reiderer M (2004) Tomato fruit cuticular waxes and their effects on transpiration barrier properties: functional characterization of a mutant deficient in a very-long-chain fatty acid-ketoacyl-CoA synthase. *J Exp Bot* 55:1401–1410
- Wiśniewska SK, Nalaskowski J, Witka-Jeżewska E, Hupka J, Miller JD (2003) Surface properties of barley straw. *Colloids Surf B Biointerf* 29:131–142
- Yephremov A, Wisman E, Huijser P, Huijser C, Wellesen K, Saedler (1999) Characterisation of the FIDDLEHEAD gene of Arabidopsis reveals a link between adhesion response and cell differentiation in the epidermis. *Plant Cell* 11:2187–2201