Detection of natural plant activators

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Abstract:

Agriculture and forestry are utilizing transgenic approaches to improve plant performance. An important aspect of plant performance is resistance against herbivores and microbial pathogens. More than seventy years ago Chester (1933) demonstrated for plants that immunity to diseases can be induced. In a recent review Karban and Kuc (1999) ask whether we have recognized the potential contribution of simple, non patented, environmentally safe compounds as inducers of resistance. Noteworthy, utilizing an information code of a given crop or tree cultivar for resistance induction would yield broad spectrum resistance in this cultivar. Any successful defense process starts at the cellular level. At the margin of an attacked tissue site information on the cellular challenge or on the occurred damage must be submitted to neighbouring cells. This offers the chance for directly grasping messages from the intercellular space. The project will establish microanalytical methods for testing liquid obtained from the extracellular space at wound margins with respect to its potential to induce resistance. These methods focusing on the flow of chemical information between plant cells bear the potential for developing efficient, cultivar-specific activators of plant resistance. The project approach is based on the detection of extracellular signals as well as the detection of highly responsive genotypes. The project supports an integrated concept of pest management using genotypes together with their specific activators. Investigation of a broad range of cultivars is essential for the approach, whereas knowledge on responsible genes is not required. This renders the project competitive to transgenic approaches for improved resistance.

Detection of natural plant activators

1. Scientific background and novel chances

Several reviews have summarized the extensive knowledge about induced resistance in plants (e.g. Sticher et al., 1997; van Loon, 2000). Agricultural applications of plant activators have been presented by Karban and Baldwin (1997) and Lyon and Newton (1999). An example of commercial development of plant activators was described by Tally et al. (1999). 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. With the exception of tobacco, farmers in Europe and in the US are still more convinced by conventional pesticides.

The limited economic success of plant activators may be overcome by adopting the above-mentioned strategy of joint marketing of inducible cultivars together with their specific activators. The prospect for the detection of numerous natural activators is based on the wide distribution of induced resistance in various plant taxa. Against herbivores it has been shown in more than 100 plant species; against pathogens induced resistance was demonstrated in more than 30 species (Karban and Kuc, 1999). It is reasonable to assume that in the course of evolution a large number of genotypes was convergently realized, in which genotype-specific transport compounds contribute as natural activators to plant fitness.

2. Objectives

Based on progress in analytical chemistry this project focuses on the intercellular flow of chemical information within the plant leaf using indirect and direct methods. (1) Indirect methods are based on displaying the "chemical memory" (Bray, 1995) of leaf cells by bringing them into contact with a defense elicitor, a chemical compound which initiates defense responses. The focus will be on extracellular defense responses against pathogens which have been shown to be modified after plant immunisation with pathogens and with herbivores (Sticher et al., 1997; Conrath et al., 2001; Heil and Bostock, 2002). A prominent example of an endogeneous resistance activator is salicylic acid (Delaney et al., 1994; Kauss and Jeblick, 1995; Kästner et al., 1998; Tenhaken and Rübel, 1997). Another type of stimulus for defense responses arises from wounding (Graham and Graham, 1994). The extracellular space is readily accessible to state-of-the-art ion-sensitive microelectrodes and amperometric microsensors (Wang, 2000). Hence, speed and magnitude of extracellular defense responses can be monitored to read the "chemical memory" after preceding activator treatments. (2) Direct methods to uncover novel infochemicals will try to identify these compounds in extracellular fluids which have proven to induce resistance. A special focus will be on phenolic compounds (Lynn et al., 1990; Hammerschmidt and Smith-Becker, 1999).

The research of this project will be directed to willow and poplar, fast-growing woody plants, for three reasons: (1) These plants tend to have a high potential for induced resistance (Lambers et al., 1998) (2) They are cultivated for biomass production for energy (European Commission, 1997; Verwijst, 2001). The White Paper: "Energy for the future: Renewable sources of energy" aims at doubling the share of renewable energy in energy consumption from 6% in 1997 to 12% in 2010. Short rotation forestry on abandoned agricultural lands within the EU is one component of the strategy to reach this goal. (3) Fast-growing woody plants are threatened by various pests (e.g. blue willow beetle, gall midge, lepidoptera species, rust fungi, bacterial diseases caused by *Pseudomonas* and *Erwinia* species). Application of conventional pesticides in forestry is difficult and raises environmental concerns. On the other hand, public acceptance of short rotation forestry could be raised by conserving indigeneous species simply by utilizing them.

3. Scientific approach

3.1 Principal structure

The scientific approach starts with a two-step selection of appropriate genotypes. The first step will be guided by an analysis of phylogenitically proven success as documented by geographic plant distribution. Thus, genotype collections with indigeneous varieties will preferentially be considered, e.g. the Belgique collection of Black Poplars which were characterized in the EUROPOP project (Instituut voor Bosbouw en Wildbeheer, Geraardsbergen, Belgium). In a second selection step varieties will be investigated with respect to their ability to trigger a systemic resistance induction after herbivore or pathogen attack. The selection procedure is followed by microphysiological experiments to start the search for endogeneous molecules which are released from leaf cells upon wounding or pathogen attack and which accelerate and amplify defense responses within the leaf. These experiments will utilize micropipettes for liquid sampling and for liquid application. Local biochemical analysis will be performed at wound margins by using novel microsensors. The sensors will allow to detect, whether a sample liquid conveys alarming information to leaf cells and to what extent leaf cells are able to detect chemical signals. Online-monitoring of chemical changes at the wound margin will also direct the decision when samples should be collected for remote analysis. Remote analysis by mass spectrometry then will be used to uncover the chemical nature of activating compounds. Identified compounds will be validated in micropysiological experiments for their activator properties. The final test level within the project will be to study whether pretreatments with the identified compounds affect the plant susceptibility to a selected pest.

3.2 Herbivore experiments

The focus of the proposed study is on within-plant mechanisms of defense, yet it is on the level of the organism that plant protection must function. Rigorous testing of genotypes for their ability to trigger systemic-induced resistance (SIR) to different insect species will be conducted. SIR is often demonstrated through analyses of chemical compounds (e.g. Heil and Bostock, 2002). However, it must be emphasized that an induced response, i.e., induction of a particular chemical, does not necessarily mean that an induced resistance (i.e., reduced performance of the attacker) also exists (Larsson, 2002). Whether or not SIR results in enhanced resistance may depend on, e.g., the degree of host plant specialization in the attacker, other biochemical cascades acting antagonistcally, degree of inducer specificity, or virulent genotypes of the attacker (Walling, 2000; Hatcher et al., 2004).

One important part of the research will be on *Salix viminalis* and the monophaguous gall midge *Dasineura marginemtorquens*. There is an unusually strong, genetically determined, resistance in *S. viminalis* against this insect (Strong et al., 1993; Ollerstam et al., 2002). Totally resistant, susceptible, and intermediate (both living and dead larvae occurring on the same plant) genotypes occur within one and the same full-sib *S. viminalis* family. Resistant genotypes respond to gall initiation attempts with symptoms that are very similar to hypersensitive response (HR) (rapid induction of cell death, induction of salicylic acid, induction of hydrogen peroxide) (Ollerstam et al. 2002; Ollerstam & Larsson 2003). For the first time it will be investigated whether this local HR-like response is accompanied by the spreading of a systemic signal.

A second line of research will be on Black poplar clones and putative systemic induction by a leaf beetle, *Phratora vitellinae*. It is known that hybrid poplar (*Populus deltoids* X *P. nigra*) respond systemically to insect feeding by increased activity of cell wall invertase and condensed tannin biosynthesis (Arnold and Schultz, 2002). Whether or not this kind of induction results in increased resistance to subsequent feeding is however not known. Prior feeding by gypsy moth larvae in *Populus nigra* elicited a systemic plant response that retarded

performance of gypsy moth larva (Havill and Raffa, 1999; Glynn et al., 2003). Leaf beetles (*Phratora vitellinae*, *Chrysomela populi*, *C. tremulae*) are common pests in European poplar plantations. Variation exists among poplar clones with regard to constitutive resistance against these insects (e.g. Augustin et al., 1993), but it is not known if there is clonal variation in SIR. Leaf beetles will be employed as excellent model insects for studies of plant resistance in woody plants (Larsson et al., 1986; Denno et al., 1990; Häggström and Larsson, 1995).

3.3 Microphysiology

Creating wound situations

Two types of experimental wound situations in leaves will be employed, both requiring microscopy and micropositioning systems for pipettes and sensors. The first type is created by local flooding of the intercellular air space, a pathological situation which occurs at wound margins (Fink, 1999; Felix et al., 2000). Flooding of a few neighbouring substomatal cavities can be achieved without damaging any leaf cell by guiding solution through micropipettes which have been inserted in open stomal pores. This procedure has been demonstrated for barley leaves and is also suitable for poplar species because of their large stomata (Westerkamp and Demmelmeyer, 1997). The flooded substomatal cavity serves as a microreactor; biochemical changes in the reactor can be monitored with microsensors navigated through neighbouring pores (Hanstein and Felle, 2004). Due to small stomatal pores, flooding of substomatal cavities with micropipettes and insertion of microsensors is cumbersome for willow leaves. Thus the leaf has to be mechanically wounded. A cylindrical hole in a 300 µm thick leaf with a diameter of 300 µm filled with 20 nl solution will be an excellent microreactor in terms of pipette access and sensor access as well as sufficiently short diffusion pathways.

Chemical indicators of resistance state and defense stage

In order to display the readiness for defense after application of a natural activator, pathogen attacks will be simulated and the quickest known extracellular defense responses will be monitored: (1) changes in ion fluxes at the plasma membrane (Felix et al., 1993; Boller, 1995; Schweizer et al., 1996; Jabs et al., 1997; Kuchitsu et al., 1997; Hanstein and Felle, 2004) and (2) the oxidative burst driving rapid cell wall fortification (Bradley et al., 1992; Lamb and Dixon, 1997; Grabber et al., 2000). For both response manifestations it has been shown that they are accelerated and amplified after different types of sucessful resistance induction including wounding. Near open wound margins the cellular readiness for defense against pathogen invaders becomes particularly important. The ability to reinforce the cell wall upon first recognition of the pathogen has indeed a high impact on resistance (Barber and Ride, 1988; Barber et al., 1989; Matern et al., 1995; Lamb and Dixon, 1997; Sticher et al., 1997; Grabber et al., 2000; Niks and Rubiales, 2000; Hückelhoven and Kogel, 2003; Collins et al., 2003). In the project, pathogen attacks will be mimicked by delivering appropriate elicitors to leaf tissue. Four main parameters will then be monitored to evaluate the strength of the initial defense responses: extracellular voltage, pH, hydrogen peroxide and vitamin C as the most important extracellular antioxidant (Horemans et al., 2000). The first three parameters are also fast indicators whether an extracellularly applied solution contains chemical information for the investigated cell population.

3.4 Analytical tools (nanotechnology)

Commercially available sensors based on quartz-platinum fibers will be tailored for simultaneous analysis of extracellular voltage, pH, H_2O_2 and ascorbate with a single microelectrode. This novel tetrode will considerably save analysis time and will also be interesting to other application fields, e.g. in neuroscience. For preparation of the ascorbate sensor a novel composite polymer will be used which yields stable sensor performance in solutions with fluctuating pH and variable phenolics content. Miniaturisation of the tetrode will provide a unique tool to display defense stages in the flooded substomatal cavity (chemical eye). The key argument for preferring the small substomatal cavity as a microreactor to produce defense activators compared to wound holes is that it remains free from any cellular debris. Thus, the number of non-infochemicals in samples taken for activator identification from the cavity is reduced.

Measurement of pH and H_2O_2 will be based on nanostructured materials. Fabrication of nanostructured films will be performed via directed deposition within a liquid crystal (LC) template (Attard et al., 1997) which yields a highly porous metal layer. The pore diameter is typically 2-5 nm and the pore walls are 2-5 nm thick, while their depth depends on the charge passed during electrodeposition and can reach micrometers. Their electroactive area is up to three orders of magnitude greater than that of the bare metal electrode, indicating that the pores are entirely accessible to the solution. This huge area yields unprecedented properties beneficial to analytical measurements. For example, nanostructured microdiscs were found to retain the diffusion typical of microelectrodes but with much increased electrocatalytic activity and dramatic improvements were observed for the amperometric detection of hydrogen peroxide (Evans et al., 2002). Nanoscale liquid chromatography in combination with mass spectrometry (nano-LC-ESI-MS) or comparable techniques will be used for remote analysis of phenolics in nanoliter samples taken from the extracellular solution of stimulated cell populations (see Wuhrer et al., 2004, as an example of femtomole detection of oligosaccharides).

3.5 Risk elements

Failure to induce resistance: Even lack of systemic induced resistance in any of the investigated genotypes will leave phenomena of locally induced resistance (LIR) for exploitation. LIR does not spread through the whole plant but still involves mediator molecules of physiological transformation in the neighbourhood of a pest attack. In microphysiological experiments, the risk of failure in inducing resistance will be minimized by applying various test configurations including different nutritional state and age of test leaves, different elicitors (Felix et al., 1993; Schweizer et al., 1996), extracellular fluids from various sources and various defense stages as activator candidates (ranging from extracellular solution of defending tissue to collected xylem sap) and exogeneous activators (e.g. derived from commercially available fungal and algal products).

Failure to detect the altered cellular defense state by means of extracellular observations after a simulated pathogen attack: Four important extracellular defense parameters will be used to characterize the cellular "behaviour". If resistance induction still remained hidden, two basic escape routes could be used: expanding the extracellular chemical tongue for simultaneous monitoring of seven different chemical parameters (heptode microelectrodes, pattern recognition tools) or directing the biotest to intracellular compounds.

Failure to miniaturize the tetrode for the flooded substomatal cavity: Experiments with wound holes will act as a surrogate approach, in which the commercially available tetrode size will be used.

Failure to detect activating components in samples taken from the substomatal cavity: If the secreted amount of signaling compound is too small, the wound hole approach has to be used to

collect samples. If the active ingredients within an activating extracellular cocktail cannot be identified at all, upscaled preparations of cocktails have to be used for validation in pest experiments.

Risks associated with activator preparation for validation in microphysiological and pest experiments: It is planned to upscale preparation of plant wound cocktails in order to have sufficient material for further purification of active ingredients. An escape strategy will be to synthesize identified active ingredients, which will require a satellite project.

4. Project impact

Developing a conventional plant protectant costs around 160 million US-Dollar (Lyon and Newton, 1999). Development of the first plant activator by the route outlined in this proposal will require 1 million US-Dollar. Subsequent developments using established analytical tools should be possible for 0.2 million US-Dollar per activator. Activators will be derived from and marketed for region-specific cultivars. Incurring costs for activator development will pay off by a higher farming profit in the long-term due to the long-term stability of plant protection by resistance induction. The investments will support high-tech enterprises producing microanalytical equipment, applied ecology research centers and microphysiology service laboratories.

Market segments: The first segment addressed with this project, forestry, will continue to develop from the increasing need to produce renewable energy and to counteract global warming. Protection of food crops would also benefit from the discovery of additional endogeneous resistance activators. Substantial breeding programs are conducted in order to improve pathogen resistance in crop plants. The German government spends at least 250 million EURO per year for green gene technology (http://deutschland.dasvonmorgen.de/pub/bufo2000_englisch.pdf). In companies an equal amount is spent for biotechnology research in the areas of agriculture and food (Moch and Tappeser, 2002). However, in a number of crop species commercial biotechnology approaches do not yield durable pathogen resistance, e.g. in cereals against mildew and rust fungi (Niks and Rubiales, 2002).

Plant protection based on knowledge and exploitation of endogeneous plant signals will reduce pesticide application. The integrated strategy of promoting region-specific plant varieties together with their endogeneous activators will utilize and conserve regional genetic resources.

5. Resources

Estimated total project cost: 990,000 EURO
Total requested grant to the budget: 930,000 EURO

Partner	Personnel resources (man-month)	Cost items exceeding 100,000 EURO
HE-1	46	220,000 [1)]
HE-2	36	
RES-3	36	

[1)] Subcontract to SME (sensor development)

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