THESIS OF DOCTORAL DISSERTATION

Abiotic and biotic stress effects on barley and tobacco plants

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BACKGROUND AND OBJECTIVES

Introduction

Empirical studies show that the human species – apart from a negligibly scarce number of light eating specimens – can be considered to be heterotrophic, thus relies basically on the ingestion and processing of carbon-fixing autotrophic plants to sustain its life processes. Moreover, it does not only use plants directly, for consumption, but also – in a rather energy wasting manner – indirectly, already pre-processed by other heterotrophic organisms at the cost of great energy losses (feed production). It also relies on plants in order to be able to change its environment (construction materials), to provide its ecological optimum (heating), furthermore, to practice specific behavioural patterns (travel, "bio"-fuel, ethanol) characteristic to this sole species only, that are far beyond the basic natural needs concerning subsistence and race preservation. The human species proliferating on the Earth at an ever increasing rate requires constantly growing quantities of plant material produced on an agriculturally cultivable area of rather limited size, meanwhile climatic conditions are changing in a less and less favourable manner (salinity, aridity), caused exactly by the many environmentally harmful human activities. The fact that other species (pests and pathogens) utilise the larger proportion of plants produced by humans much quicker before it can, gives rise to further problems. Even though there is constant demand for larger crop yield, the Earth can not be exposed to an ever growing quantity of artificial fertilisers and pesticides – or else we need to urgently search for another planet to live on. It is thus unquestionably essential to strive at better understanding how plants can tolerate environmental stresses and resist pathogen attack, to develop environmentally acceptable plant production and protection methods that can help to compensate for yield losses caused by environmental damage to plants.

Oxidative stress and antioxidants

Abiotic and biotic stresses originating from the environment that cause tissue damage to plants practically all result in the rapid accumulation of reactive oxygen species (ROS) leading to an oxidative microenvironment in cells. The protection of cells from damage caused by the different reactive oxygen compounds (e.g. superoxide, hydroxyl radical, hydrogen peroxide, singlet oxygen) is the important task of antioxidant systems in plants. ROS produced in plant cells – that play important roles in signal transduction processes under stress-free conditions – can be detoxified through several ways: either by electron donors such as glutathione, ascorbate, carotenoids and tocopherol, or via chemical reactions catalysed by enzymes like superoxide dismutase, peroxidases, glutathione S-transferase and the enzymes of the ascorbate−glutathione cycle.

The concentration and activity of enzymatic and non-enzymatic antioxidants significantly changes with the physiological state of plants; the age, the availability of nutrients, or the existence of a response to an earlier stress, greatly influence their oxidative stress tolerance. The antioxidant capacity of plants and thus tolerance to oxidative stress normally diminishes with ageing. The senescence of tissues can be artificially retarded: juvenility of plants is increased by large nitrate nitrogen doses, decapitation or treatment by cytokinins, or the introduction of genes into the plant genome that increase antioxidant capacity directly or through the augmentation of juvenility.

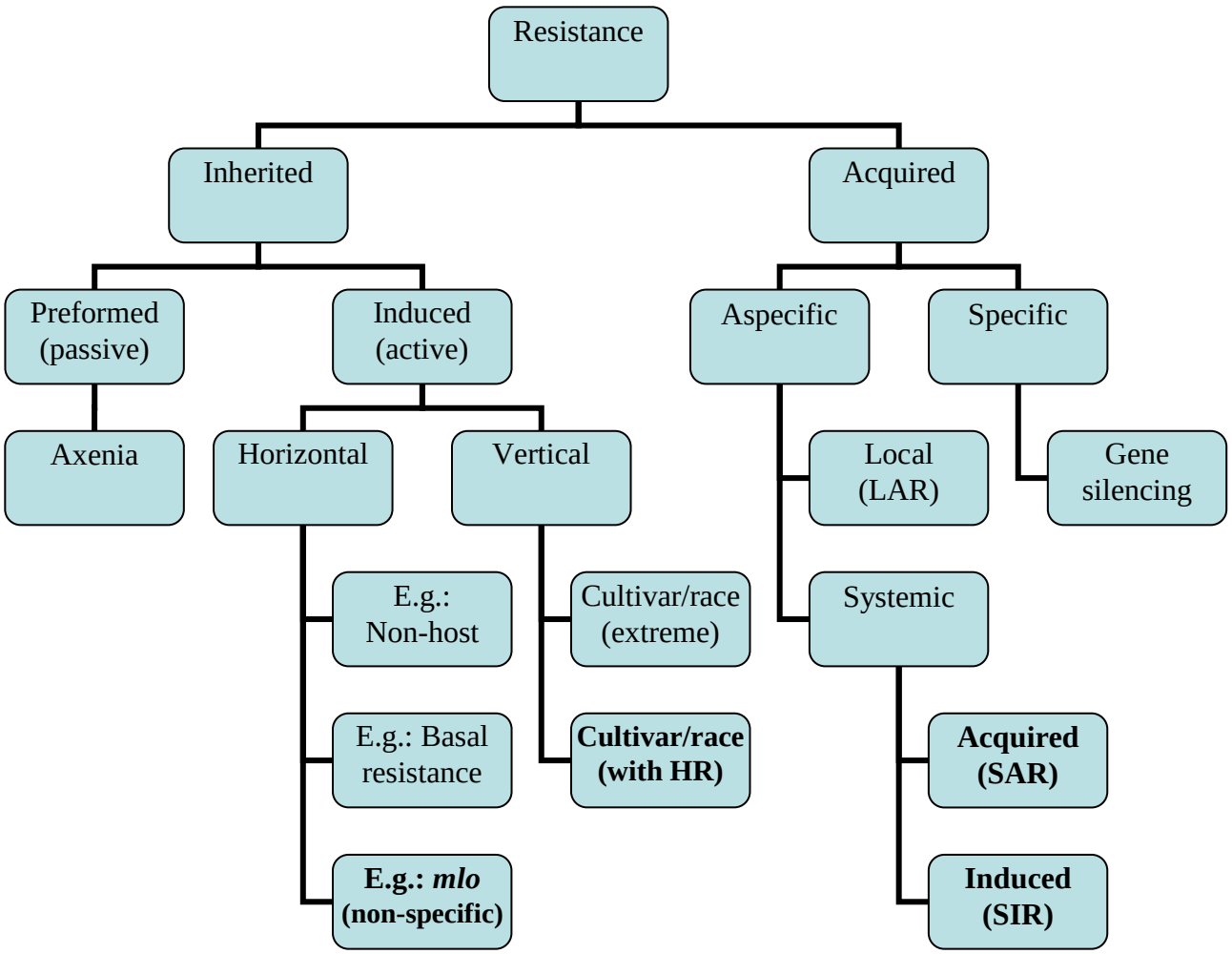
Abiotic stress

A typical example of abiotic stress is oxidative damage caused to plants by heavy metals. Over the last decades different human activities resulted in increased heavy metal accumulation in soils of industrial zones and cities world-wide. Heavy metals can interfere with the activity of certain enzymes in several ways: either through direct inhibition by the metal or through the indirect effect of other processes such as changes typical under oxidative stress conditions. This results in increased hydrogen peroxide production and lipid peroxidation in both leaves and roots.

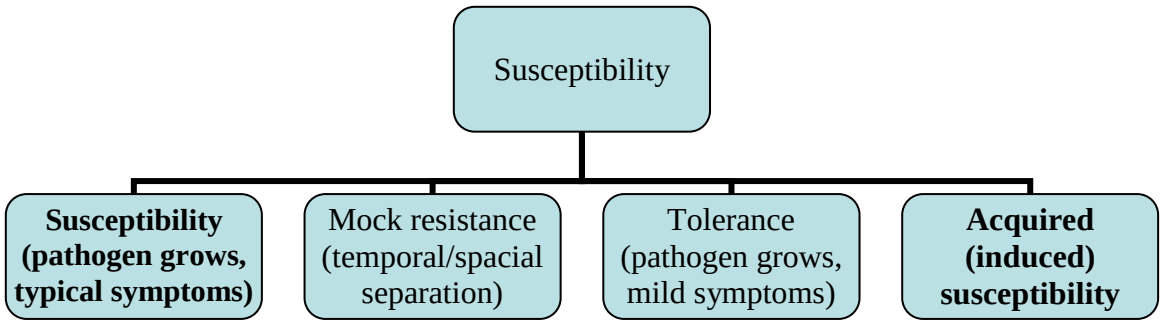
Salt stress is another abiotic stress of anthropogenic origin that poses problems increasing parallel with the growth of areas affected by aridity. High salt concentrations in soil and irrigation water are a major threat to agricultural production in arid and semiarid regions. The presence of excess ions in the rhizosphere causes injury to plant roots, followed by their gradual accumulation in the aerial parts with heavy damage to plant metabolism, which leads to stunted growth and reduced yield. Plants have developed complex mechanisms to avoid NaCl toxicity and low water potential in soil caused by salinity as well as drought. Furthermore, mutualistic symbiosis with mycorrhizal and endophytic fungi can confer salt tolerance to plants and decrease yield losses in cultivated crops grown in saline soils.

Biotic stress: plant–pathogen interactions

The many different possible outcomes of plant–pathogen encounters can be, of course, classified in several ways. Basically, a certain plant can be resistant to a given pathogen (incompatible interaction; the most common case in nature) or susceptible to it (compatible interaction; this occurs much less frequently). The different forms of plant resistance and types of susceptibility to pathogens are illustrated in the following figures:



Types of plant resistance



Types of plant susceptibility

The resistance and susceptibility forms in bold letters are the ones that are dealt with in this present study, so I will only focus future attention on discussion of details about these.

Vertical (race specific) and horizontal (race-nonspecific) inherited resistance

Several types of inherited resistance occur in cultivated barley cultivars against the *Blumeria graminis* f. sp. *hordei* (*Bgh*) fungus responsible for the powdery mildew disease of barley. This biotrophic parasitic fungus keeps host plant cells alive during infection and favours young tissues that provide the fungus optimal environment for growth and proliferation. The genes *Mla12* and *Mlg* mediate race specific (vertical) resistance to *Bgh* race A6, while the recessive *mlo5* allele of the *Mlo* locus confers complete, race-nonspecific (horizontal) resistance to barley against various barley powdery mildew isolates. These genes interfere with different stages of fungal development, thereby resulting in distinctive interaction phenotypes. Functional alleles of the *Mla* locus mediate fungal arrest by inducing a hypersensitive reaction (HR) in the underlying mesophyll cells after the fungal penetration of the target cell. The gene *Mlg* mediates resistance via single-cell HR of the attacked epidermal cells, while the gene *mlo5* controls penetration resistance through an effective papilla formation, leaving the attacked cell alive. On the other hand, on susceptible barley leaves, powdery mildew spores germinate, grow an appressorium and penetrate epidermal cells where fungal haustoria, needed for the uptake of nutrients from the plant cells, are formed. Later the surface of the plant is covered with hyphae of powdery mildew that eventually sporulates.

One of the earliest responses of plant cells to pathogen attack is the accumulation of reactive oxygen species, such as superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2). Specific staining and microscopic analysis have indicated that powdery mildew infection can induce a strong, early accumulation of H_2O_2 in attacked leaves of Mla-, Mlg- and mlo-type resistant, but not in susceptible barley lines. Here we addressed the question whether these differences in accumulation of H_2O_2 can be confirmed by quantitative methods. We further aimed at investigating the antioxidant changes accompanying powdery mildew inoculation of the resistant barley lines and the susceptible cultivar.

Systemic acquired resistance

Local infections in plants often induce a systemic response that affects the whole plant and this wide-spectrum resistance can last for weeks or months against viral, bacterial and fungal pathogens. This form of induced disease resistance is called systemic acquired resistance (SAR). An interesting feature of this resistance form is that infection by different pathogens induces the same type of resistance, suggesting that common routes are involved in the many different pathogen recognition pathways during the reaction processes induced in the plant. The phenomenon of SAR is correlated tightly with the rise in salicylic acid content and the accumulation of PR (pathogenesis-related) proteins.

Tobacco mosaic virus (TMV) infection is localized in resistant tobacco plants, such as *Nicotiana tabacum* L. cv. Xanthi-nc that contains the *N* gene for hypersensitive response. A

coordinated programmed cell death in virus-inoculated leaves of Xanthi-nc plants is accompanied by the restriction of virus proliferation to small zones around the sites of infection and by the induction of SAR. The first TMV infection of Xanthi-nc tobacco enhances the localizing response to a second attack by TMV, resulting in the appearance of fewer and smaller lesions on the leaves distal to the primary infection site. SAR depends on the accumulation of salicylic acid, which is produced locally and systemically throughout the plant after TMV inoculation. Our earlier results show that increased antioxidant activities also contribute to the attenuation of necrotic symptoms during SAR.

Xanthi-nc tobacco plants expressing the bacterial *nahG* gene encoding salicylate hydroxylase, an enzyme that converts salicylic acid to catechol, contain reduced amounts of salicylic acid. Expression of *nahG* gene is sufficient to abolish the SAR response in these transgenic plants. NahG tobacco plants are rendered more susceptible to TMV, characterised by larger necrotic lesions as compared to the wild-type Xanthi-nc plants, indicating that increased production of salicylic acid is critical for resistance to TMV.

Systemic induced resistance

Symbiosis with mycorrhizal and endophytic fungi can confer tolerance to plants against stress originating from unfavourable environmental conditions. The mutually beneficial relationship can result in systemic induced resistance of the plant against pathogen attack. Recently, a root-endophytic basidiomycete, *Piriformospora indica*, has been shown to improve plant resistance against root and leaf diseases and alleviate salt stress in barley. We studied metabolic activity and antioxidant defence induced by the endophyte during the development of systemic induced resistance in barley.

Acquired (induced) susceptibility

A previous stress can often render plants resistant to a later following second stress, however, in certain cases, the opposite holds true. In barley cv. 'Ingrid' susceptible to powdery mildew and in near-isogenic resistant barley lines, susceptibility can be increased and resistance can be broken to induce susceptibility to *Bgh* by heat shock treatment.

Objectives

1. One aim of our work was to study the correlation between the physiological state (juvenility or senescence) of tobacco plants and their resistance against oxidative stress of abiotic origin either caused by heavy metal stress (mercuric chloride), or by externally applied hydrogen peroxide.

2 We compared biochemical responses (metabolic activity, fatty acid composition, lipid peroxidation) to TMV infection in Xanthi-nc tobacco and its salicylic acid-deficient transgenic line (NahG) which can not develop a SAR response to TMV inoculation.

3. Parallel to the quantitative determination of hydrogen peroxide production in response to powdery mildew inoculation, our goal was to gain more information on the ascorbate–glutathione cycle in barley defence reactions against powdery mildew in susceptible cv. Ingrid and near-isogenic, resistant lines. We were also interested in whether accumulation of H_2O_2 correlates with membrane damage and with the accumulation of ethylene, a secondary messenger plant hormone.

4. Biochemical mechanisms underlying *P. indica*-mediated salt tolerance were studied in barley. Physiological markers for salt stress, such as metabolic activity, fatty acid composition and lipid peroxidation were assessed to characterise the response of salt tolerant and salt sensitive barley cultivars to strong salt stress with or without symbiosis with *P. indica*.

5. We studied the heat shock induced susceptibility to *Blumeria graminis* f.sp. *hordei* in the resistant mlo barley. After exposing mlo barely to heat treatment and *Bgh*-inoculation, the symptomless complete resistance was broken and we investigated the changes in the expression of several genes known to play a role in plant defence responses by RT-PCR technique.

MATERIALS AND METHODS

Tobacco

Several tobacco cultivars were grown in soil under greenhouse conditions until exposure to different treatments or until time of sampling, for the purpose of subjection to comparative study in the following pairs:

a) *Nicotiana tabacum* L. cv. Samsun: older leaves (in the fifth position from the hypocotyl) versus younger leaves (in the tenth position).

a) *Nicotiana tabacum* L. cv. Petit Havana SR1 (SR1 from now on) and its cytokinin-overproducing transgenic line (CTKm). Here, older (the fifth) leaves were used for experiments in both cases. CTKm tobacco was created in the Plant Protection Institute of the Hungarian Academy of Sciences by transformation with *Agrobacterium tumefaciens* GV 3101 (pMP9 ORK) containing CaMVp35S *ipt*. The *ipt* gene encodes isopentenyl transferase, a key enzyme in cytokinin biosynthesis.

c) *Nicotiana tabacum* L. cv. Xanthi-nc (Xanthi from now on), containing the *N* gene from *Nicotiana glutinosa* for resistance against TMV and its transgenic NahG line that is impaired in its ability to accumulate salicylic acid. The transgenic Xanthi-nc/NahG-10 tobacco line (NahG from now on) was provided by NOVARTIS Agricultural Biotechnology Center (Research Triangle Park, NC, USA).

Barley

a) For inoculation with barley powdery mildew *Hordeum vulgare* L. cv. Ingrid, and the backcross lines Ingrid Mla12, Ingrid Mlg and Ingrid mlo5 (kindly supplied by Lisa Munk from the University of Copenhagen, Denmark) were grown in soil under greenhouse conditions.

b) Seeds of salt-sensitive barley cv. Ingrid and salt-tolerant cultivar California Mariout were used in our experiments concerning salt stress. After surface sterilisation seeds were germinated for two days, then one part of the germinating seeds was transferred to pots and grown in expanded clay in a growth chamber.

Symbiotic treatment

The other part of the seeds was inoculated with the endophytic fungus *Piriformospora indica*: developing roots of 2-day-old germinating seeds were immersed in *P. indica*-homogenate before transferring to pots and grown under the same conditions. *P. indica* was propagated in liquid *Aspergillus* minimal medium. Root colonisation was determined microscopically in 1-week-old plants after staining root fragments with acid fuchsin in lactoglycerol.

Abiotic stressors

a) **Mercuric chloride** solution was applied to the surface of young and old Samsun tobacco leaves. 24 hours after the treatment with $HgCl_2$ leaf discs were cut from plants for assays.

b) Leaf discs were cut from young and old leaves of Samsun tobacco and from SR1 and CTKm tobacco, and then exposed to oxidative stress by exogenous **hydrogen peroxide** through floating them on H_2O_2 solutions of different concentrations.

c) Uncolonised and *P. indica*-infected Ingrid and California Mariout barley plants were exposed to **salt treatment** from the age of 3 weeks, continuously bottom-watered with sterile water containing 100 or 300 mm NaCl for 2 weeks.

d) **Heat shock treatment** (49°C for 45 sec) was performed on 7-day-old Ingrid, Mla, Mlg and mlo barley. One part of the plants was inoculated with *Bgh* 24 hours later.

Biotic stressors

a) ***Tobacco mosaic virus*** (TMV) strain U1 was inoculated onto resistant Xanthi and salicylic acid-deficient transgenic NahG tobacco plants. The third and fourth true leaves of 8-week-old tobacco plants were mock-inoculated or inoculated with TMV. A subsequent TMV challenge was applied on the upper leaves (fifth and sixth leaf positions above the hypocotyl) 2 weeks later. All measurements were performed on the upper fifth and sixth true leaves of 10-week-old plants.

b) **Barley powdery mildew fungus** (*Blumeria graminis* f. sp. *hordei* race A6) was inoculated onto 7-day-old Ingrid, Mla, Mlg and mlo barley plants. Our measurements were all performed on primary leaves.

Investigation of membrane damage

a) Oxidative damage caused to plant cell membranes by senescence, stress or disease, and the accompanying increase membrane permeability was detected by the amount of **electrolytes leaking** from the cells. The conductivity of the bathing solution of leaf discs or segments was recorded at several time points with a conductometer.

b) Lipid peroxidation was detected through the analysis an end product; thermally produced **ethane**. In tobacco or barley leaf samples *in situ* decomposition of ω -3 unsaturated hydroperoxy fatty acids into ethane was accelerated by a brief heat treatment. Ethane content of samples was analysed in a gas chromatograph compared to standard ethane gas.

Analysis of ethylene by gas chromatography

Gas samples taken for ethylene determination from test tubes containing primary leaves of powdery mildew-inoculated and healthy barley plants was analysed by gas chromatography.

Lipid analysis

Total lipid was extracted from leaf tissues with chloroform-methanol. After phase separation the organic phase was dried under nitrogen and resuspended in chloroform, fractionated by silicic acid column chromatography, and methyl esthers of fatty acids were prepared. Phospholipids were analysed by gas chromatography for fatty acid composition. An internal standard of heptadecanoic acid (C17:0) was added to extracts. Sterols were acetylated by using acetic anhydride and pyridine and separated using cholestan as an internal standard.

Calorimetry

Leaf segments were cut from tobacco or barley for calorimetric measurements. Maximum emission rate of heat in mW was calculated per g dry weight basis.

Hydrogen peroxide content measured by fluorometry

The fluorescent compound 2′,7′-dichlorofluorescein is produced from the oxidation of 2,7′-dichlorofluorescin diacetate (DCFH-DA) by H_2O_2 in the presence of cellular peroxidases. Solution of DCFH-DA was introduced into intercellular spaces of barley leaves by vacuum infiltration, and then the relative fluorescence of leaf extracts was measured by spectrofluorometer.

Spectrophotometric determination of enzymatic and non-enzymatic antioxidants

Activities of superoxide dismutase (SOD), guaiacol-dependent peroxidases (POX), catalase (CAT), ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), glutathione reductase (GR) and glutathione S-transferase enzymes were detected in plant tissue extracts spectrophotometrically. Concentration of ascorbate and dehydroascorbate was determined using ascorbate oxidase enzyme, oxidised and reduced forms of glutathione were measured employing GR enzyme.

Gene expression measurements

Total plant RNA was extracted from barley leaves on silica membrane column after guanidine isothiocyanate lyses. Quantity and quality of RNA was inspected spectrophotometrically and by gel electrophoresis. To detect low copy number transcripts we used semi-quantitative RT-PCR method. The amplification of cDNA was terminated in the exponential phase, that is, in case of each gene we determined the PCR cycle number that allowed for visualisation of expression differences between samples after ethidium bromide staining. We analysed the following genes by RT-PCR: *HvHsp70*, *BAX inhibitor 1* (*BI-1*), *HvWRKY1* and *HvWRKY2*.

RESULTS AND DISCUSSION

The correlation between juvenility and high antioxidant capacity

Compared to older leaves, young tissues in plants have been observed to exhibit augmented tolerance to biotic stress caused by necrotrophic pathogens, so we investigated the effect of senescence in case of abiotic stress caused by heavy metal ions or by direct oxidative stress. Heavy metal stress such as mercuric chloride treatment not only inhibits growth in plants by direct toxicity, but also causes oxidative damage to plant cells through the generation of reactive oxygen species. We exposed young and old leaves of Samsun tobacco to oxidative stress by externally applied mercuric chloride or hydrogen peroxide treatment. Membrane integrity as an indicator of oxidative damage caused to plant cells by various abiotic stresses was measured by the ion leakage method.

Electrolyte leakage as an indicator of membrane damage caused by mercuric chloride-induced heavy metal stress produced a much less significant increase in electrolyte leakage on young leaves than it did on old ones compared to their water treated controls. Similarly we have observed that membrane damage caused by hydrogen peroxide (externally applied ROS, that is, direct oxidative stress) resulted in much weaker electrolyte leakage from young leaves than from old ones. Parallel to higher stress tolerance, we have found that the H_2O_2-detoxifying catalase enzyme was twice as active in young leaves as in old leaves. Another key enzyme of ROS-detoxification, superoxide dismutase, that catalyses the breakdown of superoxide into hydrogen peroxide and oxygen, was also 50% more active in young leaves. These results are in accordance with the greater antioxidant capacity of younger leaves. Supporting these previous data, membrane integrity cytokinin-overproducing CTKm tobacco tissues with inhibited senescence was less affected by hydrogen peroxide treatment than that of the control, non-transgenic SR1 tobacco leaves. The *ipt* gene inserted after a constitutive promoter providing continuous gene expression in CTKm tobacco, encodes isopentenyl transferase, a key enzyme in cytokinin biosynthesis, so CTKm plants are characterised by constant cytokinin-overproduction. This results in long-term juvenility of CTKm tobacco. Our earlier results have shown enhanced antioxidant capacity in CTKm tobacco as well, that is probably responsible for the higher degree of stress tolerance.

In conclusion the above data strengthened our hypothesis that juvenile plant tissues with higher antioxidant capacity can better tolerate oxidative damage generated by biotic or abiotic stressors.

The role of salicylic acid in systemic acquired resistance of tobacco

Previous reports have shown that TMV inoculation of Xanthi-nc tobacco leaves results in local and systemic increases in salicylic acid and is associated with changes in heat production, lipid

peroxidation and lipid composition. The aim of this study was to characterise the role of salicylic acid in these metabolic responses with the help of a salicylic acid-deficient NahG transgenic line of Xanthi-nc tobacco. NahG tobacco plants expressing the bacterial *nahG* gene (from *Pseudomonas putida*) encoding salicylate hydroxylase, an enzyme that converts salicylic acid to catechol (1,2-dihydroxyphenol), are not able to accumulate salicylic acid.

The rise of endogenous salicylic acid in TMV-inoculated leaves of Xanthi-nc tobacco is associated with thermogenesis and energy expenditure. High level of salicylic acid, either externally applied or accumulated upon TMV infection, has also been observed to induce an increase in lipid peroxidation in tobacco. As to lipid composition, the saturation level of fatty acids decreased in wheat plants during frost hardening in correlation with an increase in salicylic acid content. The involvement of lipids and lipid compositional dynamics in SAR in plants is also supported by genetic studies in *Arabidopsis thaliana* suggesting a role for fatty acid desaturase activity. These experiments provide evidence for roles of salicylic acid in thermogenesis, lipid peroxidation and composition as well.

Since membranes play a crucial role in HR of plant cells, **fatty acid composition** of the phospholipid fraction and **sterol composition** were determined by gas chromatography. The degree of unsaturation of fatty acids has been shown not to be affected by the induction of SAR in Xanthi-nc plants. We have also found that TMV inoculation itself did not change significantly the fatty acid composition in either of the tobacco lines. Only in non-inoculated plants did we find a lower proportion of trienoic fatty acid in NahG transgenic plants as compared to Xanthi tobacco. It has been shown with transgenic methods that a decrease in the ratio of trienoic fatty acids increases TMV-induced lesions as compared to wild type plants. As regarding sterol composition, salicylic acid-deficient NahG plants contained slightly higher levels of campesterol than Xanthi plants. Virus infection did not affect the sterol composition in leaves of Xanthi plants. However, ratio of stigmasterol to sitosterol increased in NahG plants by almost 40% as a result of TMV inoculation. Since aging of tobacco leaves has also been found to be accompanied by an increase in the ratio of stigmasterol to sitosterol, our result suggests that TMV inoculation induces senescence in NahG plants. Changes in lipid composition can affect membrane fluidity and permeability, but fluorescence polarisation measurements showed that untreated Xanthi and NahG plants, as well as Xanthi plants that had established SAR maintained their appropriate membrane fluidity.

Lipid peroxidation and heat production have been known to accompany the hypersensitive response of tobacco plants to TMV infection. In order to further elucidate the role of salicylic acid in lipid peroxidation and thermogenicity, we analysed ethane and heat emission in TMV-inoculated leaves of Xanthi tobacco plants expressing SAR and of transgenic NahG tobacco plants that are

impaired in the SAR response. This system is useful for examination of salicylic acid-mediated and salicylic acid-independent responses of tobacco to TMV inoculation.

It has been demonstrated earlier that the severity of hypersensitive symptoms in TMV-inoculated tobacco leaves is closely correlated with the level of lipid peroxidation. In contrast, we found that lipid peroxidation is comparable in TMV-inoculated leaves of Xanthi and NahG tobacco plants as demonstrated by the **emission of ethane**. Furthermore, ethane release was similar in Xanthi plants after the primary and secondary inoculation with TMV, although we expected lipid peroxidation, like HR symptoms, to decrease in tobacco plants after establishment of SAR. These results support previous observations suggesting that salicylic acid potentiates the oxidative burst and increases cell death program in combination with reactive oxygen species. Accordingly, activation of SAR by TMV inoculation induced single-cell microscopic lesions that appeared on both uninfected leaf areas and distal leaves of TMV-inoculated Xanthi tobacco plants. Our results indicate that ethane emission may arise from TMV-induced single-cell lesions in addition to that from visible necrotic spots. This hypothesis is supported by evidence that salicylic acid and its biologically active analogues induced a dose-dependent increase of lipid peroxidation in tobacco. Furthermore, level of ethane released from uninfected leaves of NahG plants was lower by 30% than that in Xanthi tobacco. Consistent with this result, catechol has been found to be a potent inhibitor of lipoxygenase activity in mammalian cells. Increased lipoxygenase activity is characteristic of TMV-inoculated leaves of hypersensitively reacting tobacco plants and lipoxygenase-mediated lipid peroxidation has been found to be responsible for about 95% of the total lipid peroxidation in TMV-infected Xanthi-nc tobacco leaves. Therefore, we need to consider that salicylic acid-dependent and salicylic acid-independent processes equally contribute to lipid peroxidation in TMV-inoculated tobacco leaves at the time when hypersensitive lesions appear.

An enhanced heat production has previously been observed in TMV-infected leaves of Xanthi-nc tobacco plants during the formation of TMV-induced necrotic lesions. In TMV-infected tobacco, the development of necrotic lesions is known to be accompanied by an increase in oxygen uptake and an oxidative burst. We have shown previously that the oxidative burst is correlated with the number and size of TMV-induced necrotic lesions in our TMV–tobacco system. **Exothermic heat production** reflects the general metabolic activity of tobacco leaf tissue; therefore our results obtained by calorimetry consist of both salicylic acid-dependent and salicylic acid-independent heat effluxes. The amount of heat emitted by non-inoculated leaves of control NahG plants was significantly lower than that of Xanthi tobacco. Two to three days after inoculation when TMV-induced necrotic symptoms have already developed, heat efflux was apparently induced in the virus-inoculated leaves. Rates of heat flow from the TMV-inoculated leaves of NahG plants and Xanthi plants expressing or not expressing SAR were not significantly different, in spite of the large

differences in visible necrotic leaf damage. Salicylic acid itself has been demonstrated to induce heat efflux caused by higher oxygen consumption through stimulation of the alternative respiratory pathway and by the disruption of the proton gradient. The elevated level of heat efflux detected in the TMV-inoculated NahG leaves indicates that heat production can be independent of salicylic acid. We assume that the low rate of heat flow caused by a shortage of salicylic acid in NahG tobacco can be compensated for by a strong oxidative burst. In contrast, heat efflux augmented by elevated salicylic acid levels in Xanthi plants may be compensated for by a down-regulated oxidative burst. This hypothesis is supported by previous studies from this laboratory showing that the higher the rate of TMV-induced tissue necrotisation, the higher the level of oxidative burst in tobacco leaves.

In conclusion, a substantial body of evidence indicates that salicylic acid can induce lipid peroxidation and heat efflux. Therefore, the visual inspection of TMV-induced necrotic symptoms can yield misleading conclusions about the benefit of SAR in tobacco. We assume that the biological cost of mild tissue damage in TMV-inoculated tobacco leaves expressing SAR may be comparable to that of severe tissue damage in NahG tobacco impaired in their ability to mount an SAR response. Energy requirements of resistance responses can impose costly detrimental effects on plants resulting in early senescence or retarded growth.

Antioxidant changes in different barley−powdery mildew interactions

Bgh-induced changes in the antioxidant status of barley have been studied extensively in the past decades, especially in susceptible and Mla-type resistant plants. The novelty of our study is to have extended these observations to the poorly studied Mlg and mlo backcross barley lines. Activities of antioxidant enzymes were assayed in whole-leaf extracts following *Bgh*-inoculation at “early” stages of the interaction: samples were taken after 15 hours (before fungal penetration of epidermal cells), 30 hours (after penetration attempt) and 60 hours (after establishment of pathogenesis or HR). To characterise “late” stages of the pathogenesis we took leaf samples after 4 days (before fungal sporulation), 5 days and 7 days (after sporulation).

Most of the tested antioxidant enzymes (POX, SOD, APX, GST and GR) excluding catalase and DHAR showed similar activity changes following powdery mildew inoculation. After 5 to 7 days, the activities of POX, SOD, APX, GST and GR were significantly induced in susceptible barley compared to its uninfected control. As compared to susceptible plants, we found very similar but less pronounced changes of enzyme activities in near-isogenic resistant Mla plants. One can suppose that this is due to the similar initial development of *Bgh* in the susceptible and the Mla-type resistant barley plants during the first 30 hours of infection. In the Mlg and mlo lines even weaker, or no induction of SOD, GST, APX and GR was detectable upon inoculation.

We found a definite induction of **POX activity** in all of the barley lines after *Bgh*-inoculation. POX activity increased most markedly (to more than 5-fold by the later stages of infection) in attacked susceptible barley leaves, in Mla and Mlg leaves it peaked at 4-fold activity 5 days after inoculation, while in mlo at 2-fold of the non-inoculated control 3 days after inoculation. Earlier spectrophotometric and native gel electrophoretic enzyme activity measurements have shown a pronounced and continuously growing induction of POX over a time course of 1 to 5 days following *Bgh*-inoculation both in susceptible and in Mla-type resistant barley. We may thus conclude that the role of peroxidases in defence responses does not seem to be specific to the interaction type.

Along the time course no markedly significant changes in CAT activity could be observed in any of the tested barley lines. Other researchers have published slightly controversial results on *Bgh*-induced catalase activity in susceptible and Mla-type resistant barley, but nevertheless, their measurements were all performed within the first 24 hours after inoculation. However, in other earlier results concerning wheat, no changes in catalase activity have been found after inoculation with powdery mildew (*Blumeria graminis* f. sp. *tritici*).

There are earlier reports on the increase of monodehydroascorbate reductase and APX enzyme activity in susceptible barley 4 days after *Bgh*-inoculation, which was not detectable in the Mla line. **APX enzyme activity** was also enhanced by the later time points in our experiments, **DHAR activity**, on the other hand, transiently declined in powdery mildew-attacked susceptible Ingrid, Mla and Mlg plants, reaching its minimum 4 days after inoculation in the susceptible and 5 days after inoculation in the Mla and Mlg lines. In the mlo line DHAR activity did not change significantly. Our findings, that DHAR activity decreased in the susceptible interaction 4 days after inoculation and then recovered, are consistent with earlier results and suggest that in our case the regeneration of ascorbate takes place through monodehydroascorbate reductase activity, because activity of this enzyme may counteract the low DHAR activity, keeping the oxidation of ascorbate under control.

We found that powdery mildew inoculation did not result in significant changes of ascorbate levels or in the redox state in either resistant or susceptible Ingrid barley plants at the later stages of the interaction. Earlier studies demonstrated unchanged total foliar ascorbate content 24 hours after *Bgh*-infection with a slight shift towards the reduced from in the susceptible line, but not in the Mla-type resistant barley. Others have found a 50-60% decrease of ascorbate levels 4 to 6 days after inoculation in highly susceptible barley cultivar Emir attacked by powdery mildew fungus. Resistance to powdery mildew has been shown to correlate with increased foliar glutathione content 18 to 24 hours after inoculation. In our study, total glutathione content increased significantly 7

days after inoculation in the susceptible leaves, whereas other researchers have observed such an increase in glutathione concentration 4 days after powdery mildew attack.

The early **accumulation of H_2O_2** in barley leaves is well documented in response to powdery mildew, whereas changes in H_2O_2 levels at later stages of interaction has so far been poorly investigated. Upon inoculation with powdery mildew, we found slightly elevated H_2O_2 levels in Mla, Mlg and mlo barley lines, however in susceptible barley leaves H_2O_2 concentration was reduced significantly 7 days after inoculation. Enhanced H_2O_2 production seems to be closely associated with plant defence against the powdery mildew fungus; furthermore, it has been demonstrated to play an important role in plant resistance to other pathogens as well. The external application of ROS sources has been shown to protect susceptible plants from powdery mildew and rust fungi by inhibiting or killing the pathogens. Toxic effects of ROS to the pathogen can be prevented by the simultaneous application of antioxidants. The hypothesis that H_2O_2 may play a pivotal role in resistance of barley to powdery mildew is further supported by our finding that in contrast to resistant barley lines, H_2O_2 level decreased in leaves of susceptible Ingrid plants after inoculation with powdery mildew fungus.

There was no correlation between accumulation of H_2O_2 and **membrane damage measured by leakage of electrolytes**. This is probably due to the minimal number of affected cells compared to the whole leaf in the resistant interactions. In case of the *Mla*-mediated resistance, a slight increase of leakage was detected, but Mlg and mlo lines showed no increase in electrolyte efflux after powdery mildew attack. On the other hand, two days' growth of the fungus was enough to induce significant electrolyte leakage, and 5 days after infection the leakage was about 8 times higher from infected than from control leaf segments of susceptible Ingrid plants.

Production of the ethylene stress hormone showed a similar tendency as ion leakage in powdery mildew-attacked barley leaves. Probably again due to the limited number of affected cells, no changes of ethylene production were detected in Mlg and mlo plants and only a slight increase was found in Mla leaves even at 4 days after inoculation. However, at only 3 days after inoculation, significantly more ethylene was produced in powdery mildew-attacked susceptible leaves than in control, uninfected leaves. In accordance with our results, in powdery mildew-infected wheat varieties, exhibiting various levels of quantitative resistance, ethylene evolution was correlated with the number of pustules on the leaves inoculated with *Blumeria graminis* f. sp. *tritici*.

It is also noteworthy that POX activity and leakage of electrolytes was significantly higher in case of the uninfected mlo barley as compared to other tested barley lines bearing the wild-type *Mlo* gene (susceptible, Mla and Mlg barley), strengthening earlier observations that mutation of the *Mlo* gene induces early senescence of barley leaves.

In conclusion, powdery mildew inoculation resulted in a significant induction of antioxidant defence in the susceptible barley line. One can suppose that this up-regulation of antioxidants could prevent the accumulation of ROS in powdery mildew-attacked susceptible plants. Indeed, we found decreased H_2O_2 levels in leaves of infected susceptible barley. Based on our observations we can not exclude the possibility that the powdery mildew fungus itself produces antioxidant substances or enzymes to defend itself against ROS. This idea is supported by previous findings detecting extracellular catalase secretion from haustoria of *Bgh* during infection of barley. We could not find higher catalase activity in powdery mildew-infected susceptible barley; however, we measured catalase activity in total leaf extracts and not locally, at the host-pathogen interface. The question, whether high antioxidant activities in mildewed susceptible barley were generated mainly by the plant cells or by the fungus, awaits further investigations.

Salt stress tolerance of barley mediated by symbiosis

Cultivated barley is a relatively salt-tolerant crop but there is a rather high variability among barley cultivars in this trait. Two contrasting genotypes, the salt-tolerant cultivar California Mariout and the salt-sensitive cultivar Ingrid were chosen for this study to define antioxidant responses.

Recently, a root-endophytic basidiomycete, *Piriformospora indica* has been shown to improve plant resistance against root and leaf diseases and alleviate moderate salt stress (100 mM NaCl) in barley. Here we could show that *P. indica* protects barley even from high salt stress (300 mM NaCl). In order to get a better understanding of the impact of *P. indica* on the establishment of salt tolerance, we assessed biochemical markers for salt stress such as metabolic activity, fatty acid composition and lipid peroxidation.

Previous studies have demonstrated a salt-induced increase in lipid peroxidation and a marked reduction in metabolic heat production in salt-sensitive plants, while these parameters were unaltered in salt-tolerant cultivars. Thus, calorimetrical determination of heat output can serve as a valuable tool for screening plants for salt tolerance. Lipid peroxidation is associated with cellular membrane damage elicited by salinity stress. NaCl treatment resulted in higher rates of lipid peroxidation in salt-sensitive plants than in salt-tolerant cultivars. These observations suggest that the rate of lipid peroxidation can also be used to characterize how effectively *P. indica*-treated plants cope with salt stress. Fatty acid desaturation is associated with salt stress in plants as well: it has been found previously that ω-3 desaturase genes are induced in roots of maize under high salt conditions. In agreement with this result, it has been shown that linolenic acid plays a pivotal role in the tolerance of tobacco plants to salt stress. Therefore, composition of fatty acids was analysed in leaves of uncolonised and *P. indica*-colonised salt-sensitive barley plants under salt stress conditions to characterize fatty acid desaturation.

Barley plants irrigated with saline water for 2 weeks showed stunted growth and underwent early senescence. High salt stress (300 mM NaCl) caused a substantial decline in **shoot fresh weight** of every type of plant in the experiments: uncolonised and *P. indica*-colonised cv. Ingrid and cv. California Mariout plants as well. Compared to uncolonised plants, shoot fresh weight of *P. indica*-colonised barley cv. Ingrid was enhanced about two-fold under both control and saline conditions. Among plants grown in highly saline environment, shoot fresh weight of salt-tolerant cv. California Mariout was significantly higher compared to the uncolonised cv. Ingrid, but the highest shoot biomass production was detected in *P. indica*-colonised Ingrid plants.

We provide clear evidence in our present study that salt-induced responses indicated by heat emission and ethane production in the *P. indica*-infected salt-sensitive barley cv. Ingrid resemble those found in salinity-tolerant plants. While infection of roots with *P. indica* did not cause significant changes in metabolic activity of leaves under non-saline conditions, our calorimetric studies indicated that the **rate of metabolic heat production** of leaf samples was reduced by about 30% when Ingrid plants were exposed to 300 mM NaCl for 2 weeks, however, it increased in leaves of *P. indica*-infected plants after salt treatment. Therefore, the endophyte seemed to overcompensate the salt-induced inhibition of leaf metabolic activity. Previous results have shown that the extent of natural herbicide-resistance of wild oat biotypes is tightly correlated with the rate of heat production upon herbicide exposure due to the activation of metabolic pathways required for defence responses. This suggests that enhanced tolerance to salt stress can be associated with higher metabolic activity in *P. indica*-colonised barley.

Previous studies have shown that exogenously applied unsaturated fatty acids can protect barley during NaCl-induced stress. Thus, lipid desaturation could be an important component of plant tolerance in response to salt stress. *P. indica* colonisation leads to a significant reduction in the proportion of oleic acid in barley leaves, as it was previously found in salt-treated barley roots. Similar to salinity, *P. indic*-infection slightly increased the proportion of C18:3 fatty acid in the phospholipid fraction isolated from barley leaves. With one exception (C16:1), *P. indica* induces changes in **fatty acid compostion** similar to those induced by salinity. Such effects on the fatty acid composition of host plants may display a symbiotic adaptive strategy mediated by the endophyte to cope with salt stress in hostile environments. We speculate that *P. indica* might induce similar effects on fatty acid composition of the host plants in its original habitat, the arid Thar desert.

High salinity stress induced the peroxidation of membrane lipids as demonstrated by the emission of thermally produced ethane derived from the decomposition of the 16-hydroperoxide of linolenic acid. *P. indica* itself did not affect the emission of ethane from leaves of cv. Ingrid. Salt-induced lipid peroxidation was, on the other hand, significantly attenuated in *P. indica*-treated plants. Cellular membrane damage due to salt stress is associated with an accumulation of ROS.

According to a recent report, endophytic fungi characterized by their broad host ranges can confer effective tolerance to ROS under abiotic stress conditions such as salinity.

An increasing body of evidence suggests that high salinity induces oxidative stress in plants that is at least partly responsible for tissue damage. Several studies have demonstrated that salinity increases antioxidant activities of salt-tolerant plants above the levels found in salt-sensitive plants. It has been previously shown that *P. indica* also induces antioxidants: the **amount of ascorbic acid**, the ratio of reduced to oxidized ascorbate and the activity of dehydroascorbate reductase were elevated in barley roots. Since ascorbate was not found in *P. indica*, we can assume that the fungus induces the accumulation of ascorbate in plant root cells. Under high salt stress condition, *P. indica*-infected Ingrid plants maintained efficient redox balance of ascorbate and contained higher ascorbate level than the unsalinized control, although the concentration of reduced ascorbate decreased over time in roots of salt-treated infected plants. Strikingly, ascorbate content and the ratio of reduced to oxidized ascorbate dramatically decreased in roots of salt-treated uninfected plants as soon as after 1 week of salt exposure. These findings are consistent with those that show that ascorbate content or the ratio of ascorbate to DHA decreased in the salt sensitive plants under salt stress, while it declined to a lesser extent only or even increased in salt tolerant plants. Earlier studies have suggested that tolerance of plants to salt stress is associated with the induction of antioxidant enzymes. We found that NaCl increased the activities of the examined antioxidant enzymes in roots of salt-stressed barley. Although enzyme activities decreased after an initial induction in both salt-sensitive and tolerant plants, their decline was delayed and less pronounced in *P. indica*-colonised Ingrid barley and in the salt-tolerant cv. California Mariout.

The mechanism responsible for *P. indica*-mediated up-regulation of the plant antioxidant system is not known. It has been shown recently that *P. indica* is able to produce auxin when associated with plant roots. On the other hand, *P. indica* increased the amount of methionine synthase which plays a crucial role in biosynthesis of polyamines and ethylene. Transgenic tobacco plants overproducing polyamines also have enhanced tolerance toward salt stress, and salt treatment induces antioxidant enzymes. *Sebacina vermifera*, an endophyte closely related to *P. indica* down-regulates ethylene production in *Nicotiana attenuata*. Interestingly, our preliminary results suggest that *P. indica* induces ethylene biosynthesis in barley roots. Ethylene signalling may be required for plant salt tolerance and ethylene may induce some antioxidant enzymes when plants are exposed to heat stress. However, further experiments are necessary to clarify the function of phytohormones in *P. indica*-induced salt tolerance in barley.

In conclusion, our results demonstrated that high saline environment is well tolerated by salt-sensitive barley when previously inoculated with the mutualistic basidiomycete *P. indica*. This endophyte appears to confer tolerance to salt stress, at least partly, through the up-regulation of

ascorbate and antioxidant enzymes. Our observations are only correlative but supported by the fact that elevated antioxidant activities are also demonstrated under saline conditions in barley cv. California Mariout which is genetically tolerant to salt. However, several possible symbiotic mechanisms could account for salt tolerance. For example, root endophytes may act as a biological mediator allowing symbiotic plants to activate stress response systems more rapidly and strongly than non-symbiotic plants. Since *P. indica* has a broad host range among both monocot and eudicot plants and can easily be propagated in axenic culture on a large scale, we emphasize the high potential of the endophyte in protecting crops against salt stress in arid and semiarid agricultural regions.

Gene expression studies in heat shock-induced powdery mildew susceptibility

An earlier stress such as heat shock can develop resistance in barley to powdery mildew infection. However, it is very important to note that the exact parameters of the heat treatment greatly influence its outcome. In our experiments, the temperature and duration of heat, and the time elapsed between the heat shock and inoculation lead to an exactly opposite result: susceptible 'Ingrid' barley became even more heavily infected by *Bgh*, that is, we found more sporulating powdery mildew colonies on the heat-treated and inoculated primary leaves. In Mla barley with HR-accompanied resistance the size of necrotic lesions became larger, and a certain degree of sporulation was observed. Mlg-resistance that is manifested through single-cell HR of the attacked epidermal cells was also broken by our treatment. The most surprising result, however, was the successful infection of barley carrying the gene *mlo* with complete, wide-spectrum resistance. So, we initiated investigations of gene expression changes in the mlo barley to gain information on underlying molecular processes.

Transcription of our first gene of interest, the gene of the Hsp70 heat shock protein was stimulated already 24 hours after heat treatment, and was induced in an additive manner by the two types of treatment. Similarly, the gene of the BI-1 protein (Bax inhibitor 1, a programmed cell death inhibitor) was induced by both treatments, that is, it reacted to both heat shock and *Bgh*-infection. Over a time course of several days increasing *BI-1* expression was detected earlier and the expression of the gene *BI-1* was found to be affected by powdery mildew inoculation differently in susceptible and resistant barley, which supports our plans to include the other near-isogenic lines (susceptible Ingrid, Mla and Mlg) in our investigations.

We studied the expression patterns of the genes of two WRKY proteins that are transcription factors known to play a role in pathogen defence and stress responses in plants. We found that *WRKY1* was induced in a less pronounced way by inoculation and more evidently by heat, and the two treatments together resulted in weaker expression than heat treatment alone, in other words, the

heat shock diminished the effect of *Bgh*-inoculation. Expression of the gene *WRKY2* only reacted to pathogen infection. Having undergone both treatments the induction of *WRKY2* in mlo barley leaves was less strong, suggesting that in this case, the heat treatment seemed to attenuate the effect of powdery mildew infection on gene expression.

In summary, our present results can be regarded as preliminary experiments of my next research project, but in any case, they have already proven to be an interesting and promising possibility for future investigations. In a recent study, exciting linkage was discovered between Mla-type race specific resistance and the basal or general resistance in plants. The signal transduction pathways of these two very different resistance mechanisms are interconnected exactly through the activation/repression of *WRKY* transcription factors, which we have began to investigate in our experiments as well. These results leave us no doubt about our next candidate barley line to be inspected by gene expression studies.

New scientific results

1. In our studies concerning abiotic stress, by comparing membrane damage caused either by oxidative damage through toxic heavy metal salt stress (mercuric chloride) or directly by ROS (hydrogen peroxide) to young versus old tobacco leaves, and cytokinin juvenile hormone-overproducing transgenic CTKm tobacco plants versus non-transgenic controls, we have proven that juvenility is accompanied by enhanced antioxidant capacity and thus protection against abiotic oxidative stress.

2. Heat efflux and ethane emission from *Tobacco mosaic virus* (TMV)-inoculated Xanthi tobacco and salicylic acid-deficient transgenic NahG tobacco plants that are not able to establish SAR against a second inoculation by TMV, were found to rise to similar levels. However, under non-inoculated conditions, these values in leaves of NahG tobacco were found to be significantly lower than from Xanthi plants.

3. In susceptible cv. Ingrid barley the examined antioxidant levels were enhanced after infection with powdery mildew, whereas in the resistant Mla, Mlg and mlo lines these values changed much less. The level of hydrogen peroxide, on the other hand, increased considerably in the resistant barley lines. Thus our results support the theory of pathogen-induced susceptibility, i.e. the hypothesis that the powdery mildew fungus, in a compatible relationship with barely, may induce antioxidants in the plant in order to avoid the damaging effect of ROS produced by the plant against the invading pathogen.

4. Symbiotic association with *Piriformospora indica* root endophytic fungus compensated for the NaCl-induced biomass-reduction and attenuated the elevated lipid peroxidation, metabolic heat efflux and fatty acid desaturation in leaves of the salt-sensitive barley cultivar Ingrid during severe salt stress.

5. Heat shock-induced susceptibility in barley was found to show correlations with the induction or repression of several genes known to play a role in abiotic or biotic stress responses (*BI-1*, *HSP70*, *WRKY1*, *WRKY2*).

MOST IMPORTANT PUBLICATIONS FROM THE SUBJECT OF THE DISSERATION

Journal articles:

HARRACH B. D., FODOR J., POGÁNY M., PREUSS J., BARNA B. (2008): Antioxidant, ethylene and membrane leakage responses to powdery mildew infection of near-isogenic barley lines with various types of resistance. *European Journal of Plant Pathology* 121: 21–33.

BALTRUSCHAT H., FODOR J., HARRACH B. D., NIEMCZYK E., BARNA B., GULLNER G., JANECZKO A., KOGEL K.-H., SCHÄFER P., SCHWARCZINGER I., ZUCCARO A., SKOCZOWSKI A. (2008): Salt tolerance of barley induced by the root endophyte *Piriformospora indica* is associated with a strong increase in antioxidants. *New Phytologist* 180: 501–510.

FODOR J., HARRACH B. D., JANECZKO A., BARNA B., SKOCZOWSKI A. (2007): Metabolic responses of tobacco to induction of systemic acquired resistance. *Thermochimica Acta* 466: 29–34.

POGÁNY M., HARRACH B. D., HAFEZ Y. M., BARNA B., KIRÁLY Z., PÁLDI E. (2006): Role of reactive oxygen species in abiotic and biotic stresses in plants. *Acta Phytopathologica et Entomologica Hungarica* 41: 23–35.

Conference proceedings, abstracts:

HARRACH B. D., HÜCKELHOVEN R., KOGEL K.-H., BARNA B. (2007): Gene expression changes in heat-induced susceptibility of barley to powdery mildew. XIII. International Congress on Molecular Plant–Microbe Interactions (21–27 July 2007, Sorrento, Italy) Abstr. p. 333.

HARRACH B. D., BARNA B. (2004): Hydrogen peroxide and mercury tolerance of tobacco with altered antioxidant properties. 14th FESPB Congress (23–27 August 2004, Cracow, Poland) *Acta Physiologiae Plantarum* Vol. 26, No. 3 Suppl., p. 216.

HARRACH B. D., BARNA B. (2003): Stress resistance and antioxidant capacity in tobacco plants. 4th International Conference of PhD Students (11–17 August 2003, Miskolc, Hungary) Proc. pp. 41–47.