Methods

Nanoinfusion: an integrating tool to study elicitor perception and signal transduction in intact leaves

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Received: *8 July 2003*
Accepted: *1 October 2003*

doi: 10.1046/j.1469-8137.2004.00971.x

Summary

• To study elicitor effects in intact leaves of *Hordeum vulgare* cv. Ingrid, chitin fragments were delivered to substomatal cavities with a micropipette. Responses were monitored by a calibrated reference microelectrode and a pH-sensitive microelectrode, simply positioned below neighbouring open stomata in the air-filled space of the substomatal cavities.

• Flooding of a leaf spot of approx. 600 × 300 μm with physiological aqueous solutions caused an immediate transient polarization of the extracellular solution in the order of –25 ± 12 mV. Immediately after the pipette solution was consumed, the extracellular solution was again polarized, still before the cavities dried out. In dry cavities, the extracellular equilibrium potential was –34 ± 10 mV. On flooding, the extracellular pH rose to 5.7 ± 0.3 after approx. 12 ± 7 min and returned to a stable level of 5.2 ± 0.3 after 30 min.

• Sequential infusion on the same leaf spot, first with elicitor-free solution, then with the same solution containing 25 μM *N*-acetyl-chitooctaose, into the still-flooded cavities yielded an elicitor-specific pH maximum between 6 and 7 approx. 10 min after flooding. A pronounced pH maximum > 7 occurred between 40 and 240 min after flooding in wild-type plants.

• The use of elicitor nanoinfusion for the integrated development of resistance inducers in cereals, wine and poplar is discussed.

Key words: intact leaf, infiltration, elicitor perception, apoplastic pH, microsensors, pathogen resistance, dynamic system analysis, barley (*Hordeum vulgare*).

Introduction

Plant leaves eventually supply all human activities with energy. Competition for leaf-produced energy resources drives the evolution of plant–pathogen interactions, as well as scientific research on plant protection. One important approach to achieving higher fitness against leaf pathogens was invented thousands of years ago, when pathogen attack was translated by the attacked plant into a permanent metabolic switch to a state enabling improved defence in a subsequent attack, termed induced resistance (Agrawal *et al.*, 1999; van Loon, 2002). The improved defence state manifests itself physiologically in the accumulation of antimicrobial substances and in the acceleration and amplification of defence responses once a pathogen starts its offensive again (Conrath *et al.*, 2001). Often the switch occurs not only in the attacked organ, but throughout the whole plant (systemic). The natural success of the different types of induced systemic resistance is impressive. 'The adaptive significance to plants of inducing and coordinating these different systemic responses is great. Their variety ensures that the plant is primed and can therefore respond more effectively to subsequent pathogen attack.' (Hammond-Kosack & Jones, 2000). It is not surprising that utilizing natural mechanisms to improve crop resistance has been

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recognized as a scientific challenge. However, the desired switch to improved resistance states is based on a complex regulatory network, and the hitherto selected trigger substances from the class of salicylic acid analogues proved to be weak activators under a variety of field conditions, particularly in cereals. Farmers in Europe and the USA are still more convinced by conventional pesticides.

The field of biotechnology impressively illustrates the impact of technological improvements on the feasibility of genome-wide or proteome-wide investigations aimed at understanding complex regulatory networks. The renaissance of systems biology (Kitano, 2002) documents the importance of investigations on the integration of mechanisms and regulatory circuits in the living organism, in order to achieve a 'whole-istic' understanding of system behaviour. Nowadays, molecular biological research on the mechanisms of durable pathogen resistance in crops focuses on the interplay of several genes, which can be investigated by high-throughput biochemical technologies and by novel statistical and machine learning tools provided by systems biology. In a similar manner, development of resistance inducers could benefit from technical tools that add to a systems-level understanding through standardized analysis of system dynamics (Kitano, 2002).

The outcome of plant–microbe interactions is determined by the speed and quality of transition to the cellular defence program. Observation of the laws obeyed by the attacked plant cell is substantially improved by *in situ* monitoring techniques that uncover cellular switch points in response to defined biotic signals in the intact cellular environment. In plant–pathogen interactions, microbial elicitors act as alarm signals that trigger complex defence cascades (Nürnberger *et al.*, 1994; Boller, 1995; Kuchitsu *et al.*, 1997). In this paper a modular, open analytical platform is described that allows a dynamic analysis of the initial stages of defence processes triggered by elicitors.

It has been shown that extracellular defence responses triggered by elicitors are sensitive to resistance inducers (Kauss & Jeblick, 1996; Shirasu *et al.*, 1996). Thus observation of the extracellular defence processes in an intact leaf could become a versatile tool to display the resistance state of a leaf. Here we present data on extracellular pH waves induced by chitin fragments. This type of analysis does not require an extraction procedure, as the leaf is an open book offering microsensor access through stomatal pores to substomatal cavities, and specific microsensors are increasingly available for a variety of substances that mark and govern stages in the transition to defence. A major drawback of microsensors with respect to handling and statistical analysis is alleviated by a nanoliter infusion method changing the substomatal air space into a solution-filled microreaction chamber, which mirrors the cellular processes in all the surrounding cells. Apart from this, nanoinfusion is a convenient vehicle to convey chemical stimuli to the test system, allowing biochemical manipulation, model development and model testing.

In combination with pretreatment procedures, the technique presented can be used for *in vivo* testing of a wide range of single drugs and drug mixtures as resistance inducers with reasonable speed. In this paper the analytical platform for leaf nanoinfusion assays is introduced, an example of the discriminatory power between resistance states is given, and perspectives for integrated research in basic and in applied fields are outlined.

Materials and Methods

Plants and leaf preparation

Two near-isogenic lines of *H. vulgare* L. cv. Ingrid, wild type (WT) and the *mlo* mutant, were supplied by the Institute of Phytopathology and Applied Zoology, Giessen University. Seeds of *H. vulgare* cv. Golf (Fig. 4e,f) were obtained from the Plant Nutrition Laboratory, The Royal Veterinary and Agricultural University, Copenhagen, Denmark. Seeds were germinated in the dark at room temperature on filter paper soaked in demineralized water. After 4 d, nine seedlings were transferred to 1.5 l nutrient solution (Murashige and Skoog diluted 1 : 5; Serva GmbH, Heidelberg, Germany), adjusted with Fe-EDTA (Fluka, Neu-Ulm, Germany) to a final iron concentration of 0.26 mM. Lower iron concentrations cause Fe deficiency symptoms in leaves of the *mlo* mutant. Iron uptake into plant cells requires Fe reduction at the plasma membrane, where the MLO protein is located. The solution was changed on a weekly basis. Plants were grown in a growth chamber at 20°C with a 12 h light period (photosynthetic photon fluence rate (PPFR) 300 μmol m^{-2} s^{-1}) and the relative humidity ranged between 50 and 70%. Investigations on the leaf defence state were performed with primary leaves after 5–14 d in nutrient solution. In the morning, leaves were cut with a razor blade a few millimeters above the ligula (unless intact plants were used, as shown in Fig. 3) and immediately transferred to the solution reservoir of the plant cuvette; the solution contained 1 mM KCl, 0.1 mM NaCl and 0.1 mM $CaCl_2$. The leaf blade was gently fixed on a closed-pore polymer foam by a sealing compound (plastic-fermit; Nissen und Volk, Hamburg, Germany). A leaf area approx. 5 cm^2 was enclosed in a 2 mm high mini-chamber (Fig. 1; www.transmit.de Projektbereich Phytosensor-Technologie) connected to a CO_2 controlled gas supply. The cuvette allowed access of microsensors and micropipettes. After 1 h at a PPFR of approx. 150 μmol m^{-2} s^{-1}, the photon flux was adjusted to 300 μmol m^{-2} s^{-1}. The temperature was 22 ± 2°C. Three hours after cutting the leaf surface was exposed to a CO_2 concentration of 380 p.p.m. and a relative air humidity > 90% to promote stomatal opening. The cuvette air was mixed as described previously (Hanstein & Felle, 2001) and supplied at a rate of 500 ml min^{-1}. A stomatal aperture of 6–9 μm was achieved. Barley stomata are arranged in rows, and the distance between stomata in the same row is approx. 200 μm. Three neighbouring

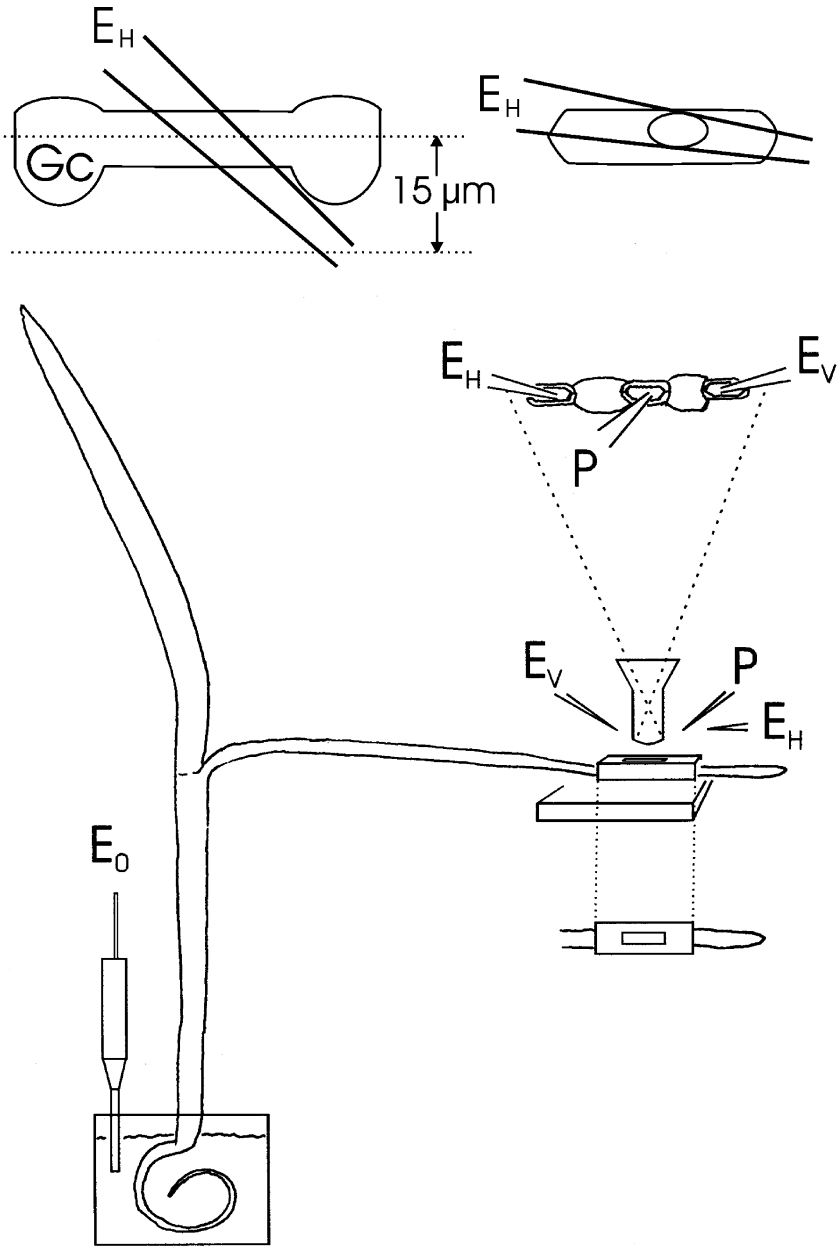

Fig. 1 Arrangement of nanoinfusion pipette (P) and electrodes (E_0, E_V, E_H) for pH monitoring in the substomatal cavity of *Hordeum vulgare* leaves. The solution in which roots (or cut leaf ends) are immersed is grounded by E_0. Part of the leaf is enclosed in a cuvette with an upper window for microelectrode access. Under the microscope, P and the microelectrodes for voltage and pH monitoring (E_V and E_H) are positioned below three neighbouring stomatal pores. Top left: side view on E_H and on one guard cell (Gc) with the electrode tip hanging at a depth of 15 µm in the free space of the substomatal cavity. Top right: projection from above into an open pore with inserted E_H. Under the microscope, the tip is hidden below the guard cells. The ellipse shows the electrode margins lying in focus with the pore edges.

stomata were used for the infusion assay: the middle stoma served as port for nanoliter infusion, and two microelectrodes were positioned in the two neighbouring stomata.

Working stage

Micropipettes and electrodes were positioned under a binocular microscope equipped with a long-distance objective (EF L 20/0.32; Leica microsystems GmbH, Bensheim, Germany) at a magnification of 200 or 250. Only those microscopes in which focusing is done by moving the objective are suitable. The leaf surface was illuminated from opposite sides at an angle of 45° by a fiber cold-light source (KL1500; Leica). The microscope and three micromanipulators (DC-3K; Marzhauser, Wetzlar, Germany) were placed on an air-buffered working stage (60 × 120 cm) from Spindler & Hoyer (Göttingen, Germany; now Linos AG). The control units for the manipulators (STM3 or MS314; Marzhauser) were placed on a mechanically separate stage within the home-made Faraday cage.

Nanoliter infusion

The micropipettes without filament were pulled from glass capillaries with 1.5 mm OD and 1.2 mm ID (Hilgenberg, Malsdorf, Germany) on a patch-clamp puller (LM-3/P-A; List Medical, Göttingen, Germany). Micropipettes were

silanized internally using a solution of 0.2% (v/v) tributylchlorosilane in chloroform (Fluka, Neu-Ulm, Germany). For this purpose they were placed with the tip upwards in a metal block and heated up to 200°C in an oven. About 10 µl silane solution was drawn into the back end by dipping it into the solution. The pipettes were again placed for 30 min in the oven. For preparation of the infusion solutions, aqua purificata (Ph.Eur.; Roth GmbH, Karlsruhe, Germany) was used. Unless stated otherwise, the infusion solution contained 3 mM KCl and 0.1 mM $CaCl_2$ (standard solution). The chitin oligomers octa-*N*-acetylchitooctaose (#O6383), hepta-*N*-acetylchitoheptaose (#H1271) and penta-*N*-acetylchitopentaose (#P6967) were purchased from Sigma-Aldrich (www.sigma-aldrich.com). They were added to the infusion solution from 1 mM aqueous stock solutions.

Approximately 2 µl infusion solution was sucked into the tip of the micropipette. For sequential infusions with two different solutions, the second solution was filled into the pipette from the back end leaving a distance of at least 3 mm between both solutions. The pipette was connected to a PV830 Pneumatic PicoPump (WPI, Sarasota, USA), mounted on a micromanipulator, and navigated through an open stomatal pore to roughly 15 µm below pore level, avoiding contamination of the tip from cuticular waxes.

Measuring circuit, microsensors and calibration

The electrical circuit used for measuring extracellular ion activities in leaves with potentiometric microsensors is described by Hanstein & Felle (1999). It is based on correction of the signal from the ion-sensitive electrode by subtracting the extracellular voltage signal. The ion-sensitive electrode and the voltage electrode were connected to a high-impedance amplifier (FD 223; WPI) which performs the analogue subtraction procedure. Outputs of the original signals and the difference signal were recorded on a chart recorder (W + W Recorder Model 314; Kontron, München, Germany). The solution containing the cut end of the leaf (or in experiments with whole plants, the root) was connected to circuit ground. The home-made ground electrode was a plastic pipette tip with an agar diffusion barrier filled with 0.5 M KCl and combined with a Ag/AgCl half cell.

pH microsensors were prepared as described (Felle *et al.*, 2000), with the following modifications: the outer tip diameter was 3 µm, the tip was not heat polished, and the KCl concentration in the reference solution was 0.1 M. An unsilanized micropipette with a 3 µm wide, unpolished tip filled with 0.1 M KCl was used for extracellular voltage recording in the leaf. The pH microelectrode could be used for up to 40 experimental days. A new voltage microelectrode was prepared every day. The voltage microelectrodes were tested for interference from natural fluctuations in the KCl concentration of the extracellular solution in the leaf (tip potential). Increasing the KCl concentration from 1 to 10 mM changed the voltage signal by < 12 mV, and the resulting potential error in pH measurements remained below 0.2 pH units.

Once a day the pH microelectrode was calibrated with two buffer solutions containing 10 mM KCl (pH 5 and 6.5; 10 mM MES, adjusted with 10 mM Tris to the respective pH). The sensitivity was at least 55 mV per pH unit. Before each experiment the signal of the voltage microelectrode was measured in a 1 mM KCl solution. This signal was subtracted from the signals of the pH microelectrode in the buffer solution. The resulting values were used for scaling the difference trace recorded by the chart recorder. This procedure rules out underestimation of the extracellular pH in case of slight KCl interference with the voltage microelectrode.

Microelectrode positioning

Microelectrodes for voltage and pH were mounted on the motor-driven manipulators at a vertical (*z*) angle of 45°. The final position of an electrode at a guard cell is illustrated in Fig. 1 (top left). For stomatal pores of barley primary leaves, the pore length (*x*-direction) was approx. 30 µm and the pore width (*y*-direction) varied between 6 and 9 µm. Through the basic arrangement of the micromanipulators on the working stage, horizontal projection of the electrode axis was aligned along the pore's long side with a maximum deviation of 15° (Fig. 1, top right). The first step in positioning was to place the electrode tip within a pore at pore level approx. 6 µm from the pore corner (5 min procedure). Three more or less time-consuming modes of further positioning were applied. (1) Crude positioning: all positioning steps were performed with a joystick controller (STM3), and the step size of movements was controlled by optical inspection at 250-fold magnification. The tip was first lowered by approx. 15 µm (*z*-direction), which was derived from the distance between the former tip position and the position where the electrode shank was focused. Then the tip was moved in small steps in the *x*-direction until electrical contact was made with the extracellular fluid of a guard cell. The target *z*-level 15 µm below pore level ensured that the sharp electrode end did not come into direct (harmful/contaminating) contact with guard cells. (2) Improved positioning: All positioning steps within the substomatal cavity were performed with the MS314 controller with its smallest step size of 0.5 µm in the same sequence (*z*, *x*) as before until electrical contact was made. (3) Simple positioning (also used for infusion pipette): the electrode was lowered by 15 µm without further movement. Since electrical contact was made through the infusion step, this positioning mode is available only in combination with the permanent infusion technique.

Data processing and illustration

Statistics were performed on curve extrema using EXCEL (Microsoft Office 97; Microsoft Corporation). Data denote

mean values ± SE. The comparison of the chitin oligomer response between WT and the *mlo* mutant of *H. vulgare* cv. Ingrid (Fig. 6) is based on five experiments in each species (octa-*N*-actetylchitooctaose, $n = 2$; hepta-*N*-actetylchitoheptaose, $n = 2$ penta-*N*-actetylchitopentaose, $n = 1$). For presentation purposes the original recordings were displayed unless stated otherwise. The original analog recorder data were converted to image files using CORAL DRAW Select Edition Version 7 (Coral Corporation, Dublin, Ireland) according to the following procedure. Scaled voltage and pH traces were scanned (SNAPSCAN 1212p, Agfa-Gevaert, Mortsel, the Netherlands), contrast and brightness were optimized using CORAL PHOTO PAINT, and the image file was stored in cpt format for two separate further processing steps. (a) a mask comprising labels, gridlines and a pointer to a specific point of the traces was added with CORAL DRAW. The figure was printed out, and the mask stored as a cdr file. (b) The original cpt file was loaded again to PHOTO PAINT and the original handwritten scaling removed with the eraser tool. With lasso and mask functions, the line width and colour of the traces were modified as required, and the traces were stored as new cpt files. Finally, the mask cdr file was opened in CORAL DRAW and the new cpt file imported. After fitting the cpt image to the mask, the pointer was removed.

Results and Discussion

Nanoliter infusion enables reproducible elicitor delivery and facilitates response recording

Highly hydrophobic cuticles on plant leaves restrict access of hydrophilic agents to the leaf interior (Schönherr & Bauer, 1996). In order to overcome these delivery barriers for hydrophilic microbial elicitors, we utilized the natural gates into the leaf – the plant stomata – as pipette ports. A micropipette tip was positioned within the primary leaf of *H. vulgare* approx. 15 µm below the level of the stomatal pore. The internal surface within the leaf was found to be sufficiently hydrophilic to permit complete flooding of one to several substomatal cavities when the back pressure on the pipette solution was raised. A minimum pressure of 0.5 bar was required to expel solution from the narrow capillary tip. In barley leaves, pores are arranged regularly in rows. The physiological experiments required a minimum flooding area of three adjacent substomatal cavities as destinations for (1) the infusion pipette; (2) a microelectrode for extracellular voltage recording; and (3) a pH-sensitive microelectrode. A pressure of 1.1 bar held for 0.5 s filled about three to five cavities of one row: the infusion space was $0.3 \times 0.3 \times 0.6$ mm, roughly equivalent to 30 nl flooding solution (based on an intercellular air-space volume of 50% of total leaf volume). A pressure of 2 bar held for 0.5 s yielded macroscopically visible flooded leaf areas up to 2 mm long. The physiological reactions, reflected by pH changes in the flooding solution were identical for both infusion areas.

Two modes of infusion were performed. Keeping the pipette in the flooded cavity maintained the flooded state, as solution taken up by the transpiring leaf tissue was replaced from the pipette reservoir (permanent infusion). Removing the pipette on liquid expulsion yielded the natural (air-filled) cavity state within a few minutes on infusion (transient infusion). In some leaves the flooding solution was taken up more slowly, or cavities remained flooded for experimental periods of 1 h, reflecting differences in the water demand of leaf tissue. Water demand, in turn, is a result of the water supply through the xylem vessels and the water loss through more-or-less open stomata. (The water potential of the extracellular solution appears to feed back on the extracellular voltage.)

Electrochemical microsensors are among the most promising tools to perform continuous monitoring of osmotica, metabolites, toxins and signalling compounds in intact organisms with excellent spatial resolution. The sample transmutes into an electrochemical cell, the quality of which determines the quality of the measurement. By means of a permanent infusion a convenient liquid cushion is created, which picks up the physiological activity not just of one, but of a number of cells around the substomatal cavity. The electrochemical sensors can be elegantly connected to this microreactor without advanced micromanipulator technology, which would be needed to gently approach a single cell.

Reproducible measurement of electrical potential in the extracellular solution

In potentiometric sensors, the potential difference between the sample solution and the internal electrode solution is used as a measure of activity of the analyte in the sample solution. Hence, in addition to the electrical potential of the internal electrolyte solution, the electrical potential of the sample solution has to be determined. As in previous investigations, the electrical potential of the extracellular solution was measured in relation to the electrical potential of the grounded leaf base (Hanstein & Felle, 1999; Felle *et al.*, 2000). For the sake of simplicity the potential difference between both positions is referred to as 'voltage' here.

Upon nanoinfusion, a sustained voltage drop by 27 ± 11 mV ($n = 39$) occurred. It took approx. 15 min until the voltage between the flooded cavities and the grounded leaf base disappeared. In parallel voltage recordings in two neighbouring flooded cavities connected by infusion solution, a pronounced voltage difference was obtained in several cases when crude electrode positioning was performed (see Materials and Methods; Fig. 2a, Difference). This finding was of great analytical importance as, because of space limitations, the voltage at the site of the pH microelectrode has to be inferred from a voltage analysis in a neighbouring cavity (see Materials and Methods). The duration of the undesired voltage difference between flooded cavities could be

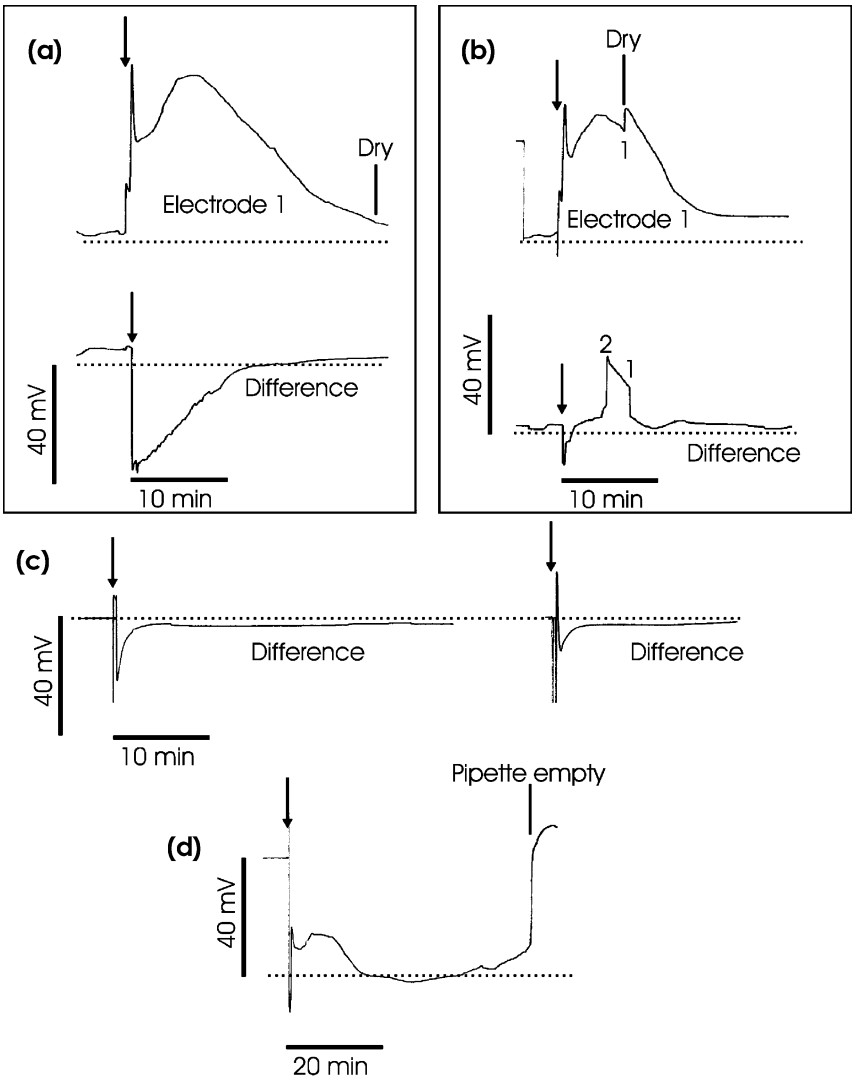

Fig. 2 Effects of nanoinfusion on electrical potential in the substomatal cavity of *Hordeum vulgare* primary leaves. The tip of a silanized micropipette was filled with a solution containing 3 mM KCl and 0.1 mM $CaCl_2$. A volume of approx. 30 nl was injected through a stomatal pore into three to five neighbouring substomatal cavities by applying a pressure pulse (1.1 bar for 500 ms) to the micropipette. The electrical potential was measured in relation to the potential of the solution, in which the cut leaf end was immersed. Dotted lines denote zero. (a) Crude electrode positioning at guard cells (see Materials and Methods) in combination with transient infusion. Upper trace: directly on expelling solution into cavities (arrow), the pipette was removed and the cavities dried out approx. 25 min later (Dry). Lower trace: To test for the reproducibility of the measurement, the potential was simultaneously measured with a second voltage electrode in a neighbouring flooded cavity. The signal of electrode 1 was automatically subtracted from the signal of electrode 2 by the electrometer, and the resulting difference was recorded as shown (Difference). In the case of full reproducibility, the difference should be zero (dotted line) over the whole experimental period. (b) Improved electrode positioning at guard cells (see Materials and Methods) in combination with transient infusion. Again the pipette was removed on expelling the solution and the cavities soon dried out (Dry). First the cavity of electrode 2 dried out (Difference, 2); then the cavity of electrode 1 (1). Note that the time between both events corresponds to a different flooding state at both electrodes (electrode 1, still flooded; electrode 2, not flooded). (c) Simple positioning of electrode tips in the free space of the cavity in combination with permanent infusion. The sensors were switched on at the moment of infusion, as no good electrical contact or no contact at all exists before infusion. Only the difference signal between two simultaneously recording voltage electrodes is shown. After the initial pressure pulse the pipette was left in the cavity, which enables permanent liquid supply through the pipette by cohesion and capillary forces. Two sequential infusions on different areas of the same leaf are displayed. (d) Potential measurement in a permanently flooded cavity, during which the infusion solution was completely consumed (Pipette empty). The signal upon 'Pipette empty' was recorded before the cavity finally dried out (n = 2).

substantially reduced by improved electrode positioning at guard cells with controlled 500 nm steps (Fig. 2b) or by simple electrode positioning in the open space of the cavity in permanently flooded cavities (Fig. 2c). Simple electrode positioning was used in the experiments of Figs 3–6. When flooding occurred only in the neighbouring cavity, not in the cavity where the electrode was placed, no voltage transients upon flooding were observed (n = 3).

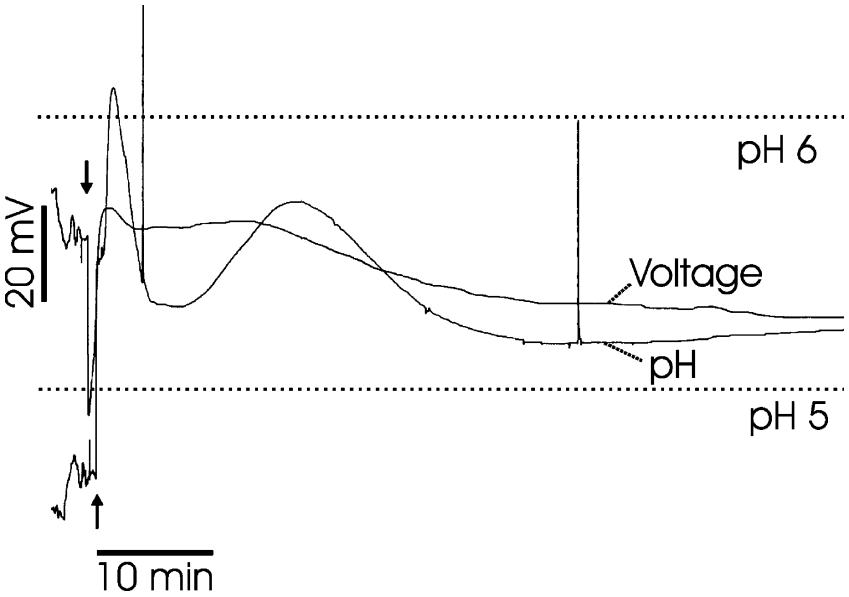

Fig. 3 Long-term voltage and pH monitoring in the substomatal cavity on permanent infusion with standard solution (3 mM KCl, 0.1 mM $CaCl_2$) in combination with simple electrode positioning (Fig. 2c). The infusion procedure is described in the legend to Fig. 2. Arrows indicate the start of infusion with respect to the voltage and the pH trace. The small horizontal offset between the two arrows, both representing the same event, is because original traces from a chart recorder are shown. (With simple electrode positioning the initial signals before infusion are of no analytical value because of poor electrode contact. Two spikes in the pH trace represent artifacts caused by interference from the power supply.)

A key observation on the extracellular voltage was that a sustained voltage drop in the order of 30–40 mV occurred after the solution in the pipette was consumed (Fig. 2d). It should be stressed that at the time of the voltage drop the cavities were still flooded, which rules out artifacts caused by changes at the electrode–solution interface. Similarly, a voltage drop often occurred at the moment when the cavity dried out (Fig. 2b, Dry). The equilibrium voltage in the extracellular solution of nonflooded cavities was approx. −34 mV, when improved electrode positioning techniques were used (Table 1, Transient infusion/improved positioning). Taking these observations together, the extracellular water potential appears to be an important factor of electrical potential in the extracellular solution. Maintaining a high water potential in flooded cavities by water supply through the pipette in the long term minimizes the potential difference between cavity and grounded leaf base (Table 1, Permanent infusion). Once the pipette water supply stops (Fig. 2d), the water potential in the cavities decreases because of permanent water uptake into the surrounding tissue, which then induces a net release of negative charges into the extracellular solution. Plasma membrane depolarization has been suggested to be involved in sensing of osmotic pressure changes in tomato cells (Felix *et al.*, 2000).

pH changes on nanoinfusion

Before applying microbial elicitors, we investigated the influence of nanoinfusion itself on the extracellular pH by infusing a standard solution that resembled the native extracellular solution (3 mM KCl, 0.1 mM $CaCl_2$). As Fig. 3 shows, two characteristic pH peaks occurred after infusion of standard solution, referred to as max 1 and max 2. Max 2 did not occur, when the initial pH was already in the region of 5.7. This finding illustrates that determination of the absolute pH is crucial for evaluation of elicitor effects, which implies proper measurement of the extracellular voltage. Furthermore, it is important to avoid a high extracellular pH, which occurs at a cell that is deformed by the sensor body. Could the observed pH oscillations result from mechanical stress exerted on guard cells by the electrode during the flooding procedure? In this respect it is noteworthy that the data shown in Fig. 3 were recorded during permanent infusion with electrode tips placed in the free space of the substomatal cavity; the sample liquid was in contact not only with guard cells, but with a larger number of cells surrounding the flooded cavity.

Table 2 lists pH kinetics observed with different infusion techniques. Compared with permanent infusion, transient infusion – in which the intercellular air space is restored within a few minutes – does not alter the pH kinetics. Furthermore, an infusion pressure of 2 bar proved not to burden the pH profile. A pressure above the standard value of 1.1 bar used in our experiments may be required when the intercellular air space connections between adjacent cavities are narrow, long or curvy.

The second pH maximum persisted (data not shown) regardless of several modifications of the infusion solution's composition: a KCl of 15 mM corresponding to KCl activities frequently reported from extracellular solution extracted from leaves; omission of $CaCl_2$, which is known to be a trigger of plasma membrane depolarization and to promote plant defence responses; higher osmotic potential by addition of 50 mM sorbitol, which has been routinely used to prevent water uptake from the extracellular solution into the vacuole during extraction of extracellular solution; measurement behind closed stomatal pores, which avoids a transient alkalinization originating from stomatal closure. We conclude that

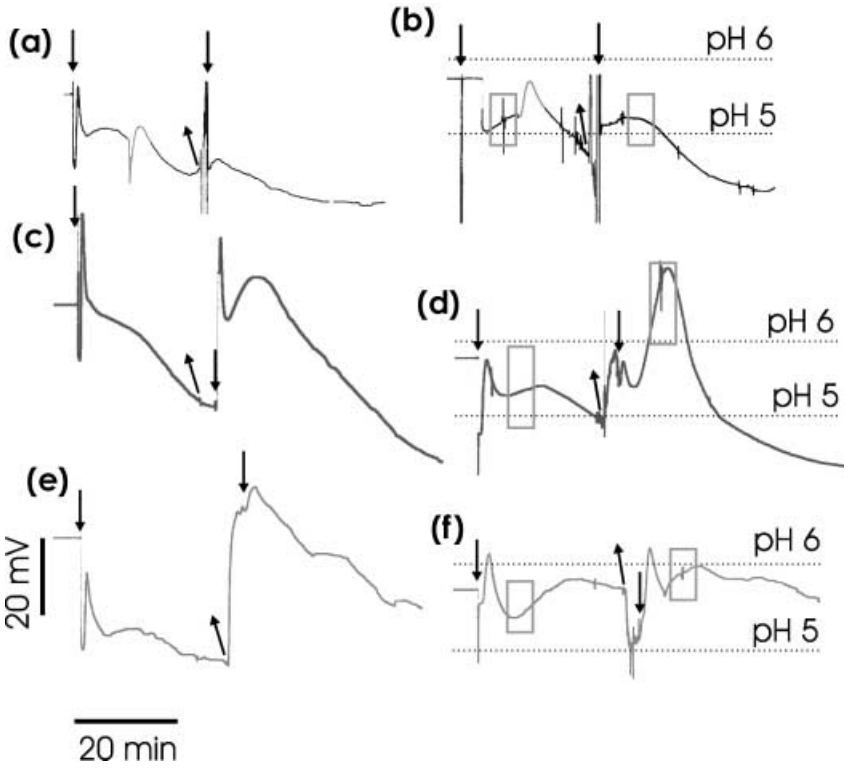

Fig. 4 Sequential infusions (vertical arrows) at the same leaf spot. A second permanent infusion was started when substomatal cavities were still flooded from the first infusion. The experiment is shown for three different leaves (top, middle, bottom curve pair). Traces on the left represent the extracellular electrical potential in the substomatal cavity (a,c,e); those on the right show the simultaneously recorded extracellular pH value (b,d,f). Electrode tips were simply positioned in the free space of neighbouring substomatal cavities. Electrodes were switched on at the moment of the first infusion (vertical arrows on the left of each trace). The first infusion was always performed with standard solution (3 mM KCl, 0.1 mM $CaCl_2$). Before the second infusion (second vertical arrow in each trace), the pipette had to be removed (upwards arrows). The first solution was expelled completely and the pipette repositioned in the still-flooded substomatal cavity. The second infusion was performed with standard solution (a,b) or after addition of octa-*N*-acetylchitooctaose at a concentration of 25 μM (c–f). To facilitate the comparison of infusion effects between subsequent infusions, time windows have been added to the pH trace representing the period between 6 and 11 min after starting the infusion. [The pH trace of (b) is affected by two artifacts. (1) The start of pH recording was delayed by 5 min as the cavity of the pH sensor had not been flooded. A signal overshoot when the sensor was switched on upon infusion (first arrow) showed that flooding was not successful. The sensor was switched off, repositioned in another flooded cavity, and switched on again. (2) The pronounced peak following the first time window results from mechanical irritation of guard cells by electrode vibration.]

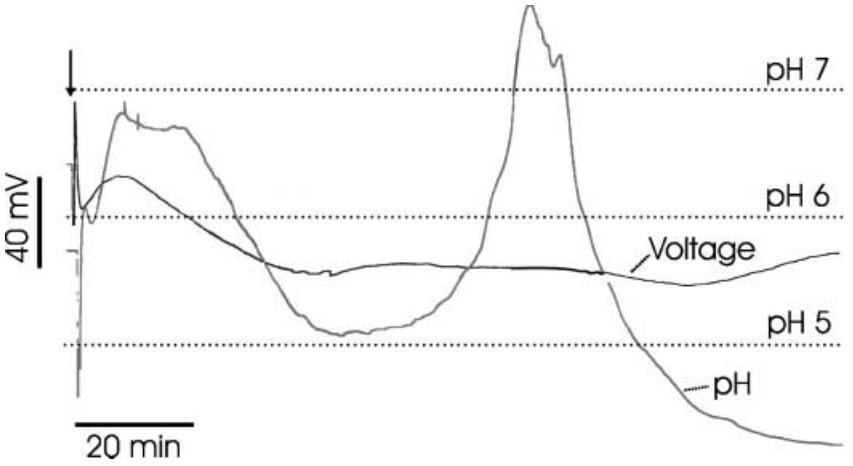

Fig. 5 Long-term voltage and pH monitoring in the substomatal cavity of *Hordeum vulgare* cv. Ingrid WT on permanent infusion with a solution containing 3 mM KCl, 0.1 mM $CaCL_2$ and 25 μM octa-*N*-actetylchitooctaose.

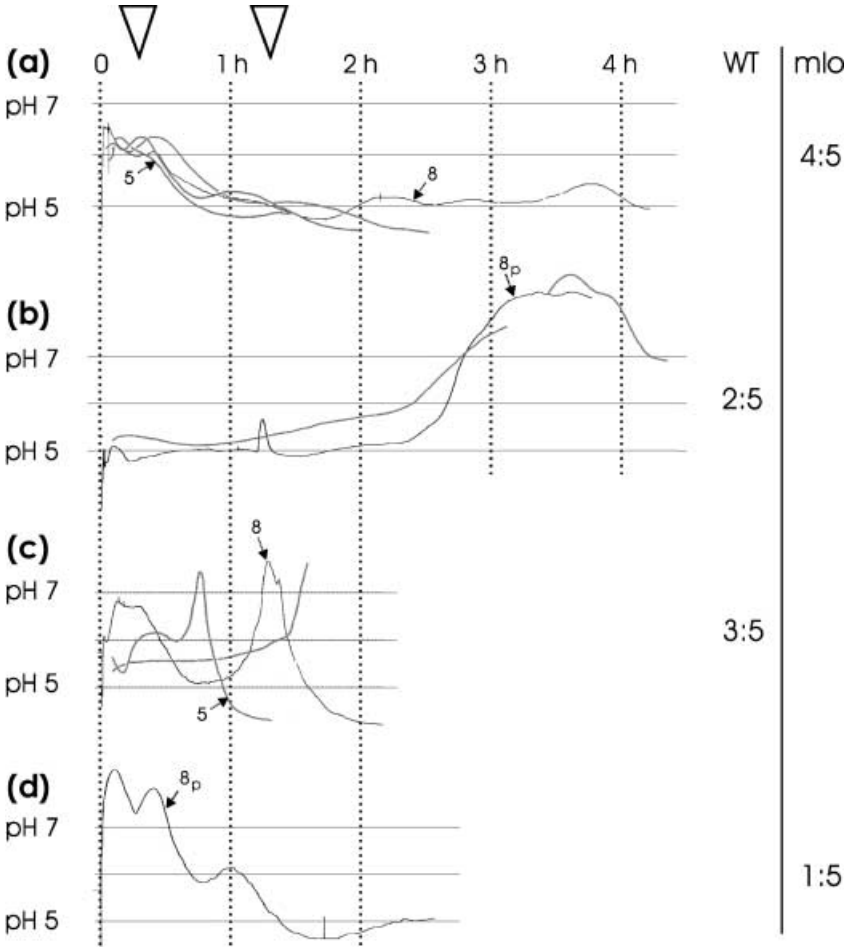

Fig. 6 Bandwidth of extracellular pH responses to chitin oligomers in primary leaves of near-isogenic lines of *Hordeum vulgare* cv. Ingrid (WT and *mlo* mutant). The four response types (a–d) are arranged according to increasing velocities of the alkaline transition. Oligomers were supplied by permanent nanoinfusion, as explained in the legend to Fig. 2. Numbers to the right of response types quantify the frequency of the response. Five primary leaves of each genotype were challenged by permanent infusion with a solution containing 25 μM chitin oligomers. Numbers close to the traces indicate the applied chitin oligomer: 8, octa-*N*-acetylchitooctaose; 5, penta-*N*-acetylchitopentaose; remaining traces, hepta-*N*-acetylchitoheptaose. Traces denoted by 8_P were obtained from a parallel analysis on the same day of two plants (WT, *mlo*) grown in the same nutrient solution. Thin black lines represent original recordings; bold grey lines were redrawn from originals.

Table 1 Extracellular voltage recording in the substomatal cavity of barley leaves (Hordeum vulgare cv. Ingrid)

Infusion treatment[a]	Polarization (mV)	Equilibrium voltage (mV)
Permanent infusion[b]	–25 ± 12 (21)	–4 ± 17 (21)
Transient infusion[c], crude positioning	–32 ± 13 (10)	–5 ± 11 (10)
Transient infusion[c], improved positioning	–29 ± 9 (8)	–34 ± 10 (8)

Transient voltage change induced by nanoliter infusion (polarization) and final equilibrium voltage. Average ± SE (number of replicates). [a]Standard infusion solution contained 3 mM KCl and 0.1 mM $CaCl_2$, and was delivered at a pressure of 1.1 bar held for 0.5 s. [b]Stationary pipette technique: voltage measured in infusion solution of flooded cavities. [c]Pipette removal technique: voltage recorded at guard cells.

Table 2 pH changes induced by nanoliter infusion into the substomatal cavity of barley leaves (Hordeum vulgare cv. Ingrid)

Infusion treatment[a]	Time of 2nd pH maximum (min)	2nd pH maximum	Equilibrium pH
Permanent infusion[b]	11.5 ± 6.8 (12)	5.68 ± 0.25 (12)	5.18 ± 0.26 (12)
Transient infusion[c]	13.8 ± 3.9 (6)	5.57 ± 0.27 (6)	5.05 ± 0.32 (4)
Pressure 2 bar	12.0 ± 4.2 (3)	5.67 ± 0.12 (3)	5.33 ± 0.05 (3)

Average ± SE (number of replicates). [a]Standard infusion solution contained 3 mM KCl and 0.1 mM $CaCl_2$, and was delivered at a pressure of 1.1 bar held for 0.5 s if not stated otherwise. [b]Stationary pipette technique, simple positioning: cavities remained flooded. [c]Pipette removal technique: pipette was removed directly on infusion and intercellular air space was restored within 15 min.

the pH oscillations of Fig. 3 represent a physiological response on flooding, and cannot be avoided even on infusion of a single cavity. As for the voltage oscillation, the pH oscillation remained restricted to the flooded cavity. No oscillations were observed in nonflooded neighbour cavities ($n = 3$). Extracellular alkalinization appears to be a common response to osmotic pressure changes (Felix *et al.*, 2000). With nanoinfusion, the equilibrium pH is restored after 30 min, while it takes several hours until the original pH is restored upon infiltration of the whole leaf (Felle & Hanstein, 2002). The equilibrium pH that soon establishes upon nanoinfusion was found to be stable for at least 4 h (data not shown). Accepting the transient pH effect of nanoinfusion as inevitable physiological background for elicitor studies in intact leaves, we point to permanent infusion as being superior to transient infusion because of the convenience of sensor positioning.

Variability upon nanoinfusion

Unstable positioning of microelectrodes may cause pH maxima (Fig. 4b). Thus avoiding unstable positioning is mandatory for proper results. It is crucial to check microscopically the stability of micromanipulators and of the leaf position for the given observation period. The remaining biological variability of the second pH maximum occurring during infusion of standard solution (Table 2, Permanent infusion, SE 0.25 pH units) suggests that several replicates have to be performed for comparisons between different infusion solutions. The degree of variability remains similar when different spots on the same leaf are probed (data not shown). As some stomata closed on infusion while others remained open, the standard errors given in Table 1 include the pH variability arising from contrasting guard cell ion transport (Felle *et al.*, 2000). Despite standard errors in the order of 0.3 pH units, small elicitor effects on the second pH maximum can be demonstrated in a single experiment on the background of a preceding control infusion at the same leaf location.

pH responses on elicitor nanoinfusion

Short-term responses The infusion pipette was filled with two solution portions separated by air; only the second solution contained elicitor. About 25 min after the first infusion (Fig. 4d, left arrow) the pipette was removed (upwards arrow), the rest of the first solution was expelled, and the pipette was again carefully placed in the previous cavity for the second infusion (Fig. 4d, right arrow). The small frames denote a time window between 6 and 11 min on infusion, which was selected to facilitate comparisons between experiments. *H. vulgare* cv. Ingrid WT displayed a pronounced increase in max 2 after addition of octa-*N*-actetylchitooctaose (Fig. 4d). Figure 4f shows an example of a small elicitor effect, which was observed in *H. vulgare* cv. Golf. The control experiment shown in Fig. 4a,b was performed with two identical infusion solutions; after the second infusion the pH remained close to 5.

Long-term responses Figure 5 demonstrates one example of the long-term response of *H. vulgare* cv. Ingrid WT on infusion of 25 μM octa-*N*-actetylchitooctaose. An impressive pH maximum of 7.7 occurred approx. 80 min after starting the infusion (max 3). As the flat voltage trace during max 3 shows, charge transport across the plasma membranes of adjacent cells was negligible. Figure 6 gives an overview of response types observed in 10 barley leaves. The four levels of Fig. 6a–d are arranged according to increased velocities of the alkaline transition. The complete absence of an alkaline transition over 4 h was found once (Ingrid *mlo*; Fig. 6a). Three further leaves (Ingrid *mlo*) were observed only for a period of 2 h, but were grouped in Fig. 6a because of their fast pH increase on the start of infusion. In two leaves (Ingrid WT) the neutral point was passed later than 120 min on the start of infusion. Three leaves (Ingrid WT) showed an increase in pH beyond 7 between 30 and 120 min after the start of infusion (Fig. 6c). In one instance an immediate, strong alkalinization was observed (Ingrid *mlo*; Fig. 6d).

Figure 6 points to differences between Ingrid WT and Ingrid *mlo* at the times indicated (triangles): The *mlo* mutant is characterized by four very similar measurements showing pH values around 6.2 approx. 20 min after the start of infusion (first triangle). One hour later (second triangle) all five replicates of the *mlo* mutant are in line, while WT is characterized by pH values ranging from 4.4 to 7.7. With more replicates and a different elicitor concentration, nanoinfusion could reveal differences in elicitor perception and signal transduction between WT and *mlo* mutant plants. Both genotypes display a considerably different resistance phenotype. The *mlo* mutant is more resistant to powdery mildew infections, and is successfully cultivated in European agriculture on a large scale (Büschges *et al.*, 1997; Piffanelli *et al.*, 2002). So far no bioassay has been reported that discriminates between WT and the *mlo* mutant within 2 h following elicitor application. Recently an infusion method was presented which discriminates between both genotypes 36 h after injecting toxins of *Bipolaris sorokiniana* (Kumar *et al.*, 2001).

Variability of elicitor responses

The pH increase to values > 7 occurred at variable times. Two experimental factors may have contributed to the variable long-term elicitor effect. (1) Leaves were from hydroponic plant cultures, and the nutrient solution was replaced weekly. Differences in leaf age and nutritional status may feed back to the elicitor response. (2) The flow rate from the micropipette into the leaf spot was variable. This can be derived from the observation that transient infusions lasted between 5 and 25 min (Fig. 2a,b, Dry). In order to control the elicitor dose during permanent infusion, the elicitor solution should be

replaced by standard infusion solution after starting the infusion (sequential infusion, see Materials and Methods). Furthermore, the plant parts outside of the small leaf cuvette (Fig. 1) should be kept in a standardized atmosphere to control the water status of the infusion area. When working with detached leaves, the easiest method is to cover these leaf parts with a wet cloth.

For screening purposes, only permanent infusion is feasible because the positioning procedure is completed within 30 min. An observation period of 60 min is required for an analysis focusing on the chemoperception process (Fig. 4), while an observation period of 120 min would be sufficient for evaluating the pattern and speed of secondary responses (Fig. 6). As leaves have to be equilibrated for 3 h in the leaf cuvette to open the stomatal pores, a batch of four leaves in different cuvettes could be prepared in the morning and investigated sequentially in the afternoon by one person. This given number of experiments per day cannot compete with high-throughput screening methods using suspension cultures (Boller, 1995). However, the strength of the nanoinfusion tool is that it enables direct analysis of the chemoperception capacity and of the elicitor response pattern in intact leaves, whenever basic differences in these parameters between suspension cultures and intact leaves cannot be ruled out.

Apart from experimental factors, the observed variability could indicate that the chosen chitin oligomer concentration was close to the physiological threshold level for triggering extracellular alkalinization. The use of this elicitor concentration in plants with induced resistance should yield a clear acceleration of the alkaline transition. Thus, for the given plant material, this concentration range would be suited to a sensitive bioassay on the success of resistance induction. In suspension-cultured cells, much lower chitin oligomer concentrations have been sufficient for triggering extracellular alkalinization (Kuchitsu *et al.*, 1997; Felix *et al.*, 1993). In these systems, responses occur within a few minutes. It remains to be elucidated whether leaf cells embedded in their natural environment tend to have higher threshold values and longer lag times for elicitation of defence responses. In cucumber hypocotyls it was clearly demonstrated that competence for elicitation of defence responses has to be induced (Kauss & Jeblick, 1996).

Perspectives

Modelling the transition to acute defence In order to stop invading pathogens, the plant leaf has to change its physiological characteristics at the plant–pathogen interface. The transition to the state of acute defence is controlled by signal transduction cascades (Suzuki, 2002) embedded in their specific metabolic and cellular environment. In the current situation of limited mechanistic and kinetic information on the intercellular interaction in intact leaves, a sensor-based input–output analysis combined with mathematical models would be useful to create new hypotheses for further experimental testing. In fact, models may uncover key physiological parameters with a large impact on the system output. This has been shown for growth processes on different system levels, for example for cell-cycle control (Borisuk & Tyson, 1998), or for activation of MAP kinase cascades (Schoeberl *et al.*, 2002). Model (software) development, and further development of the sensory hardware for measuring the variables, parameters and responses of the system, should benefit from each other. The extracellular pH in the substomatal cavity as an analytical starting point has been shown to be linked to induction of the oxidative burst; affects the degradation of the superoxide anion radical; and is, in turn, affected by the oxidative burst (Lamb & Dixon, 1997). Other variables to be determined in the future are H_2O_2 and ascorbate (Horemans *et al.*, 2000) (sensor development currently funded by the German ministry of education and research, BMBF grant 0312006A). Combining *in situ* oligo-parameter analysis, remote multicomponent analysis of extracellular solution sampled through micropipettes, and mathematical modeling tools could finally unravel the complex regulatory interaction of signaling molecules, antioxidants and proteins in the extracellular space of the intact leaf.

Application fields

The experimental approach presented in this paper uses the plant stomata to install fine measuring tools within the leaf for research purposes. These natural gates are sufficiently large in vine and poplar leaves, which are currently being extensively studied at the genetic level (Vitigen AG, Siebeldingen, Germany; Oak Ridge National Laboratory, Tennessee, USA; European Forest Genomics Network GenoSilva). European vine cultivars are prone to aggressive mildew diseases originating from the USA. Abandoned agricultural areas in Europe are discussed as areas for tree plantations as a sustainable energy resource (see expression of interest for the integrated project FORESTFUEL – http://eoi.cordis.lu/search_form.cfm, search term: FORESTFUEL). Vineyards and tree plantations would benefit from soft plant protection techniques. To date it has not been disproved that potent resistance inducers could be identified for dicot species, which could successfully compete with conventional pesticides on the market. The method presented here could provide a technological background to promote inducer development in these economically important fields.

Concluding remark

Technological progress has led Leroy Hood (Institute of Systems Biology, Seattle, USA) to start an initiative in systems biology aiming at integration of the exploding knowledge in the fields of genomics and proteomics. Kitano's overview of systems biology (Kitano, 2002) accounts for two basic

approaches to a system-level understanding – those coming from component analysis, and those coming from dynamic system analysis, both benefiting from joint efforts and from technical bridges to natural complexity.

Acknowledgements

This work was supported by the German Bundesministerium für Bildung und Forschung (grant 0312006A). The authors wish to thank Ralph Hueckelhoven and Karl-Heinz Kogel for helpful discussions.

References

Agrawal AA, Tuzun S, Bent E, eds. 1999. *Induced plant defenses against pathogens and herbivores. biochemistry, ecology, and agriculture.* St Paul, MN, USA: APS Press.

Boller T. 1995. Chemoperception of microbial signals in plant cells. *Annual Review of Plant Physiology and Plant Molecular Biology* **46**: 189–214.

Borisuk MT, Tyson JJ. 1998. Bifurcation analysis of a model of mitotic control in frog eggs. *Journal of Theoretical Biology* **195**: 69–85.

Büschges R, Hollrichter K, Panstruga R, Simons G, Wolter M, Frijters A, van Daelen R, van der Lee T, Diergaarde P, Groenendijk J, Töpsch S, Vos P, Salamini F, Schulze-Lefert P. 1997. The barley *MLO* gene: a novel control element of plant pathogen resistance. *Cell* **8**: 1793–1807.

Conrath U, Thulke O, Katz V, Schwindling S, Kohler A. 2001. Priming as a mechanism in induced systemic resistance of plants. *European Journal of Plant Pathology* **107**: 113–119.

Felix G, Regenass M, Boller T. 1993. Specific perception of subnanomolar concentrations of chitin fragments by tomato cells: induction of extracellular alkalinization, changes in protein phosphorylation, and establishment of a refractory state. *Plant Journal* **4**: 307–316.

Felix G, Regenass M, Boller T. 2000. Sensing of osmotic pressure changes in tomato cells. *Plant Physiology* **124**: 1169–1179.

Felle HH, Hanstein S. 2002. The apoplastic pH of the substomatal cavity of *Vicia faba* leaves and its regulation responding to different stress factors. *Journal of Experimental Botany* **53**: 73–82.

Felle HH, Hanstein S, Steinmeyer R, Hedrich R. 2000. Dynamics of ionic activities in the apoplast of the sub-stomatal cavity of intact *Vicia faba* leaves during stomatal closure evoked by ABA and darkness. *Plant Journal* **24**: 297–304.

Hammond-Kosack K, Jones JDG. 2000. Responses to plant pathogens. In: Buchanan BB, Gruissem W, Jones RL, eds. *Biochemistry and molecular biology of plants.* Bognor Regis, UK: John Wiley.

Hanstein S, Felle HH. 1999. The influence of atmospheric NH_3 on the apoplastic pH of green leaves: a non-invasive approach with pH-sensitive microelectrodes. *New Phytologist* **143**: 333–338.

Hanstein S, Felle HH. 2001. Miniaturised carbon dioxide sensor designed for measurements within plant leaves. *Sensors and Actuators B* **81**: 107–114.

Horemans N, Foyer CH, Asard H. 2000. Transport and action of ascorbate at the plant plasma membrane. *Trends in Plant Science* **5**: 263–267.

Kauss H, Jeblick W. 1996. Influence of salicylic acid on the induction of competence for H_2O_2 elicitation. Comparison of ergosterol with other elicitors. *Plant Physiology* **111**: 755–763.

Kitano H. 2002. Systems biology: a brief overview. *Science* **295**: 1662–1664.

Kuchitsu K, Yazaki Y, Sakano K, Shibuya N. 1997. Transient cytoplasmic pH change and ion fluxes through the plasma membrane in suspension-cultured rice cells triggered by *N*-acetylchitooligosaccharide elicitor. *Plant Cell Physiology* **38**: 1012–1018.

Kumar J, Hückelhoven R, Beckhove U, Nagarajan S, Kogel K-H. 2001. A compromised *MLO* pathway affects the response of barley to the necrotrophic fungus *Bipolaris sorokiniana* (teleomorph: *Cochliobolus sativus*) and its toxins. *Phytopathology* **91**: 127–133.

Lamb C, Dixon RA. 1997. The oxidative burst in plant disease resistance. *Annual Review of Plant Physiology and Plant Molecular Biology* **48**: 251–275.

van Loon LC. 2002. *Systematic induced resistance.* In: Slusarenko AJ, Fraser RSS, van Loon LC, eds. *Mechanisms of resistance to plant diseases.* Dordrecht, the Netherlands: Kluwer Academic.

Nürnberger T, Nennstiel D, Jabs T, Sacks WR, Hahlbrock K, Scheel D. 1994. High affinity binding of a fungal oligopeptide elicitor to parsley plasma membranes triggers multiple defense responses. *Cell* **78**: 449–460.

Piffanelli P, Zhou FS, Casais C, Orme J, Jarosch B, Schaffrath U, Collins NC, Panstruga R, Schulze-Lefert P. 2002. The barley *MLO* modulator of defense and cell death is responsive to biotic and abiotic stress stimuli. *Plant Physiology* **129**: 1076–1085.

Schoeberl B, Eichler-Jonsson C, Gilles ED, Muller G. 2002. Computational modeling of the dynamics of the MAP kinase cascade activated by surface and internalized EGF receptors. *Nature Biotechnology* **20**: 370–375.

Schönherr J, Bauer P. 1996. Effects of temperature, surfactants and other adjuvants on rates of uptake of organic compounds. In: Kerstiens G, ed. *Plant cuticles – an integrated functional approach.* Oxford, UK: BIOS Scientific Publishers, 134–154.

Shirasu K, Nakajima H, Rajasekhar VK, Dixon RA, Lamb C. 1996. Salicylic acid potentiation of an agonist-dependent gain control amplifying pathogen signals in the activation of defense mechanisms. *Plant Cell* **9**: 261–270.

Suzuki K. 2002. MAP kinase cascades in elicitor signal transduction. *Journal of Plant Research* **115**: 237–244.