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Effect of sulfur deficiency on the resistance of oilseed rape to fungal pathogens and expression profiling of the glutathione *S*-transferase family of *Arabidopsis thaliana*.

Thesis

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Abstract

The reduction of $S0_2$ atmospheric pollution in the early nineties caused sulfur-deficiency problems in the agriculture of northern Europe. Sulfur is essential for plant development and sulfur containing compounds such as sulfur rich antifungal proteins, phytoalexins and glucosinolates play an important role in plant defense against pathogens. Sulfur starved *Brassica napus* with no visible symptoms showed a strong decrease in total sulfur and glutathione content and an increased susceptibility to the blackleg fungus *Leptosphaeria maculans*, to the generalist necrotroph *Botrytis cinerea* and to the oomycete *Phytophthora brassicae*. To test the cause of this increased susceptibility, a methanol extract containing secondary metabolites and a water extract of soluble proteins of plants grown with and without sulfur fertilization were used in fungal growth inhibition tests. MeOH extract of normally grown plants showed strong antifungal activity and this activity was almost totally lost in extracts of S-starved plants. Plants preinoculated with *B. cinerea* did not contain an increased antifungal potential indicating that phytoalexins do not contribute to this activity. The loss of antifungal activity correlated with a strong reduction of the glucosinolate content of the methanol extract suggesting that the reduced level of glucosinolates might be the cause of the reduction of the antifungal potential. However, no causal link could yet be demonstrated. The general loss of fitness of sulfur-starved plants could play an additional important role in the reduction of resistance.

Plant secondary metabolism significantly contributes to defense against adverse environmental cues. To investigate stress-induced alterations at the transcriptional level, a DNA array (MetArray) harboring gene-specific probes was established, which combined *Arabidopsis thaliana* effector gene families encoding enzymes acting consecutively in secondary metabolism and defense reactions. It contained the complete set of genes encoding 109 secondary product glycosyltransferases and 63 glutathione-utilizing enzymes along with 62 cytochrome P450 monooxygenases and 28 ABC transporters. Their transcriptome was monitored in different organs of unstressed plants and in shoots in response to herbicides, UV-B radiation, endogenous stress hormones, and pathogen infection. A principal component analysis based on the transcription of these effector gene families defined distinct responses. Methyl jasmonate and ethylene treatment was separated from a group combining reactions towards two sulfonylurea herbicides, salicylate and an avirulent strain of *Pseudomonas syringae*. The responses to the herbicide bromoxynil and UV-B radiation were separate from both groups. A few genes were diagnostic in their specific response to two herbicide classes used. Interestingly, a subset of genes induced by *P. syringae* was not responsive to the applied stress hormones. In addition, small groups of comprehensively induced effector genes may be part of defense mechanisms activated by several converging pathways. The differentiating expression patterns detected by the MetArray provide a framework of information regarding the function of individual genes and argue against widely redundant functions within the large gene families analyzed.

Plant glutathione S-transferases are multifunctional enzymes encoded by a large gene family containing 47 members in *Arabidopsis thaliana*. A member of the Phi class GST, *At*GSTF8 (At2g47730), is upregulated by various treatments including oxidative stress and exhibits GSH-peroxidase activity. The chloroplastic localisation of GSTF8 was demonstrated by expressing a fusion protein consisting of the predicted GSTF8 signal pepetide and GFP in transgenic *Arabidopsis*. Analysis of the GST family indicated that GSTF8 is the only chlorplastic GST in *Arabidopsis*, making it a promissing candidate for functional analysis. To this end, GSTF8 over-expressing transgenic lines were produced and a T-DNA insertion knock out mutant was isolated from the SALK-collection. However, no change in phenotype could be seen under normal growth condition and under conditions of oxidative stresses conditions like treatments with hydrogen peroxide and the herbicide paraquat. This indicates that GSTF8 is either not involved in protection from oxidative stress in chloroplasts or, alternatively, that in addition to GSTF8 other mechanisms contribute to this protection.

Resumé

La réduction de la pollution atmosphérique due au dioxyde de soufre a provoqué au début des années nonante des problèmes de carence de soufre dans les champs principalement au nord de l'europe. Le soufre est un macroélément essentiel pour le développement de la plante. Des composés soufrés tels que des protéines riches en soufre, des phytoalexines et des glucosinolates, jouent un rôle important dans les mécanismes de défense. Des plants de colza carencés en soufre, mais ne montrant aucun symptôme visible, présentaient une forte réduction de leur teneur en soufre total et en glutathion. Ces plantes montraient aussi une augmentation de leur susceptibilité au pathogène de la nécrose du collet, *Leptosphaeria maculans*, au nécrotrophe généraliste *Botrytis cinerea* ainsi qu'à l'oomycète *Phytophthora brassicae*. Dans le but d'analyser les causes de cette augmentation de susceptibilité nous avons testé le potentiel antifongique d'un extrait protéique et d'un extrait au méthanol contenant des métabolites secondaires. Ces extraits ont été obtenus à partir de colza cultivé avec et sans soufre, et préalablement induit ou non au moyen d'une inoculation avec *Botrytis cinerea*. L'extrait méthanolique obtenu à partir de plantes normales avait une activité antifongique qui était presque entièrement perdue dans le même extrait obtenu à partir de colza carencé en soufre. Cette perte d'activité antifongique corrélait avec une forte réduction du contenu en glucosinolates de l'extrait. Ceci suggère que la réduction du contenu en glucosinolates pourrait être la cause de la réduction du potentiel antifongique. Toutefois aucun lien de causalité n'a encore pu être démontré. L'affaiblissement général des plantes carencées pourrait aussi jouer un rôle additionnel dans la diminution de leur résistance aux pathogènes fongiques.

Le métabolisme secondaire des végétaux contribue significativement à la défense de la plante contre divers stress environnementaux. Pour étudier les changements transcriptionels des gènes d'*Arabidopsis thaliana* impliqués dans les mécanismes de détoxification nous avons développé un « DNA array » (MetArray) contenant des sondes spécifiques pour chaque membre des familles de gènes impliquées dans les étapes successives de détoxification. Le MetArray contient des sondes pour la totalité des gènes codant pour des glycosyltransférases, pour 63 enzymes utilisant du glutathion ainsi que 62 cytochrome P450 monooxygénases et 28 ABC transporteurs. Leur expression a été analysée dans les différents organes de la plante ainsi que dans les feuilles suite à divers traitements tel que herbicides, irradiation avec des UV-B, hormones endogènes liées à la réponse au stress et infection avec un pathogène. Une analyse en composante principale basée sur le transcriptome de ces familles de gènes détermine des réponses distinctes. Les traitements avec le méthyl-jasmonate et l'éthylène forment un groupe. Le traitement avec l'acide salicylique deux herbicides sulfonylurée et l'inoculation avec une souche avirulente de *Pseudomonas syringae* en forment un autre. Enfin la réponse au bromoxynil un herbicide, et le traitement avec des UV-B forment un troisième groupe clairement séparé. Il faut relever un groupe de gènes qui était induit par le pathogène *P. syringae* mais qui ne montrait aucune réponse aux trois hormones liées aux voies de signalisation. De plus un petit groupe de gènes qui pourrait jouer un rôle dans les mécanismes de défense était régulé par diverses voies de signalisation convergentes. La régulation différenciée des divers gènes contredit l'hypothèse d'une grande redondance fonctionnelle dans ce quatre grandes familles de gènes.

Les glutathion *S*-transférases (GST) sont des enzymes multifonctionnels qui sont codés par une famille de gènes. Le génome d'*Arabidopsis thaliana* contient 47 glutathion S-transférases. Une GST de la classe phi, GSTF8 (At2g47730) est induite par divers traitements dont le stress oxydatif et possède une forte activité GSH-peroxydase. Nous avons montré que GSTF8 était localisée dans le chloroplaste en fusionnant le peptide signal de GSTF8 avec une protéine fluorescente (GFP). Comme GSTF8 est la seule GST présente dans le chloroplaste et qu'elle possède une activité GSH-peroxidase, elle constitue un candidat idéal pour une analyse fonctionnelle. Pour ce faire, une lignée transgénique surexprimant GSTF8 a été produite et un mutant insertionel (T-DNA) a été isolé. Toutefois aucun changement phénotypique n'a été observé aussi bien en croissance normale qu'après traitements avec du peroxyde d'hydrogène et du paraquat, induisant tout deux un stress oxydatif. Ceci indique que GSTF8 n'est soit pas impliqué dans la protection contre le stress oxydatif, soit qu'en plus de GSTF8 d'autres mécanismes contribuent à cette protection.

Chapter 1

General Introduction

General Introduction

1.1 The importance of sulfur for plant nutrition

Sulfur is one of the six macronutrients needed for proper plant development. Even if sulfur is only 3% to 5% as abundant as nitrogen in plants, it plays essential roles in various important mechanisms such as Fe/S clusters in enzymes, vitamin cofactors, glutathione in redox homeostasis and detoxification of xenobiotics (Leustek et al., 2000; Saito, 2000). Reduced sulfur incorporated in cysteine and methionine amino acids plays essential roles in catalytic centers and disulfide bridges of proteins (Hell, 1997). Sulfur is taken up from the soil by plants in form of sulfate by specific transporters. This sulfate is then reduced to be incorporated in cysteine and subsequently in methionine. Organic bound sulfur is mainly reduced to sulfide, but oxidised sulfur is also found in plants, a good example being the sulfolipids of the chloroplast membranes (Hell, 1997). Plant posses different specific sulfotransferases that catalyse the esterification of sulfate with secondary compounds and proteins.

Sulfur is a mobile nutrient that can move rapidly downward through the soil, especially through sandy surface layers. This easy leaching ability of sulfate leads to problems because an important part of the sulfur present in fertilizers may end up in ground water where it causes environmental problems such as eutrophication of aquatic ecosystems or a reduction in the quality of drinking water. In humid regions most of the sulfur in the surface soil is associated with organic matter. Sulfur deficiencies are less frequent in high organic matter soils, because the sulfur constantly mineralizes from the organic matter. However, under intensive crop production, the breakdown of organic matter and subsequent release of sulfate by microorganisms may not be rapid enough to meet the increased demands driven by high yields. Furthermore, the mineralization efficiency is dependent on environmental factors such as soil type, microorganisms present, temperature and humidity.

1.2. Reduction of sulfur dioxide pollution and occurence of sulfur deficiency in crops

The breeding of higher yield crop plants lead to increased need for sulfur in farming. This went unnoticed for a long time because during the same period sulfur deposition caused by atmospheric pollution by the burning of S-containing fossil fuels increased steadily (Hell and Hillebrand, 2001). In the second part of the 20th century atmospheric pollution with sulfur dioxide became a problem for industrialised countries. The major anthropogenic sources of the emission of sulfur dioxide were thermal power plants producing electricity from high sulfur containing coal or heating oils, industrial boilers and non ferrous metal smelters. Natural source of sulfur dioxide such as volcanoes or marine algae can account for 25% to 65% of the total emission (www.ourplanet.com). Domestic coal burning and vehicles can also contribute to high local ambient concentrations of sulfur dioxide. Atmospheric pollution with sulfur dioxide has two main consequences: acid rain and health problems. Acid rains has negative effects on aquatic ecosystems and lead to the so called “forest die back” in the eighties. During the London fog of 1952, the so called “peasouper” smog, levels of SO_2 reached 3500μ/m^3 (average over 48 hours) in the city centre, and remained high for a period of 5 days leading to the death of 4’000 people. Furthermore, in 2000 in large Chinese cities smog caused the premature death of about of 50’000 people. A recent study in Hong Kong demonstrated that pollution resulting from sulfur-rich fuels has an effect on death rates, especially respiratory and cardiovascular deaths (Hedley et al., 2002). In response to these pollution problems in the mid-eighties, international treaties legally enforced the drastic reduction of SO_2 emissions (Helsinki protocol 1979). Heavy investments by

power station operators in desulfurization equipment and use of S-poor fuels have cut sulfur pollution in Europe and North America by as much as 80 percent. As an unforeseen consequence of the reduction of sulfur dioxide pollution in recent years, an increased frequency of sulfur deficiency has been observed in several crops mainly in northern Europe. It was realised that sulfur may become a factor limiting yield and crop quality in agriculture (Dämmgen et al., 1998; Eriksen and Mortensen, 1999). A solution to the S-deficiency in agricultural crop production is the application of fertilizer containing sulfur. As sulfate is easily leached out by rain the tendency could be to over fertilize with sulfur which in turn would lead to ground water pollution.

Sulfur is used in agriculture since the antiquity for its protective effect against pests and diseases. Foliar application of sulfur was observed to boost the growth of crops (Pezet et al., 1986; Schnug, 1996). Positive correlation between S-fertilisation and enhanced disease resistance against fungal pathogens was observed in the field (Davidson and Goss, 1972; Schnug, 1996). However, for a long time sulfur was not regarded as a limiting factor for crop production.

1.3 Oilseed rape as model plant

The *Brassicaceae* family consists of hundreds of species containing important cultivated crops and wild species. The genome of *Brassica napus* contains n=19 chromosomes which is a combination of the parental genomes of *Brassica rapa* (n=10) and *Brassica oleracea* (n=9). *B. napus* is mainly known as oilseed rape (*B. napus* subspecies *oleifera*) but also as the horticultural crop rutabaga or swede (*B. napus* subspecies *rapifera*) (Cheung et al., 1997). Breeding for *B. napus* varieties containing no erucic acid (0 varieties) and low glucosinolate content in seed (00 = double low varieties) was performed in the seventies to avoid the antinutritional and goitrogenic effect of seed meal on mammals. Erucic acid was believed to cause cardio-vascular diseases. Hydrolysis of progoitrin and epi-progoitrin gives rise to dagradation products, oxazolidine-2-thiones which have a wide range of adverse effects such as goitrogenic effects and liver toxicity making its use in pork and poultry feeding unsuitable (Fenwick et al., 1983; Fahey et al., 2001). On the other hand in the last 20 years new evidence of the putative protective role of glucosinolate against cancer has accumulated (Fahey et al., 2001). Glucosinolates, are thioglucosides reported to be part of the plant defense mechanism against fungi and insects (Osbourn, 1996; Blake-Kalff et al., 1998). Isothiocyanates derived from the degradation of many glucosinolates discourage feeding by generalist insects, attract specislist insects and are toxic to bacterial and fungal pathogens (Mithen et al., 1986; Giamoustaris and Mithen, 1997; Manici et al., 1997)

The production of oilseed rape has increased significantly in the last twenty years (Howlett et al., 2001). In Switzerland the production of oilseed rape has increased in the nineties and stabilised in recent years to about 50’000t/year (www.agirinfo.com). Oilseed rape is an ideal model to study sulfur deficiency in plants because of its high demand for sulfur (making it particularly sensitive to S-deficiency) and its genetic proximity to *Arabidopsis thaliana* (Zhao et al., 1997; Blake-Kalff et al., 1998; Schmidt et al., 2001).

1.4 Description of the pathogens used in the study

Of the phytopathogenic fungi known to affect rapeseed, the loculoascomycete fungi *Leptosphaeria maculans* (Desm.) Ces. & de Not (anamorph: *Phoma lingam*) (Tode ex Fr.) (Desm.) causes the highest economic losses of this crop worldwide (Howlett et al., 2001). Since chemical protection is

difficult and costly, genetic resistance has become an important measure of disease control. Disease caused by *L. maculans* is initiated when sexual ascospores or asexual pycnidiospores land on a susceptible oilseed rape plant (Howlett et al., 2001). The disease is usually monocyclic and epidemics are generally initiated by airborne ascospores. Infection can also arise from infected seed, stubble and rain splashed conidia (West et al., 2001). When the ascospore or pycnidiospore germinates, hyphae infect the plant through stomate openings or through wounds. After colonizing intercellular spaces in the spongy mesophyll of the leaf lamina, the fungus reaches the vascular bundle and spreads down the petiole eventually invading the stem cortex and causing the stem canker symptom. The initial infection and the intercellular systemic phase of growth occurs in a biotrophic mode but behind the expanding hyphal front the interaction becomes necrotrophic (Hammond et al., 1985; Hammond and Lewis, 1986, 1987). The lifecycle is completed when new ascospores are generated in the necrotrophic regions caused by the fungus (Bohman, 2001). Only partial resistance exist in the important oilseed crops, *B. napus*, *B. rapa*, and in the different cabbage crops (*B. oleracea*) (reviewed in (Howlett et al., 2001) and (West et al., 2001)). Little is presently known about underlying molecular mechanisms that make plants resistant to *L. maculans*. Some studies have been performed of the response of *B. napus* to *L. maculans* inoculations and Hammond and Lewis (1986) reported the deposit of lignin-like structures and calcium accumulation. Additionally, (Roussel et al., 1999) showed that avirulent isolates induce HR and that the lumen of vessels in the HR area was occluded by fibrillar-like material. Further experiments have shown that pathogenesis related proteins accumulate differently in resistant and susceptible plants (Dixelius, 1994).

Botrytis cinerea Pers.: Fr.: (teleomorph *Botryotinia fuckeliana* (de Bary) Whetz. is an ascomycete, classified among the inoperculated Discomycetes, order of *Leotiales* and the *Sclerotiniaceae* family. *B. cinerea*, commonly named grey mould, is a characteristic necrotrophic pathogen that has a very broad host range with more than 250 potential host plants (MacFarlane, 1968) including economically important crops such as cereal crops, fruits, vegetables, and flowering plants. *Phytophthora brassicae* (previousely *P. porri*) is an oomycete infecting a wide range of *Brassicacea* plants including *Brassica napus* and *Arabidopsis thaliana* (Roetschi et al., 2001). Phytophthora species cause agronomically important diseases including the well known potato late blight and soybean root rot (Kamoun, 2003).

1.5 The “green liver” concept

The ability of plants to detoxify naturally occurring toxic substances and low molecular xenobiotics was modelled in the concept referred to as the “green liver” (Sandermann, 1992, 1994; Coleman et al., 1997). It comprises four sequential steps. In phase I the hazardous molecule is activated by either hydrolases or oxidases such as P-450 cytochrome oxidase. This activation results in the introduction of functional carboxyl- or hydroxyl-groups or in the exposition of such groups. Activated molecules are better available for further steps in the detoxification process. In phase II the activated molecules are conjugated to a highly polar molecule like glucose, malonate, sulfate or glutathione. This conjugation is catalyzed by enzyme families such as glucosyltransferases or glutathione S-transferases. Tagging harmful molecules with glucose or glutathione changes their physico-chemical properties and often strongly reduces their toxicity. In phase III the conjugated molecule is exported by specific transporters in an energy dependant manner from the cytosol to the vacuole or the apoplast. Examples of these transporters are multi-drug-resistance-associated-proteins (MRPs) that are member of the superfamily of the ATB-binding-cassette (ABC) transporters (Kolukisaoglu et al., 2002). Phase IV includes all the possible further processing, degradation and recycling reactions. For some compounds the detoxification process ends with the compartementation (step III) but for other molecules further metabolic steps take place in the vacuole.

1.6 The role of glutathione for plants in stress conditions

Glutathione is a tripeptide γ-glutamyl-cysteinyl-glycine (GSH) containing a sulfhydryl group which is highly reactive (Rennenberg, 1982). GSH is synthesized from glutamate, cysteine and glycine in two sequential ATP-dependent reactions catalyzed by γ-glutamylcysteine sythase and glutathione synthase. These enzymes are present both in the cytosol and in the chloroplast. GSH represent the major non-protein thiol in plant cells and is a storage form of reduced sulfur (Hell, 1997). GSH concentration is estimated to be between 300 and 1300 nmol/g FW in most tissues (Schnug et al., 1995). Beside its role as a central compound in the sulfur metabolism, GSH plays various important roles in the plant such as direct antioxidant, regeneration of ascorbate, redox buffering and as a precursor of phytochelatins (Noctor and Foyer, 1998). Phytochelatins are small peptides formed by γ-glutamyl-cysteinyl repeats used by the plant to chelate and detoxify heavy metals (Cobbett and Goldsbrough, 2002). The high reactivity and water solubility of GSH makes it an ideal molecule to protect the plant against various stresses including oxidative stress, xenobiotic molecules and heavy metals (Xiang et al., 2001). Furthermore, GSH is used by glutathione S-transferases to tag electrophilic molecules including many xenobiotics (see 1.4 and 1.6).

1.7 Glutathione S-transferases are multifunctional enzymes

Glutathione S-transferases (GSTs; EC 2.5.1.18) form a large and diverse family of multifunctional, dimeric enzymes that catalyze the conjugation of GSH to a large variety of lipophilic compounds with electrophilic centers. GSTs are present in bacteria, fungi, animals and plants. They were first discovered in animals due to their important role in drug metabolism and detoxification (Wilce and Parker, 1994). Their presence in plants was first recorded in maize where a GST was shown to be responsible for the detoxification of the herbicide triazine (Timmerman, 1989; Dixon et al., 2002). GSTs are abundant proteins and constitute more than one percent of the soluble proteins fraction in maize leaves (Marrs, 1996). GST protein have a molecular weight of an approximatively 25 kDa. Homo and heterodimers can form thus increasing the diversity of GST dimers to be found *in planta.*

GSTs are typically encoded by large gene families (Marrs, 1996; McGonigle et al., 2000; Wagner et al., 2002). GSTs have been extensively studied in animals, and mammalian GSTs are classified in different families such as Alpha, Mu and Pi classes involved in drug detoxification (Wilce and Parker, 1994). Sigma class GSTs are implicated in prostaglandin synthesis (Rowsey et al., 2001). Theta and Zeta class have members both in plants and animals. A prokaryote-specific beta class was discovered in bacteria and a delta class is specific to insects (Rossjohn et al., 1998; Chelvanayagam et al., 2001). The most numerous plant GST classes Phi and Tau are plant specific and their sequences are significantly different from the animal GSTs. We can now take advantage of the full genome sequence of *Arabidopsis thaliana* to understand the organisation of the GSTs in higher plants. The Arabidopsis genome contains 47 *GSTs* genes divided in four classes Phi (F), Tau (U), Theta (T) and Zeta (Z) sharing only limited sequence similarity (Edwards et al., 2000; Wagner et al., 2002). *GST* are often present in the form of gene clusters or tandem repeats. This situation seems to result from gene duplication events during the evolution. Indeed, only a quarter of these genes are found to be single genes (Wagner, 2001). Expression and regulation studies of GSTs in plant have shown that their regulation is highly variable. Furtermore GST function does not correspond to the classification based on sequence similarity (McGonigle et al., 2000; Wagner et al., 2002).

Plant GSTs were intensively studied with regard to their role in herbicide detoxification. The function of GSTs is generally believed to be the detoxification of both xenobiotics and endogenous toxic

compounds (Marrs, 1996). Surprisingly only few potential endogenous substrates have been found raising the questions of what is the real *in vivo* role of GSTs and what are their natural substrates. From an evolutionary point of view it is difficult to understand how enzymes could develop which are highly specific for xenobiotics of human origin that are present in the environment for only very limited time (Sandermann, 1994). One explanation is that GSTs have evolved to remove hazardous substances of natural origin, such as toxins from pathogens or allelopathic substances from competitor plants. From this point of view the xenobiotics from human origin might only be similar to those compounds and be detoxified by GSTs as a side activity. However, in recent years some experimental data described natural substrates of GSTs. The first report was that the aquatic plant *Ceratophyllum demersum* was able conjugate enzymatically glutathione to the toxin microcystin-LR, an inhibitor of protein phosphatases produced by *Cyanobacteria* (Pflugmacher et al., 1998). Further putative natural substrates for GSTs are the phytoalexins medicarpin (Li et al., 1997) and some isothiocyanates (Kolm et al., 1995). Some GSTs were also shown to catalyse the glutathione dependant isomerisation in the catabolism of tyrosine (Dixon et al., 2000). An interesting link between hormon metabolism and GSTs has been revealed by the observation that some auxin- and cytokinin-binding proteins are GSTs (Zettl et al., 1994; Bilang and Sturm, 1995; Gonneau et al., 1998). Furthermore, GSTs can act as glutathione peroxidase (Cummins et al., 1999; Wagner et al., 2002). Our knowledge of natural substrates of GSTs is still very limited but non transferase activities such as isomerase, peroxidase or binding of hormones can give insight in new roles for GST.

1.8 Aim of the thesis

This thesis work was divided into three main topics. The first part was the investigation of the effect of sulfur-deficiency on plant resistance to fungal pathogens. Sulfur deficiency in the field leads to yield reduction and increased disease impact. We build a controlled test system for *Brassica napus* to demonstrate that sulfur starvation decreased the resistance of oilseed rape to three different fungal pathogens. *Arabidopsis thaliana* and *Brassica napus* are both members of the *Brassicaceae* family and exhibit extensive homology to each other at the DNA sequence level (Bancroft, 2001; Schmidt et al., 2001). One goal of our study was to grow *Arabidopsis thaliana* under S-starvation condition to take advantage of the tools available for this model plant and transfer the knowledge later to *B. napus*. However, we were not able to obtain nicely growing S-starved *A. thaliana* plants at rosette stage. When S-starved, *A. thaliana* plants always showed symptoms of senescence. Therefore the initial plan to use *A. thaliana* as a model plant was abandoned. In order to investigate the possible causes of the enhanced disease susceptibility of *B. napus* and to determine which compound could be responsible for it, we have investigated the antifungal potential of two plant extracts, a methanol extrac containing secondary metabolites and a protein containing extract.

The second part was the study of the expression profiling of the whole set of *Arabidopsis thaliana* glutathione S-transferases. Glutathione S-transferases are members of multigene families. To study the expression of the 47 GSTs of *Arabidopsis thaliana* we have developed a nylon membrane based macroarray with gene specific probes for each GST. This work was done in collaboration with three other groups and the so-called MetArray focused on the analysis of the transcriptome of three additional gene families all involved in secondary metabolism and detoxification: cytochrome P450 monooxygenase, glycosiltransferases and ABC transporters. One goal was to learn more about the organ specific expression of the four gene families. In a second step we were interested to monitor their expression in leaves under different abiotic and biotic stress conditions and treatment with stress hormones.

The third part describes the functional analysis of GSTF8. We first wanted to confirm that GSTF8 was as predicted from its sequence localized in the chloroplast and that it was the only chloroplastic GST in *Arabidospsis thaliana*. Information on the peroxidase activity of GSTF8 and its upregulation after oxidative stress lead to the hypothesis that GSTF8 could act as GSH-peroxidase in the chloroplast. Because of the functional redundancy in large gene families functional analysis of the GSTs is difficult (Wagner, 2001). Nevertheless because of its unique chloroplastic localisation, GSTF8 appeard to be a good candidate for functional analysis.

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Chapter 2

Increased disease susceptibility of sulfur deficient oilseed rape is caused by a loss of antifungal potential

Increase disease susceptibility of sulfur deficient oilseed rape is caused by a loss of antifungal potential

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Abstract

The reduction of $S0_2$ atmospheric pollution in the early nineties caused sulfur-deficiency problems in the agriculture of northern Europe. Sulfur is essential for plant development and sulfur containing compounds such as sulfur rich antifungal proteins, phytoalexins and glucosinolates play an important role in plant defense against pathogens. Sulfur deficient plants with no visible symptoms showed a strong decrease in total sulfur and glutathione content and an increased susceptibility to the blackleg fungus *Leptosphaeria maculans*, to the generalist necrotroph *Botrytis cinerea* and to the oomycete *Phytophthora brassicae*. To test the cause of this increased susceptibility, a methanol extract containing secondary mrtabolites and a water extract of soluble proteins of plants grown with and without sulfur fertilization were used in fungal growth inhibition tests. The methanol extract of normaly fertilized plants had antifungal activity against a variety of fungal pathogens and this activity was almost totally lost in extracts of S-starved plants. Plants preinoculated with *B. cinerea* did not contain an increased antifungal potential indicating that phytoalexins do not contribute to this activity. The loss of antifungal activity correlated with a strong reduction of the glucosinolate content of the methanol extract suggesting that the reduced level of glucosinolates might be the cause of the reduction of the antifungal potential. However, by testing different glucosinolates for antifungal activity no causal link could be demonstrated.

Keywords: sulfur, nutrition, sulfur starvation, disease resistance, antifungal potential, glucosinolates, isothiocyanates, *Brassica napus*.

Introduction

Sulfur is one of the essential macroelements for plant life with numerous biological functions (Leustek et al., 2000). Sulfur is taken up by plants in its inorganic sulphate form from the soil or sulfur dioxide and hydrogen sulphide gases from the atmosphere. In the initial step of the biological sulfur cycle, plants assimilate and reduce sulphate to sulphide and then incorporate it into cysteine, the first organic molecule carrying reduced sulfur. Cysteine is further converted to methionine. Sulfur is contained in a variety of cellular components and plays critical biochemical roles in a number of cellular processes, such as redox cycle, detoxification of heavy metals and xenobiotics, and metabolism of secondary products. (Saito, 2000; Nikiforova et al., 2003).

Intensive farming and yield raising in crop production has increased the demand for sulfur in the last decades, but this went unnoticed in industrialized contries because of pollution by atmospheric deposition of SO_2 produced by burning of S-containing fossil fuels. In the second part of the 20th century air pollution with sulfur dioxide became a major concern. Thermal power plants burning high sulfur coal or heating oil are generally the main sources of anthropogenic sulfur dioxide emissions worldwide, followed by industrial boilers, and non ferrous metal smelters, although there are natural sources of sulfur dioxide (accounting for 25-65 percent of total SO_2 emission) such as volcanoes (www.ourplanet.com). Emissions from domestic coal burning and SO_2 from vehicles can also contribute to high local atmospheric concentrations of sulfur dioxide. Pollution resulting from sulfur-rich fuels has an effect on death rates, especially respiratory and cardiovascular deaths (Hedley et al., 2002) and was the major source of acid rains. In response to this problem in the mid-eighties governments of developed countries took policies to legally enforce the drastic reduction of SO_2 emissions (Helsinki protocol 1979). As an unexpected outcome of the reduction of sulfur dioxide pollution in recent years an increased frequency of sulfur deficiency has been observed in several crops mainly in northern Europe. Sulfur may become a factor limiting yield and crop quality in agriculture (Dämmgen et al., 1998; Eriksen and Mortensen, 1999).

It has been known since the antiquity that sulfur has protective effects against pests and diseases. Most of the knowledge is however restricted to the external effect of foliar applied sulfur. Less is known about soil supplied sulfur which has a strong influence on plant resistance by directly stimulating biochemical processes in primary and secondary metabolism (Pezet et al., 1986; Schnug, 1996). Field observations pointed at positive correlation between S-fertilisation and enhanced disease resistance against fungal pathogens (Davidson and Goss, 1972; Schnug, 1996). This lead to the question, whether sulfate availability could be a limiting factor for the ability of plants to respond to fungal infection.

In recent years oilseed rape (*Brassica napus* L.) has become the major oil crop in the EU and one of the major three oil crops worldwide (Howlett et al., 2001). Oilseed rape is particularly sensitive to S deficiency because it has a high demand for S. Oilseed rape produces seeds with a large yield of protein with relatively large quantities of S-containing amino acids (Zhao et al., 1997; Blake-Kalff et al., 1998) and the plants require S for the synthesis of glucosinolates, a group of thioglucoside compounds reported to be part of the plant defense mechanism against fungi and insects (Blake-Kalff et al., 1998). In Switzerland the production of oilseed rape has increased in the nineties and stabilises in recent years to about 50’000t/years (www.agirinfo.com).

Of the phytopathogenic fungi known to affect rapeseed, *Leptosphaeria maculans* (anamorph: *Phoma lingam*) causes the highest economic losses of this crop worldwide (Howlett et al., 2001). *Botrytis*

cinerea (teleomorph *Botryotinia fuckeliana*) commonly named grey mould, is a characteristic necrotrophic pathogen that has a very broad host range with more than 250 potential host plants (MacFarlane, 1968) including economically important crops such as cereals, fruits, vegetables, and flowering plants. *B. cinerea* is not a problem on rape but it was used in our study as a non specialized ubiquitous pathogen to compare it to the specialized *L. maculans*. *Phytophthora brassicae* (formerly *P. porri*) is an oomycete infecting a wide range of Brassicacea plants including *B. napus* and *Arabidopsis thaliana*. In our study we have used a strain expressing a green fluorescent protein as a quantitative marker (Si-Ammour et al., 2003).

The effect of S-starvation on the resistance of oilseed rape to three different pathogens was analysed. Taken together our results show a clear influence of the S-nutritional status of the plant on its resistance to disease. To find out which mechanism could be responsible for the decrease resistance of sulfur deficient plants, the antifungal potential of a MeOH extracts containing secondary metabolites and extracts of water-soluble proteins were tested. The MeOH extract of normal plants showed antifungal activity against a number of phytopathogenic fungi. This activity was lost in extracts of S-deficient plants. The loss of antifungal activity correlated with a strong reduction in glucosinolates conten., However, we were not able to link the reduction of antifungal activity to the reduction of a particular glucosinolate or derived isothiocyanate.

Materials and Methods

Organisms and growth condition

Seeds of *Brassica napus* cv Bienvenu (0) and cv Express (00) were obtained from the Swiss federal agricultural research station of plant production of Changins. Seeds were sown on vermiculite and watered first with tap water and after 10 days once with half strength Hoagland nutritive solutions containing sulfur (Hoagland and Martin, 1950). After 15 days the seedlings were transferred to pots containing quartz sand (diameter 12 cm) and further watered with 0.5x Hoagland solution with or without addition of sulfate in form of $MgSO_4$. For the S-deficient plants the $MgSO_4$ was replaced by equimolar amount of $MgCl_2$, and among micro salts $CuCl_2$, $MnCl_2$ and $ZnCl_2$ were used instead of $CuSO_4$, $MnSO_4$ and $ZnSO_4$. Plants were grown in a growth chamber at 20°C 16h light / 18°C 8h dark cycle.

Leptosphaeria maculans (anamorph: *Phoma lingam*) and *Cladosporium sp* isolates were obtained from the Swiss federal agricultural research station of plant production of Changins and grown on Potato Dextrose Agar (PDA, Difco, Detroit, USA) containing 25 µg/ml of aureomycin (chlortetracycline hydrochloride, Rectolab SA, Servion, Switzerland). *L. maculans'* pycnidiospores production was induced by growing colonies for 14 days at 15°C under 12h black light (OSRAM L3673 BLB)/12h dark cycle. Pycnidiospores were harvested according to (Hammond et al., 1985). The ascomycete *Botrytis cinerea* isolates BMM and Pellier were isolated from geranium and vine, respectively and grown on Potato Dextrose Agar (Difco, Detroit USA). Conidia from 14 days old well sporulating colonies were harvested in distilled water containing 0.2% (v/v) Tween-20. The oomycete *Phytophthora brassicae* isolate 155 expressing constitutively a green fluorescent protein (GFP) was grown on V8 agar (Si-Ammour et al., 2003).

Inoculation protocols

Leaves of 5 to 6 week old plants, at growth stage 2.4 - 2.5 of the scale devised by (Harper and Berkenkamp, 1975), were inoculated with the different pathogens. For *L. maculans* 10 µl of a spore suspension (10^6 spores/ml) in water with 0.2% Tween-20 was applied to the leaves after wounding with a needle. Plants were incubated for 5 days in 100% HR in a glass chamber, and then the lids were removed. Lesion size was measured at 12 and/or 21 dpi. For *B. cinerea* 10 µl of a spore suspension (10^4 spores /ml) in water with 0.2% Tween-20 was applied to the leaves after wounding with a needle. Plants were incubated at 100% HR and lesions size was measured at 4 or 5 dpi. Agar plugs taken from the margins of an expanding colony of *P. brassicae* were applied to the leaves after wounding with a needle. Plants were then incubated in 100% HR for 7 days for lesions size measurement. Four days after infection GFP fluorescence was determined (Si-Ammour et al., 2003). The experiments were repeated threetimes. For each repetition 32 independent measurements were done for *L. maculans*, and 16 independent measurements for *B. cinerea* and *P. brassicae*.

Measurement of total sulfur and glutathione

For total S content, leaves of 6 week old *B. napus* cv Bienvenu plants were dried for 48h at 65°C and then ground to fine powder with a mixer (Moulinex, type Y91). Two independent batches of plants were then mixed for analysis. Total S was determined using a Philips PW2400 X-ray fluorescence (X-RF) spectrometer under constant Helium flux, at the University of Lausanne according to (Schnug

and Haneklaus, 1992). Total GSH content of 6 week old *B. napus* plants cv Bienvenu was determined according to (Harms et al., 2000). Frozen leaf material (60mg) was homogenised to a fine powder and extracted for 15 min in 600 µl 0.1 N HCl at 4°C. After centrifugation (20 min, 14000g, 4°C), 120 µl of the supernatant were added to 200 µl of 0.2 M 2-(cyclohexylamino) ethanesulfonic acid (pH 9.3). Reduction of total disulfides was performed by adding 10 µl 9 mM bis-2-mercaptoethylsulfone in 200 mM Tris-HCl, 5 mM EDTA (pH 8). After 40 min at room temperature, free thiols were labelled with 20 µl 15 mM monobromobimane in acetonitrile and incubated for 15 min in the dark at room temperature. The reaction was stopped by adding 250 µl 15% HCl. The samples were analysed by HPLC (Harms et al., 2000).

Plant extractions and glucosinolates analysis

MeOH extracts (Griffiths et al., 2001) were used to quantify the glucosinolates content of the plant and in growth inhibition tests. For glucosinolate determination 20 µl aliquots were analysed by HPLC. The analytical column used was equipped with a Lichrospher (100 RP 18, 5 µm, 4 x 250 mm). The binary mobile phase system was composed of distilled water (A) and water : acetonitrile, 80 : 20 (B). The analysis was run with the following gradient program: 0 to 45 min linear gradient 0 to 100% B and then held for 5 min on 100% B. The flow rate was 1 ml/min and the detection of desulfoglucosinolates was monitored with an UV/VIS detector at 230 nm.

For protein extraction rape leaves (5g fresh weight) were ground in liquid nitrogen with a mortar and pestle. The resulting powder was extracted with 2.5 vol of 50 mM Tris/HCl pH 7.5 containing 1 mM phenylmethylsulfonyl fluoride (PMSF). Insoluble material was removed by centrifugation at 10'000g for 20 min. Protein were precipitated with ammonium sulfate (95% saturation) at 0°C for 2 h. The proteins were collected by centrifugation (20'000g, 20 min) and resuspended in 2 ml 10mM Tris/HCl pH 7.5. The extracts were desalted using Sephadex G-25 columns and filtered successively through a 0.45 and 0.22 µm membrane filters prior to application in the bioassays. For bioassays of plants after induction of defense mechanisms, detached leaves of six week old plants were wounded with a needle and inoculated with *B. cinerea* at a concentration of 3×10^3 spores/ml. After 60 hours the leaves were extracted with MeOH as described previously.

Bioassays

Fungal spores were harvested from well sporulating colonies on PDA plates and were resuspended in half-strength PDB (potato dextrose broth, Difco, Detroit, USA). The concentration of the spores suspensions was adjusted to 2 to 4 x 10^5 spores/ml. 0.5 ml from the freshly prepared spores' suspensions was spread on 9 cm PDA Petri dishes. The plates were incubated 24h at 18°C to allow the spores to germinate. At this time, 5 mm diameter sterile paper filter discs were laid on the agar surface and 40 µl of the solution to be tested was applied to the discs. After additional 48 to 72 hours incubation at 18°C the inhibition zones were measured and pictures were taken from the plates. For each treatment three repetitions with 5 replicates were done with two different extracts. For pure molecule bioassays 40µl of isothiocyanate (Fluka, Buchs, Switzerland) at concentration of 1mM and 10µM were applied to Petri dishes inoculated with *Cladosporium* sp. For sinigrin degradation products 10mM sinigrin (Carl Roth AG, Karlsruhe, Germany) in 0.33M KPO_4 buffer was incubated with 5 units myrosinase (thioglucoside glucohydrolase, Sigma Chemicals, St Louis, USA) 1 hour at 25°C. For each treatment three repetitions with 5 replicates were done. Pathogen growth inhibition tests in microtiter plates were done according to (Berrocal-Lobo et al., 2002).

Results

In the field sulfur deficient plants often show only weak or no obvious visible symptoms at all but the effect of this S-deficiency on yield and disease resistance can nevertheless be important. To produce controlled sulfur deficiency, two rape varieties, *Brassica napus* cv Bienvenu and *B. napus* cv Express, were sawn on vermiculite and watered once after 10 days with sulfur containing Hoagland nutritive solution. After 15 days they were transferred to quartz sand and watered during 3 to 4 weeks with Hoagland nutritive solution with or without addition of sulfur. After 5 to 6 weeks at the time of pathogen inoculation the S-starved plants did not show any visible sign of S-deficiency and looked healthy. At this time point plant grown on full nutritive solution (Figure 1A) looked very similar and were impossible to distinguish from S-starved plants (Figure 1B). However, when starved for eight weeks younger leaves started to exhibit strong S-deficiency symptoms (Figure 1C). The chlorosis spread over intercostal areas with anthocyan accumulation while the zones along the veins remainded green (Haneklaus and Schnug, 1992).

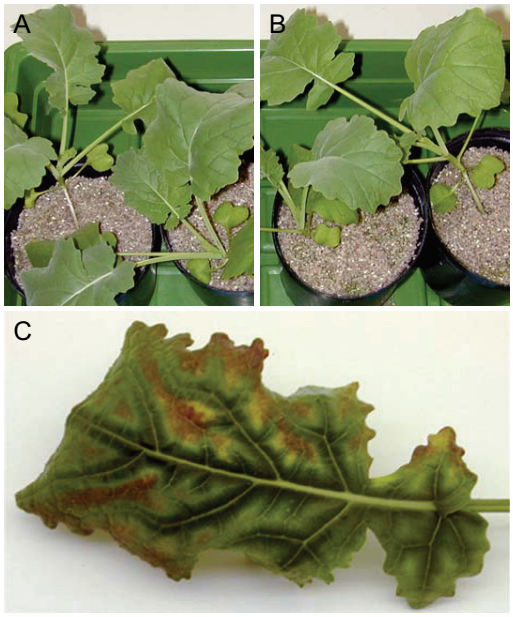


Figure1. Growth phenotype of S-deficient *B. napus*. A) Six week *B. napus* plants grown on quartz sand fertilized with Hoagland solution containing $MgSO_4$ and B) without sulfate fertilization. No S-deficiency symptoms are visible at this point. C) After 8 weeks of S-starvation plants showed clear symptoms of S-deficiency.

To confirm that plants fertilized with the solution without sulfur were indeed S-deficient, total sulfur and glutathione content were measured at the time of inoculation (Table 1). Sulfur starved plants even if they showed no visible symptoms, contained only 9.8% of total sulfur and 6.3% of GSH compared to normal plants. Plants grown on soil were included in our analysis to as comparison for plants growing in normal field conditions. Plants grown on sulfur containing Hoagland nutritive solution showed about 20% reduction in total sulfur content and about 60% reduction in total GSH content.

With the XRF analysis method used to determine total sulfur content it was also possible to obtain the plant content for Fe, Ca, Mg, K and P. We observed no significant difference for Ca, Mg and P. K was reduced in sulfur starved plants by about 40% and Fe was below detection level in all samples (data not shown).

Table 1. Total sulfur and GSH content of leaves of six week old *B. napus* and five week old *A. thaliana* plants grown on soil or on quartz sand with or without S fertilization.

	B. napus		*A. thaliana*	
	Total S (% of DW)	GSH total (nmol/gFW ±SE)	Total S (% of DW)	GSH total (nmol/gFW ±SE)
Soil	1.30	140.0 ± 18.3	0.79	54.0 ± 2.6
Sand/Hoagland +S	1.02	59.1 ± 7.5	0.74	154.9 ± 5.6
Sand/Hoagland -S	0.10	3.7 ± 1.0	0.75	147.9 ± 14.9

At the beginning of our study we have tried to grow *Arabidopsis thaliana* plants under the same conditions as rape to take advantage of the tools available for this model plant and then transfer the knowledge to *B. napus*. However, we were not able to obtain nicely growing sulfur deficient Arabidopsis plants. *A. thaliana* was very efficient in obtaining nutriment and showed a normal S content under the same S-starving condition as rape (Table 1). It only exhibited a 25% growth reduction in biomass production. After intensive washing of the quartz sand S-deficient *Arabidopsis* plants were obtained, but these plants did not grow properly. Already after 2 weeks, they started to show clear signs of senescence in form of yellowing and anthocyan production. Thus, Arabidopsis proved to be unsuitable for our experimental goals.

To study the effect of sulfur nutrition on plant disease resistance three pathogens were tested. *Leptosphaeria maculans* a specific pathogen of *Brassica* which is considered as a facultative necrotroph since the pathogen initially grows biotrophically, in the intercellular space without causing cell death. In a later phase, it promotes necrosis and can live saprophytically on dead plant material (Hammond et al., 1985; Hammond and Lewis, 1987). *Botrytis cinerea* is a very broad host range necrotrophic ascomycete. The hemibiotrophic oomycete *Phytophthora brassicae* is able to infect many crucifers including *B. napus*. Five different isolates of *L. maculans* and two isolates of *B. cinerea* exhibiting diverse aggressiveness were screened to find for both fungi a moderate aggressive isolate on normal plants. Cultivar Bienvenu was challenged with both *L. maculans* and *B. cinerea*. However due to its high resistance level to *L. maculans* cultivar Express was only inoculated with *B. cinerea*.

Figure 2A shows pictures of typical lesions triggered by each pathogen on leaves of *B. napus* grown with and without sulfur fertilization. For all three pathogens lesion size is clearly affected by the sulfur status of the plant. With both *L. maculans* and *B. cinerea* on control plants the necrotic lesion was surrounded by a black circle which defined clearly the end of the lesion. In S-starved plants this black circle was generally missing and the lesion expanded broadly.

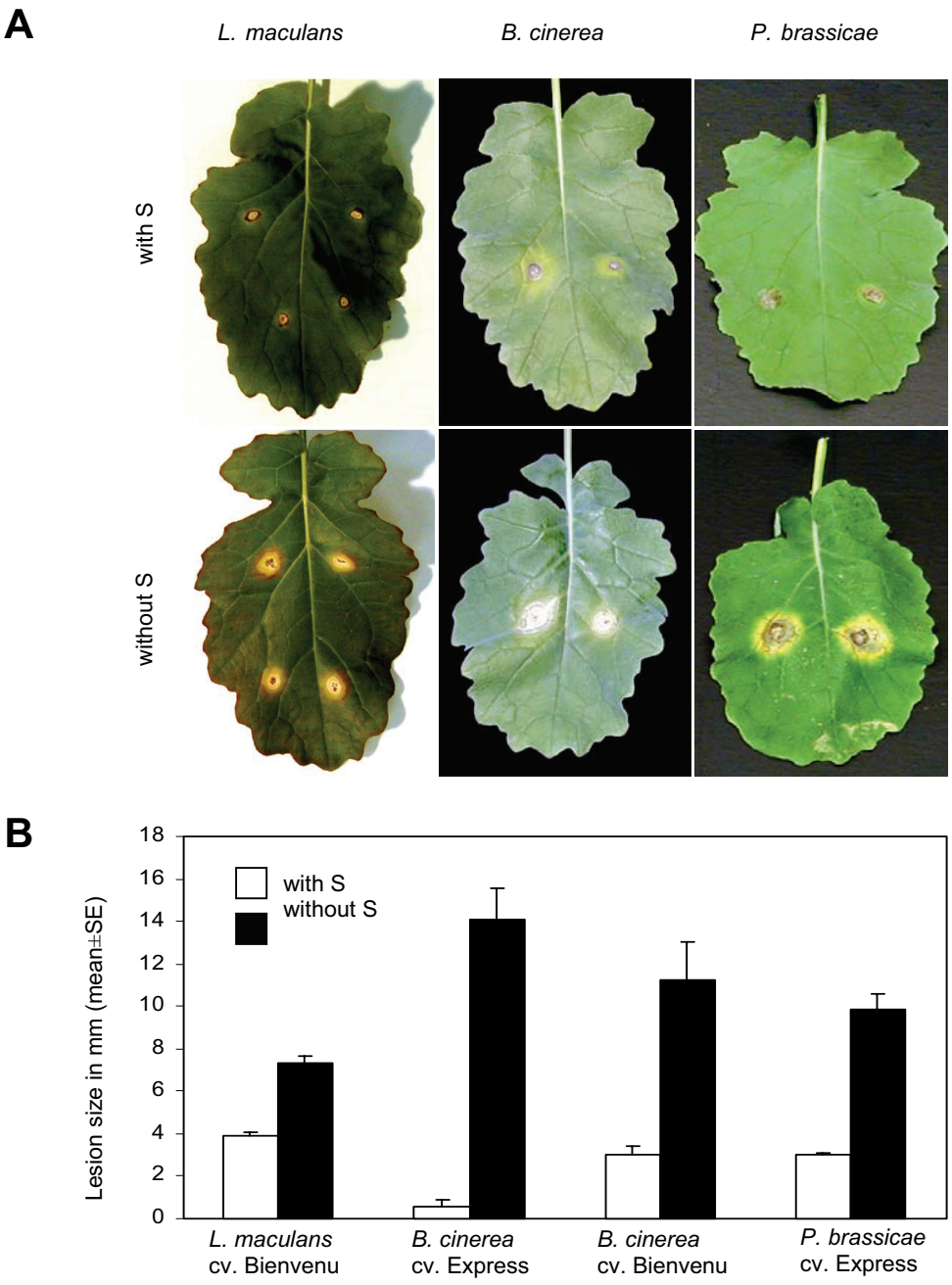


Figure 2. Disease resistance tests of fertilized and S-deficient *B. napus* plants. A) Disease symptoms on leaves of 6 week old *B. napus* caused by inoculation with *Leptosphaeria maculans* (21dpi), *Botrytis cinerea* (4dpi) and *Phytophthora brassicae* (7dpi). B) Summary of statistical analysis of the lesions size measurement. The experiments were repeated threefold and 32 independent measurements were done for *L. maculans*, and 16 independent measurements for *B. cinerea* and *P. brassicae*.

Figure 2B shows the effect of the sulfur status of *B. napus* cv Express and cv Bienvenu on disease susceptibility against the three pathogens. At 21 dpi lesions caused by *L. maculans* on S-deficient *B. napus* cv Bienvenu leaves were 1.9 times larger than in normally fertilised plants. In control plants lesion size remained the same between 12 dpi and 21 dpi whereas it still continued to increase in S-starved plants (data not shown). More pronounced differences were observed with *B. cinerea* in both *B. napus* cv Express and cv Bienvenu. In the cultivar Express lesions were at 4 days post inoculation about 24 times larger in sulfur starved plants compared to the controls. In the cultivar Bienvenu this difference was reduced to about 3.7 fold. Finally compared to control plants the disease lesions caused by *Phytophthora brassicae* were 3.3 times larger in the plants that lack adequate S-nutrition.

To quantify the fungal development by another method, we have used an isolate of *P. brassicae* that is expressing green fluorescent protein (GFP) as a quantitative marker (Si-Ammour et al., 2003). This fluorescent isolate makes it possible to quantify the pathogen biomass by measuring GFP fluorescence. Figure 3 shows that uninfected leaves (black area) had a base line fluorescence due to chlorophyll autofluorescence of 467 relative units for the control plants and 317 relative units for the S-starved plants. This reduction in autofluorescence indicates a reduction in chlorophyll content of S-starved plants. The fluorescence level was only slightly increased to 644 relative units in control plants inoculated with *P. brassicae* expressing GFP (black area) indicating that *P. brassicae* did hardly colonize these plants. In contrast, in inoculated S-deficient plants the fluorescence strongly increased to 1172 relative units. After subtraction of the background fluorescence there was a 4.8 fold increase in GFP fluorescence levels between normally fertilized plants and S-deficient plants indicating that S-deficient plants were much more susceptible towards *P. brassicae*.

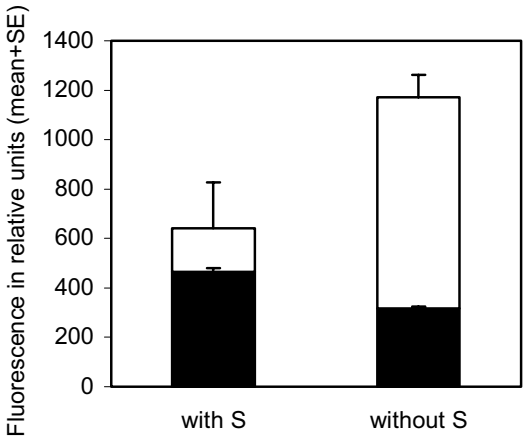


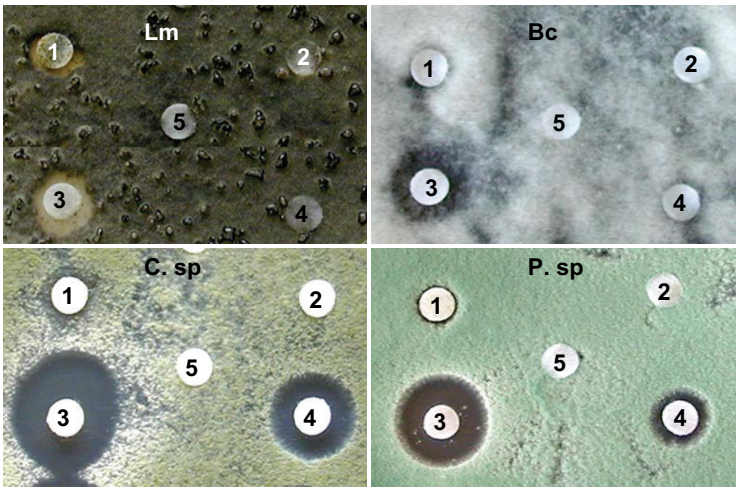
Figure 3. GFP fluorescence of differently fertilized *B. napus* cv Express infected with *P. brassicae* constitutively expressing GFP. Black area shows the level of autofluoresence of the chloroplasts. White area shows the GFP fluorescence caused by the spread of the *P. brassicae*.

Our results demonstrate that S-deficiency negatively affects disease resistance of *B. napus*. The increase in susceptibility could be caused by the specific effect of S-deficient on the accumulation of one or more S-containing defense compounds. To further analyse this hypothesis, we have tested extracts from normal and S-deficient plants for their antifungal potential.

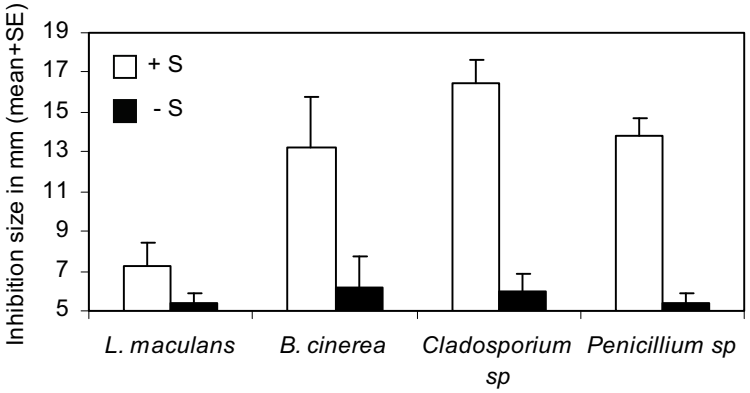
A total methanol extract used to quantify the glucosinolates from control plants was found to inhibit the *in vitro* growth of the four fungal pathogens. *L. maculans*, *B. cinerea*, *Cladosporium sp* and *Penicillium sp* as indicated in form of growth inhibition zones around the applied extract (Figure 4A). A similar extract from plants suffering S-deficiency had no or very little effect on all four fungi tested. Figure 4B shows that the inhibition zone caused by extracts from S-fertilised plants is in comparison to S-deficient plants 6.6 times larger for *L. maculans*, 6.8 times larger for *B. cinerea*, 12 times larger for *Cladosporium* and 25 times larger for *Penicillium*. Figure 4C shows the effect of the MeOH extracts on the growth of the phytopathogenic bacterium *Pseudomonas syringae* pv tomato, of *B. cinerea* and of *Penicillium sp* assayed in liquid culture in microtiter plates. Growth was measured as an increase in absorbance at 490 nm. Methanol extracts from S-deficient plants had 5.4 times lower antibacterial activity than extracts from control plants. The reduction in antifungal potential was 2.2 fold for *B. cinerea* and 2.5 fold for *Penicillium*. To test if the antifungal potential was increased following inoculation due to induced metabolites such as phytoalexins, MeOH extracts of *B. cinerea* inoculated *B. napus* leaves were analysed in fungal growth inhibition test. The plants were extracted 60 hours after inoculation. Figure 5 shows no significant difference in antifungal potential between extracts of inoculated or control plants leading to the conclusion that the antifungal potential is due to phytoanticipins and not phytoalexins. Furthermore, no inhibition was observed with protein extracts from both control and S-starved plants, challenged or not with *B. cinerea* as a pathogen to induce defense responses.

Figure 6 shows the quantification of four groups of GSL: alkene-, indolyl-, thioalkyl- and aromatic-GSLs. Table 2 shows the list of individual GSLs measured and the group to which they belong. The alkene GSLs group contains progoitrin, sinigrin, napoleiferin, gluconapin and glucobrassicanapin. The indolyl GSLs group includes glucobrassicin and neoglucobrassicin. The thioalkyl GSLs group comprises glucoiberin, sulphoraphene and glucoiberverin. The aromatic GSLs group contains gluconasturiin. The reduction of GSLs content in S-deficient plants is 14 times for indolyl GSLs, 18 times for aromatic GSL, 21 times for thioalkyl GSLs and 85 times for alkene GSLs. Thus, S-deficient had a dramatic negative effect on the glucosinolate content. There is a good correlation between reduced glucosinolates levels and reduced antifungal and antibacterial activity of the glucosinolate containing MeOH extract. To test the hypothesis that GSLs could be responsible for the antifungal potential of the MeOH extract, eight different commercially available isothiocyanates (ITC): methyl-ITC, ethyl-ITC, allyl-ITC, tert-butyl-ITC, phenyl-ITC, methoxyphenyl-ITC, 2-phenylethyl-ITC, benzyl-ITC and the degradation product of the GSL sinigrin were tested for their antifungal potential on agar plates against *Cladosporium* (Table 2). None of the eight ITC and sinigrin degradation products show antifungal activity even at very high concentration of 1mM (data not shown). It remains an open question whether the degradation products of the many other GSLs of *B. napus* possess direct antifungal activity.

A



B



C

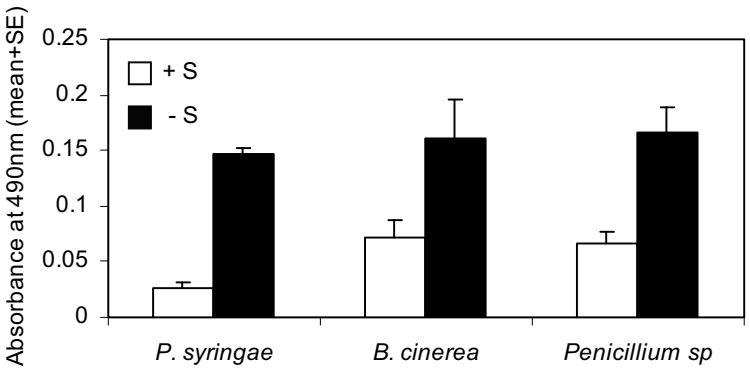


Figure 4. Antimicrobial activity of MeOH extracts from fertilized and S-deficient *B. napus* plants. A) Growth inhibition of *Leptosphaeria maculans* (Lm), *Botrytis cinerea* (Bc), *Cladosporium sp* (C. sp) and *Penicillium sp* (P. sp). 1 and 2 are extracts of plants grown without sulfur fertilization; 3 and 4 are extracts of plants grown with sulfur fertilization. 2 and 4 are 10-times diluted compared to 1 and 3, respectively. 5 corresponds to a control. B) Quantitative analysis of the growth inhibition tests. C) Inhibitory effect of the MeOH extracts on the growth of the bacteria *P. syringae* pv *tomato* and on *B. cinerea* and *Penicillium sp*. The test organisms were grown in liquid culture in microtiter plates. Quantification was done by measurement of absorbance at 490 nm. Each value is the average of five measurements. The experiments were repeated threefold.

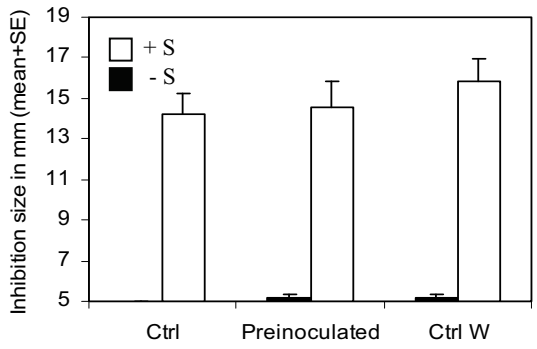


Figure 5. MeOH extracts of preinoculated plants do not have increased antifungal potential. Ctrl corresponds to extracts from non inoculated plants, Preinoculated represents extracts from plants inoculated with *B. cinerea* 60 hour prior to extraction. Ctrl W correspond to extracts of non inoculated plants from which the GSLs content was analysed (see Figure 6).

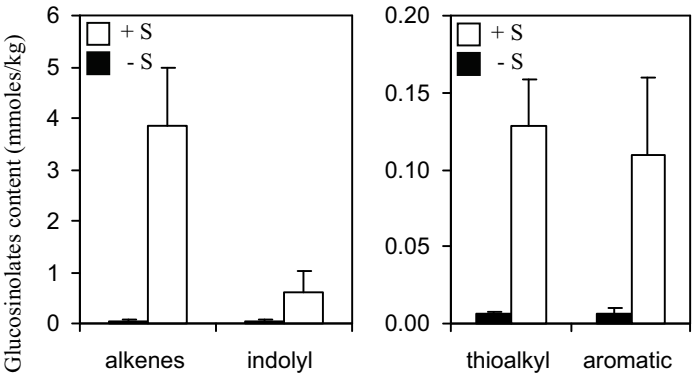


Figure 6. Quantification of glucosinolate concentration in MeOH extracts of normal and S-deficient *B. napus*. The analysed individual glucosinolates were grouped into 4 groups according to their chemical features (see Table 2). Each group of GSL shows strong reduction in of S-deficient plants. Reduction is 85x for alkene, 14x for indolyl, 21x for thioalkyl and 18x for aromatic GSL.

Table 2. List of glucosinolates measured in *B. napus* cv Express leaves and isothiocyanates (ITC) tested in bioassays. The individual GSLs analysed were grouped according to their structural similarities: A = alkene, B = aromatic, I = indolyl and T = thioalkyl.

Glucosinolates analysed		
common name	chemical name	group
progoitrin	2(R)-2-hydroxy-3butenyl	D
sinigrin	2-propenyl	D
napoleiferin	2-hydroxy-4-pentenyl	D
gluconapin	3-butenyl	D
glucobrassicanapin	4-pentenyl	D
gluconasturtiin	2-phenylethyl	G
glucobrassicin	indole-3-ylmethyl	I
neoglucobrassicin	1-methoxyindol-3-ylmethyl	I
glucoiberin	3-(methylsulfinyl)propyl	A
sulforaphene	4-methylsulfinyl-3-butenyl	A
glucoberverin	3-methylthio)propyl	A
ITCs tested in bioassays	**corresponding GSL**	
allyl-ITC	allyl-GSL	
benzyl-ITC	glucotropaeolin	
ethyl-ITC	glucolepidiin	
3-methoxyphenyl-ITC	methoxyphenyl-GSL	
methyl-ITC	glucocapparin	
phenylethyl-ITC	gluconasturtiin	
phenyl-ITC	phenyl-GSL	
ter-butyl-ITC	butyl-GSL	

Discussion

Sulfur is part of a wide variety of cellular components and plays critical roles in a number of cellular processes, such as structural and regulatory roles via protein disulfide bridges, or biochemical roles like electron transport in Fe/S-clusters, redox cycle, detoxification of heavy metals and xenobiotics, and metabolism of secondary products (Hell, 1997; Saito, 2000). The major part of reduced sulfur is channelled from cysteine into methionine, Fe/S clusters, vitamin cofactors and proteins (Hell and Hillebrand, 2001). Under S-deficient growth conditions plants try to acclimate by synthesizing high-affinity sulfate transporters that function in the efficient uptake of external sulfur and reallocation of internal sulfur sources (Takahashi et al., 1997; Leustek et al., 2000; Saito, 2000). In recent many products of the plant's response to pathogen turned out to be S-containing compounds like the phytoalexins and glucosinolates of crucifers or the sulfur rich antifungal peptides (Vignutelli et al., 1998; Tierens et al., 2001). The reduction of atmospheric sulfur pollution lead to S-deficiency in the field and had a negative impact on yield and pathogen resistance. In this study we focused on the effect of S-deficient on plant disease resistance.

Taken together our results showed clearly that oilseed rape plants grown under S-deficiency conditions exhibited enhanced disease susceptibility (Figure 2). This was true for all pathogens tested, for the specialized *L. maculans* and the ubiquitous pathogens *B. cinerea* and *P. brassicae* and for the two *B. napus* cultivars Express and Bienvenu. The enhanced disease susceptibility is apparently caused by a general mechanism because the change in susceptibility was not pathogen or cultivar specific. Field experiments pointed at a positive correlation between S-fertilisation and disease resistance of oilseed rape against fungal pathogens but no causal link was demonstrated (Schnug, 1996). There are two main ways to explain why S-deficient plants become more susceptible to pathogens. First, the increased susceptibility is caused by the specific effect of S-deficiency on the accumulation of S-containing defense compounds such as phytoalexins, glucosinolates and cysteine-rich antifungal polypeptides which play a determinant role in pathogen resistance. Second, S-deficiency leads to a general fitness reduction and a global weakening of the plant that causes general susceptibility to stress.

In our study MeOH extracts of control plants showed antifungal activity with four different fungal pathogens and this activity was almost completely lost in MeOH extracts of S-deficient plants. Furthermore, the antifungal potential was not increased by activation of induced defense mechanism. This means that the responsible compounds are phytoanticipins present in healthy plants before infection and not phytoalexins induced after infection. Interestingly, the reduced antifungal activity of extracts from S-deficient plants correlated with a strong reduction in GSLs content. Upon tissue damage GSLs enter in contact with the enzyme myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1) and the unstable alglycone generated by the action of myrosinase may then form various degradation products, including isothiocyanates, nitriles and thiocaynates, all of which are highly reactive compounds (Mithen et al., 1986; Osbourn, 1996). The major breakdown products generated in leaves of *Brassica* are isothiocyanates. GSL hydrolysis products have been demonstrated to be toxic towards a range of fungi *in vitro*, including pathogens and non-pathogens of *Brassica*. The mechanism of toxicity is not known (Mari et al., 1993; Manici et al., 1997; Hashem and Saleh, 1999). A number of pathogens of *Brassica*, such as *L. maculans* (Mithen et al., 1986) *Peronospora parasitica* (Greenhalgh and Mitchell, 1976) *Mycosphaerella brassicae* (Hartill and Sutton, 1980) and Alternaria sp (Milford et al., 1989), have been shown to be sensitive to at least some glucosinolate breakdown products. It has also been suggested that indolyl GSLs breakdown products may function as precursors to a class of indole phytoalexins that are induced in Brassica (Rouxel et al., 1989).

However, it is unclear whether there is any relationship between resistance of fungi to GSLs and ability to cause disease. Although inoculation with *L. maculans* elicited changes in the leaf indolyl-GSL profiles, no correlation was found between degree of resistance to the fungus and the level of GSLs (Wretblad and Dixelius, 2000). The role of GSLs in blackleg resistance has previously been studied, but the relevance of these coumpunds remained still unclear. Early studies correlated sinigrin content with resistance to *L. maculans* (Mithen et al., 1987). High GSL levels have been associated with resistance of oilseed rape and Indian mustard to *L. maculans* (Mithen and Magrath, 1992) and with resistance of cabbages to *P. parasitica* (Greenhalgh and Mitchell, 1976). However, in studies of crosses of *B. napus* lines with high and low GSLs levels in their leaves, resistance to *L. maculans* and GSLs profiles did not cosegregate (Mithen and Magrath, 1992). Further studies with *B. napus* lines that had contrasting GSLs profiles indicated that high levels of GSLs are unlikely to confer greater resistance to *L. maculans* in oilseed rape (Giamoustaris and Mithen, 1997). Furthermore *P. lingam* was shown to efficiently degrade the aliphatic GLSs sinigrin, progoitrin and gluconapin as well as aromatic GSLs sinalbin (Wu and Meijer, 1999). This high number of GSLs in *B. napus* makes it difficult to assess the antifungal activity of individual GSL. In addition, the nature of the breakdown products depends on the structure of the GSLs, the type of myrosinase present, and other factors, like pH, temperature, metal ion concentrations, and protein cofactors (Osbourn, 1996). It is therefore difficult, simply by assessing the relative amounts of specific GSLs present in the host plant, to predict which toxic products a pathogen is likely to encounter. Our tests with a limited number of pure isothiocyanates and the degradation products of sinigrin showed that none of the tested GSLs derived molecules exhibited antifungal activity. Apparentely some other compounds including other GSLs appear to be responsible for the antifungal activity present in MeOH extracts of S-fertilized plants. This means that some other molecules, not tested yet, are responsible for the antifungal activity.

In conclusion we demonstrated that sulfur deficiency increases the susceptibility of oilseed rape plants to fungal pathogens. S-deficient plants appear to lack one or more antifungal phytoanticipins. The increase in susceptibility correlates with the reduction of GSLs, but no causal relationship was demonstrated because none of the isothiocyanates or degradation products of sinigrin tested showed antifungal activity. The second hypothesis of a general weakening of the plant could still play an additional important role in the decrease of resistance of S-starved oilseed rape.

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Chapter 3

Transcriptome of Arabidopsis gene families related to secondary metabolism differentiates responses to groups of abiotic and biotic stressors

Transcriptome of Arabidopsis gene families related to secondary metabolism differentiates responses to groups of abiotic and biotic stressors

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Summary

Plant secondary metabolism significantly contributes to defensive measures against adverse environmental cues. To investigate stress-induced alterations at the transcriptional level of underlying genes, a DNA array (MetArray) harboring gene-specific probes was established, which combined *Arabidopsis thaliana* effector gene families encoding enzymes acting consecutively in secondary metabolism and defense reactions. It contained the complete set of genes encoding 109 secondary product glycosyltransferases and 63 glutathione-utilizing enzymes along with 62 cytochrome P450 monooxygenases and 28 ABC transporters. Their transcriptome was monitored in different organs of unstressed plants and in shoots in response to herbicides, UV-B radiation, endogenous stress hormones, and pathogen infection. A principal component analysis based on the transcription of these effector gene families defined distinct responses. Methyl jasmonate and ethylene treatment was separated from a group combining reactions towards two sulfonylurea herbicides, salicylate and an avirulent strain of *Pseudomonas syringae*. The responses to the herbicide bromoxynil and UV-B radiation were separate from both groups. A few genes were diagnostic in their specific response to two herbicide classes used. Interestingly, a subset of genes induced by *P. syringae* was not responsive to the applied stress hormones. In addition, small groups of comprehensively induced effector genes may be part of defense mechanisms activated by several converging pathways. The differentiating expression patterns detected by the MetArray provide a framework of information regarding the function of individual genes and argue against widely redundant functions within the large gene families analyzed.

Introduction

Plants feature an enormous variety of small organic, secondary metabolites, which possess diverse protective, defensive or signaling functions. Accordingly, many enzyme activities encoded by large gene families are involved in the biosynthesis, modification and compartmentation of these compounds. These gene families are assumed to have evolved to provide a broad diversification of similar biochemical reactions and flexibility of plant responses to different environmental conditions (Pichersky and Gang, 2000). Our laboratories are aiming at elucidating the function of selected gene families related to plant secondary metabolism, in particular with respect to their role as effector genes in plant xenobiotic metabolism and pathogen defense. This study is focused on analyzing the transcriptome of four gene families that cover different steps in secondary metabolism: oxidation by cytochrome P450 monooxygenases (CYP), conjugation with UDP-activated carbohydrates by secondary metabolite glycosyltransferases (UGT), conjugation with the tripeptide glutathione by glutathione transferases (GST), and eventually compartmentation via ATP-binding-cassette transporters (ABC transporters). Importantly, these enzyme classes are often acting consecutively in the synthesis of secondary compounds and in detoxification reactions. Products of CYP-catalyzed reactions are substrates for transferases such as UGTs and GSTs thus leading to tagged molecules, which may be transported to the vacuole or excreted by ATP-driven export pumps (Coleman *et al.*, 1997; Jones and Vogt, 2001; Kreuz *et al.*, 1996; Martinoia *et al.*, 2002; Sandermann, 1994; Wagner *et al.*, 2002).

CYP genes form one of the largest families with 246 genes and 26 pseudo-genes annotated in *Arabidopsis* (Werck-Reichhart *et al.*, 2002). Catalytic functions of CYPs are extremely diverse and usually result from the activation of molecular oxygen and insertion of one oxygen atom into a lipophilic substrate to produce a compound that can be further processed (Mansuy, 1998; Werck-Reichhart and Feyereisen, 2000). Plant CYPs are involved in the synthesis of precursors of polymers, pigments, signaling and defense molecules and the hydroxylation or dealkylation of exogenously applied compounds (Chapple, 1998; Kahn and Durst, 2000; Werck-Reichhart *et al.*, 2002; Schuler and Werck-Reichhart, 2003).

CYPs are defined by a common structural fold and only three strictly conserved amino acids (Graham and Peterson, 1999; Werck-Reichhart and Feyereisen, 2000). Sequence identity among plant CYPs is variable ranging from below 20% to higher than 95% in highly duplicated sub-families forming clusters of up to 13 genes at the same locus. By far the largest CYP clade is usually referred to as plant specific class A including mainly enzymes of secondary metabolism. Four to six other clades, referred to as Non-A CYPs, are related to animal or microbial enzymes involved in lipid, sterol or large isoprenoid metabolism (Paquette *et al.*, 2000; Werck-Reichhart *et al.*, 2002). The function of more than 80% of the *Arabidopsis* CYP genes is unknown.

Limited information is available on the expression of CYP genes, except for a few genes with a characterized function (Bell-Lelong *et al.*, 1997; Mizutani *et al.*, 1997; Kubigsteltig *et al.*, 1999; Mathur *et al.*, 1998; Mikkelsen *et al.*, 2003; Nair *et al.*, 2002). Several CYPs were transcriptionally activated with dual developmental and stress responsive control (for review: Werck-Reichhart *et al.*, 2002). The first attempt at a more systematic analysis of CYP expression in *Arabidopsis* using microarray technology was recently reported (Xu *et al.*, 2001). This analysis highlighted the problems caused by the use of non-gene-specific probes that was to be circumvented in our analysis.

UGTs transfer carbohydrate residues onto small organic compounds to regulate their activity, toxicity or amenability to transport. Many of these compounds such as phytoalexins, cell wall precursors and

plant hormones are important for plant defense, cellular homeostasis and signaling. UGTs constitute a large gene family of 120 members including eight putative pseudogenes annotated in *A. thaliana* Columbia (Paquette *et al*., 2003). They are defined via a conserved amino acid domain in the C-terminal region. Based on a phylogenetic alignment 14 *A. thaliana* UGT groups A to N were compiled (Jones and Vogt, 2001; Li *et al*., 2001). There is only very limited information on the function of individual UGTs. A systematic analysis of the substrate properties using recombinantly expressed proteins has been initiated. *In vitro*, Group E and L UGTs were shown to glucosylate indole-3-acetic or salicylic acid as well as hydroxycinnamate derivatives (Jackson *et al.*, 2001; Lim *et al*., 2001, 2002; Milkowski *et al*., 2000). UGTs may display a reduced substrate specificity and rather be regioselective accepting certain chemical substructures (Jones and Vogt, 2001). Importantly, interfering side-activities towards xenobiotic substrates have been described for recombinant *Arabidopsis* group E and L enzymes (Messner *et al*., 2003). The function of several UGTs in flavonoid glycosylation has been studied in other plant species (for review: Jones and Vogt, 2001). Information on the expression patterns or transcriptional regulation of *Arabidopsis* UGTs is almost completely lacking. Indole-3-acetic acid glucosyltransferase *AtUGT84B1* was found to be preferentially expressed in apical regions, whereas *AtUGT73B5* was induced during superoxide-dependent cell death (Jackson *et al.*, 2001, 2002; Mazel and Levine, 2002).

Similarly to UGTs, GSTs have been implicated in the detoxification of endogenous and xenobiotic compounds and in plant secondary metabolism (Edwards *et al.*, 2000; Marrs, 1996). GSTs are a family of multifunctional, dimeric enzymes that catalyze the conjugation of the tripeptide glutathione to a large variety of lipophilic compounds with electrophilic centers. Plant GSTs are encoded by large and diverse gene families. The 47 *Arabidopsis* GSTs are divided on the basis of sequence similarity into the phi, tau, theta and zeta classes (Dixon *et al*., 2002; Edwards *et al*., 2000; McGonigle *et al*., 2000; Wagner *et al.*, 2002). A fifth class of GST-like genes (lambda), which does not possess glutathione transferase activity (Dixon *et al.*, 2002), was not included in this study. Considering the high number of plant specific phi and tau GSTs, relatively little is known about their roles in the metabolism of plants. Some members have demonstrated roles in herbicide detoxification (Edwards and Dixon, 2000; Marrs, 1996). Phi and tau GSTs catalyze the glutathione-tagging of secondary metabolites including phytoalexins and isothiocyanates, function as glutathione peroxidases and have non-catalytic roles as flavonoid-binding proteins (Dixon *et al*., 2002; Edwards *et al*., 2000). Some of these GSTs appear to have roles as auxin- and cytokinin-binding proteins, as components of UV-inducible cell signaling pathways (Loyall *et al*., 2000) or as potential regulators of apoptosis (Kampranis *et al*., 2000; for review: Dixon *et al*., 2002). Theta and zeta GSTs have homologs in animals and fungi. Zeta GSTs catalyze an important glutathione-dependent isomerization step in the catabolism of tyrosine (Dixon *et al*., 2000). Theta GSTs may be mainly active as glutathione peroxidases reducing organic hydroperoxides produced during oxidative stress (Dixon *et al*., 1999; Edwards *et al*., 2000; Wagner *et al.*, 2002). In agreement with their postulated function in stress protection, the expression of several GSTs is enhanced upon exposure of plants to various stress situations including exposure to ozone, hydrogen peroxide, signaling molecules, heavy metals, heat shock, dehydration, wounding, senescence, biotic elicitors and microbial infection (for review: Marrs, 1996; Wagner *et al*., 2002).

Because of the important nature of glutathione-dependent reactions in plant stress responses a complete set of additional glutathione-utilizing enzymes from *Arabidopsis* was included in this study: eight glutathione peroxidases (*AtGPX*), six glyoxalases (*AtGLX*) and two glutathione reductases (*AtGR*). GPXs catalyze the GSH-dependent reduction of hydrogen peroxide and organic hydroperoxides. GPX expression is induced in many stress situations and GPXs are important to protect cells against oxidative damage (Mullineaux *et al*., 1998; Roxas *et al*., 1997). The glyoxalase

system converts 2-oxoaldehydes into the corresponding 2-hydroxy acids via a glutathionated intermediate. The process involves two consecutive reactions mediated by glyoxalase I (GLXI, lactoylglutathione lyase) and glyoxalase II (GLXII, hydroxyacylglutathione hydrolase). The primary substrate appears to be methylglyoxal, a cytotoxic compound mainly formed as a by-product of glycolysis (Thornalley, 1990). GRs catalyze the conversion of oxidized to reduced glutathione using NADPH as an electron donor and play a major role in maintaining the reduced to oxidized glutathione balance (Noctor *et al.*, 2002).

Finally, ABC transporters are involved in compartmentation of both endogenous metabolites and catabolites and in detoxification of xenobiotic conjugates. They are characterized by the presence of specific transmembrane and signature ATP-binding cassette domains. *Arabidopsis* harbors 105 predicted members, which is about twice the number found in the human genome (Martinoia *et al*., 2002). 54 members are full-size transporters containing two ATPase and two transmembrane domains each. These include 15 MRPs (multidrug resistance related protein), 15 PDRs (pleiotropic drug resistance protein) and 22 PGPs (P-glycoprotein). *AtAOH1* (ABC1 homolog) and *AtPMP* (peroxisomal ABC transporter homolog) are distinct full-size members whereas there are 51 additional half-size ABC transporters. The nomenclature of MRP, PDR, and PGP transporters is based on Martinoia *et al.* (2002); for *AtAOH1* and half-size ABC transporters the classification by Sánchez-Fernández *et al.* (2001) was used. A few members have been localized to different membranes. AtMRP2 was inserted in the tonoplast, whereas PGP and PDR members were located to the plasma membrane (for review: Davies and Coleman, 2000; Martinoia *et al*., 2002; Sánchez-Fernández *et al*., 2001).

Studies with *AtMRP1* to *AtMRP4* (Tommasini *et al.*, 1997; Sánchez-Fernández *et al*., 1998) showed that transcript levels of *AtMRP3* are highly increased after the addition of primisulfuron and three other herbicides. Interestingly, *AtMRP3* can also partially complement YCF1-deficient yeast for cadmium tolerance (Tommasini et *al.*, 1998), and is enhanced after cadmium exposure (Bovet *et al.*, 2003). Furthermore, two stress-inducible PDR-type ABC transporters from *Nicotiana* species were identified. *NtPDR1* was induced by elicitors as well as methyl jasmonate but not by ABA and salicylic acid (Sasabe *et al.*, 2002). Expression of *NpABC1* was enhanced by plant-derived fungicides that may be excreted by this transporter to attack a fungal invador (Jasinski *et al*., 2001). *SpTUR2*, another PDR-like transporter of *Spirodela polyrrhiza* was highly induced by ABA and low temperature (Smart and Fleming, 1996). Thus, these few biochemical and expression studies support the idea that ABC transporters might be involved in plant defense.

The present study was to initiate the joint functional analyses of these gene families aiming at identifying differential recruitment of individual members and groups of co-regulated genes in response to abiotic and biotic stressors. These included herbicides belonging to two different classes, UV-B irradiation, the endogenous stress hormones salicylic acid, ethylene, and methyl jasmonate, as well as infection with the avirulent bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*avrRpt2*). A DNA array, named MetArray, for parallel expression profiling of 265 members of these gene families was established by designing gene-specific probes from their 3´-regions.

Results & Discussion

Gene families and generation of probes

Genes belonging to large families are often highly homologous, particularly within their coding regions. Therefore, target sequences for hybridization to genes associated with secondary metabolism were selected from their 3´-ends including untranslated regions. Probes were designed and checked using a semi-automatic, web-based algorithm (Affenzeller *et al*., in preparation). Briefly, the specificity of probes was first tested via BLAST analyses against the whole *A. thaliana* genome sequence. In addition, FASTA sequence comparisons with all predicted *A. thaliana* ORFs of the MIPS database including 500 nt extensions at both ends were performed. These analyses identified any possible cross-hybridization with annotated genes. However, further analyses eliminated most of these potential conflicts when considering the relative orientation of genes and experimental cDNA sequences. Nevertheless, in a few cases it was not possible to exclude any cross-reactivity due to densely packed genes in tail-to-tail orientation or to the high homology of the genes. In agreement with Xu *et al.* (2001) probes exhibiting a homology of at most 70% over a stretch of 70 nt were regarded as highly specific; a considerable cross-hybridization was indicated for probes with more than 85% homology over 100 nt.

Genome-wide sets of gene-specific probes for 109 Arabidopsis UGTs including five putative pseudogenes, all 47 GSTs, eight GPX, six GLX, and two GR were designed. In addition, two subsets were chosen from *Arabidopsis* genes encoding CYP and ABC transporters. A group of 62 genes out of 246 predicted CYP members was selected for this study. Genes were arbitrarily chosen to include members fulfilling any of the following criteria: known function, high EST frequency, a sample of A and non-A type CYP genes, and a sample of genes belonging to highly duplicated subfamilies. Twenty-six out of 54 ABC transporters were randomly selected on the basis of known full-length members at the beginning of this project. The set contained nine MRP-, eight PGP-, eight PDR-type genes and the single ABC-1 homolog *AtAOH1*. Two different half ABC transporter genes were arbitrarily chosen (*AtTAP1, AtWBC3*). The phylogenetic relationships of the gene families represented on the MetArray are shown in Figure 1.

Information concerning all probe sequences, bioinformatic analyses and specificity can be accessed at mips.gsf.de/proj/thal/primerDesign/index.html. The results are summarized in Suppl. Tab. 1; specific comments regarding 35 out of 271 probes are highlighted to indicate a possible cross-reactivity. Since these bioinformatic analyses pinpointed any potential conflict, *e.g*. the highly homologous pairs *AtGSTF2*/ *AtGSTF3* and *AtGSTF6*/ *AtGSTF7*, a targeted examination by an independent technique can be specifically approached.

Organ distribution of transcripts

The MetArray was used to obtain comprehensive information about the organ-specific expression patterns of these gene families in roots, rosette leaves, stems, inflorescences and immature siliques. This knowledge will provide a framework for assessing the functions and possible redundancies of these genes.

Ubiquitously and often highly expressed genes were detected among all gene families (Fig. 1a-g). Most remarkable was the high and constitutive expression of most glyoxalases, glutathione reductases and glutathione peroxidases as well as many glutathione transferases (Fig. 1c, d, e). Among the

glutathione peroxidases *AtGPX1* and *AtGPX3* showed the highest transcription in all organs, whereas the closely related *AtGPX6* could not be detected in any tissue (Fig. 1c). All glyoxalases except *AtGLX-II.1* were expressed at a high level in all organs (Fig. 1d). A few out of 47 Arabidopsis glutathione transferases were highly and ubiquitously transcribed. This mostly pertained to members from closely related subgroups, such as the tau GSTs *AtGSTU5, AtGSTU13*, *AtGSTU19*, *AtGSTU20* and the phi members *AtGSTF2*, *AtGSTF8*, *AtGSTF9*, and *AtGSTF10*. In contrast, transcripts of highly related members were not detected or differentially expressed (Fig. 1b). Among the CYP, UGT and ABC transporter genes only single members were constitutively expressed at a higher level, *e.g. AtCYP73A5*, *AtCYP51A2*, *AtCYP81H1*, *AtCYP98A9*, *AtUGT72B1*, *AtUGT74C1*, *AtUGT80A2*, *AtUGT83A1*, *AtUGT89B1*, *AtMRP11* or *AtPDR8*. These high and wide-spread expression patterns might indicate constitutive functions. Nevertheless, it was interesting to note that a few of these genes were further enhanced in response to different stimuli, *e.g*. *AtGPX1*, *AtGPX3*, *AtGPX4*, *AtGR2*, *AtGSTF2*, *AtGSTF10*, *AtGSTU19*, *AtUGT80A2*, *AtCYP71B28*, *AtCYP73A5*, or *AtPDR8* (see below; Tab. 1, 2, 3).

Assuming that gene products within highly homologous subfamilies have at least related biochemical functions, the expression analyses supported both potentially redundant functions by similar expression patterns and diversity by organ-specific transcription. Highly related members of the large UGT subgroup L (Ross *et al*., 2001; Fig. 1f: *UGT74B1 – UGT84A4*) showed widely overlapping expression patterns, which could indicate redundant functions. However, using recombinant enzymes many of these *UGT*s had been shown to exhibit differential substrate preferences (Jackson *et al*., 2001; Lim *et al*., 2001, 2002). Similarly, the overlapping expression patterns of *AtGSTU16*/ *AtGSTU17*/ *AtGSTU18*, of *AtGPX1*/ *AtGPX3* or of *AtGR1*/ *AtGR2* (Fig. 1b, c, e) would be in agreement with redundant functions within the respective groups. However, they differed in their stress responsiveness (see below). Several genes belonging to highly related branches displayed non-redundant expression patterns *per se*, *e.g. AtUGT71C*, *AtUGT76E*, and *AtUGT79B* branches, *AtGSTF11*/ *AtGSTF12*, theta *AtGST* and several tau *AtGST* subgroups, *AtGPX2*/ *AtGPX7*, or *AtPDR1*/ *AtPDR6*/ *AtPDR7*/ *AtPDR8*. Finally, at least a single member out of small *UGT* groups with at most three members was ubiquitously expressed in all tissues, *e.g*. *AtUGT78D1* (group F), *AtUGT83A1* (I), *AtUGT86A2* (K), or *AtUGT92A1* (M; groups according to Ross *et al.,* 2001) (Fig. 1f).

CYP genes seemed to have a much stronger on/off control than most other genes (Fig. 1a). This might reflect a tighter control of gene expression, possibly related to the higher substrate specificity and their functions as rate limiting steps in many pathways. Among the other gene families studied there were only a few examples that showed similar control. Restricted expression patterns were observed for *AtUGT71C1*, *AtUGT72E2*, members of the *AtUGT76B* subgroup, several *AtGSTU* members, or *AtPDR7* and *AtPDR11* (Fig. 1).

These observations support the functional significance of the diversity provided by many subgroups and individual members within these gene families and argue against true redundant functions. This analysis greatly extended the knowledge on the organ-specific expression within these genes families. In most cases previous information on the expression pattern of single genes was in agreement with the array expression analysis. However, when comparing the array data with other existing information it is important to bear in mind that developmental and environmental differences may contribute to discrepancies. A technical difference could account for the observation that the array expression levels in leaves tended to be lower. To exclude a dilution of the total RNA by ample organellar rRNAs, RNA samples were quantified prior to hybridizations based on 25S/ 18S rRNA fluorescence (Experimental procedures). Nevertheless, a considerable dilution of transcripts by high

amounts of photosynthetic gene mRNAs may still affect hybridization in a different way than RT-PCR analyses.

In accordance with Northern and promoter-reporter analyses (Bell-Lelong *et al.,* 1997; Mizutani *et al*., 1998) *AtCYP73A5* was a highly expressed CYP gene showing highest levels in roots and stems. This pattern is in agreement with its essential role as cinnamate 4-hydroxylase acting upstream in the phenylpropanoid pathway in the biosynthesis of lignin, flavonoids and other phenolic compounds. Another CYP gene showing high constitutive expression in most plant organs was *AtCYP51A2*. It encodes 14_-demethylase having a housekeeping role in the biosynthesis of steroids. Interestingly, *AtUGT80A2,* which glucosylates sterols *in vitro* (Warnecke *et al*., 1997), appeared to be co-expressed as one of the highest and ubiquitously transcribed UGT genes. The prevalent expression and role of *AtCYP51A2* over that of the related *AtCYP51A1* is in agreement with the observation that *AtCYP51A2* antisense plants were strongly compromised in their growth and development (Kushiro *et al*., 2001). *AtCYP79Bs* and *AtCYP83B1* coding for enzymes catalyzing different steps in the synthesis of glucosinolates were co-ordinately expressed at their highest levels in roots. The expression profiles of *AtCYP83B1* and *AtCYP76C1* were in complete agreement with that previously observed by RNA blot analysis (Mizutani *et al.*, 1998). High expression of *AtCYP79B2* in roots was reported by Mikkelsen *et al.* (2000), however, the MetArray did not detect the lower levels in other organs.

Knowledge of the organ-specific expression patterns of UGT and GST genes was almost completely lacking. The expression of an indole-acetic acid glucosyltransferase *AtUGT84B1* in siliques was in agreement with RT-PCR analyses showing strong signals in siliques along with weak expression in inflorescences and roots (Jackson *et al.*, 2001). A more extended comparison was possible with recently published RT-PCR analyses of the MRP and PDR subgroups of ABC transporters (Kolukisaoglu *et al*., 2002; van den Brûle and Smart, 2002). In a qualitative manner, the MetArray expression patterns matched most of the reported RT-PCR data. Variations in developmental stages and growth conditions as well as technical differences (see above) may account for quantitative discrepancies such as the high expression of *AtMRP5* observed in immature siliques (Fig. 1g) in contrast to Kolukisaoglu *et al.* (2002). It might reflect slight developmental shifts since *AtMRP5*-promoter reporter plants indicated that *AtMRP5* was highly upregulated during the early stage of grain filling (Klein and Martinoia, unpublished).

Additional examples comparing array data and independent analyses are provided as supplementary information (Suppl. Tab. 2).

Principal component analysis of transcriptional responses to chemical, biological and physical challenges

Gene families acting in secondary metabolism may be involved as effector genes in response to diverse environmental cues. Therefore, the MetArray was used to monitor transcriptional changes in response to a spectrum of abiotic and biotic stimuli and to elucidate both stress-specific responses and overlapping crosstalks. The analyses were focused on the reaction of *A. thaliana* leaves 24 hours after application of the stimuli. As chemical stressors two different classes of herbicides were chosen (Fig. 2). Bromoxynil [BXN], a photosystem II targeting herbicide and two different sulfonylurea herbicides, primisulfuron [PRI] and prosulfuron [PRO], interfering with the biosynthesis of branched amino acids were sprayed at sublethal doses. UV-B irradiation [UVB] was used as a physical stressor

provoking oxidative damage and defense reactions (Surplus *et al*., 1998). Infection by the avirulent bacterial pathogen, *Pseudomonas syringae* pv. *tomato* DC3000 (*avrRpt2*) [PSavir] was included as a biological stressor. Finally, plants were treated with the three plant stress hormones salicylic acid [SA], ethylene [ETH] and methyl jasmonate [MeJA] (Dong, 1998; Kunkel and Brooks, 2002). Only those genes were kept for further analyses, whose expression was detected in at least three treatments or showed a greater than 2-fold change in at least one condition. This measure reduced the number of genes from 265 to 134 (Suppl. Tab. 4). The complete set of data including replica experiments can be accessed as supplementary data (Suppl. Tab. 5, 6).

To identify differences between and correlations among the transcriptional responses to the various stimuli the data were subjected to a principal component analysis (PCA; Fig. 3). Transcriptional reactions after treatment with the signal molecules MeJA and ETH gave one highly correlated group. Responses to SA were associated with an independent group that included genes induced by the avirulent *P. syringae* pv. *tomato* DC 3000 (*avrRpt2*) [PSavir]. Interestingly, this group also contained genes responsive to two different sulfonylurea herbicides, primisulfuron and prosulfuron. The response patterns established by treatment with another herbicide, bromoxynil or UV-B radiation were different from both groups (Fig. 3). Although the correlation of UVB and bromoxynil was less significant in the PCA, it could indicate the defense against oxidative damage, which is common to both stressors. The UVB experiment, however, differed from all other treatments because it was designed to analyze the long-term effects on plants grown in the presence of UV-B (Götz *et al*., in preparation).

The separation of the ETH and MeJA response from the reaction to PSavir and SA was not surprising *per se*. Several genetic analyses and gene expression studies revealed a mutually antagonistic nature of the SA-dependent and jasmonate- or ethylene-dependent defense pathways but also substantial overlap between these different signaling pathways (for review: Kunkel and Brooks, 2002). However, it is important to emphasize that the correlations demonstrated here were based on a small, non-biased set of effector gene families related to secondary metabolism lacking classical marker genes for these pathways like pathogenesis-related proteins or components of signaling cascades (Dong, 1998; Kunkel and Brooks, 2002). Thus, the enormous genetic diversity of secondary metabolism may endow the plant with the ability to recruit differential consortia of effector genes in response to various stimuli. Nevertheless, responses to different biotic and abiotic stimuli may also converge as indicated by the co-induction of genes by several inputs (see below).

A subset of genes was extracted from the PCA that most significantly contributed to the differentiation or association of stress responses (Fig. 4; Experimental procedures). Among these genes two small subgroups were co-induced by several stimuli indicating their involvement in comprehensive reactions. Both groups identify effector genes that are likely to be activated by converging, yet differentiating defense pathways.

One group comprised genes that were not induced or even repressed by ethylene or methyl jasmonate but activated by several other stimuli (Fig. 4; yellow group). This group contained several distinct members of the CYP and UGT families as well as *AtGLX-II.1*, *AtGSTF8*, and *AtGPX7*. All genes except the putative salicylate-glucosyltransferase *AtUGT74F2* (Lim *et al*., 2002) encoded enzymes of so far unknown biochemical or physiological functions. Another group combined genes that were widely induced by sulfonylurea herbicides, pathogen inoculation and the three stress hormones SA, MeJA and ETH. In contrast, no enhancement was found after BXN application and UV-B irradiation (Fig. 4, blue group). In addition to *AtCYP98A9*, *AtCYP72A8*, *AtGSTZ1*, *AtUGT86A1*, and *AtPDR8*, this group also contained *AtCYP71B15*, *AtGSTF2*, *AtGSTF6*, and *AtGSTF7*. The latter genes are

distinct members of subfamilies that were found to be associated with the yellow group. Therefore, these expression patterns do indicate different functions fulfilled by homologous members in a specific context instead of redundancy.

The induction of *AtGSTF2* and of the homologous *AtGSTF6* and *ATGSTF7* by several stress signals had been already described (Maleck *et al.*, 2000: *ERD11 = AtGSTF6*, *GST11 = AtGSTF7*; Wagner *et al.*, 2002). Parallel RT-PCR analyses revealed that both *AtGSTF6* and *AtGSTF7* were regulated in a similar way whereas only *AtGSTF2*, but not the homologous *AtGSTF3*, was responsive to multiple stimuli (Tab. 4). The biological functions of these GSTs and most other genes of this group are not known. *AtGSTZ1* encodes a maleylacetone isomerase catalyzing a key step in the catabolism of tyrosine that was induced by MeJA and pathogens (Dixon *et al*., 2000; Wagner *et al.*, 2002). The activation in additional stress situations as demonstrated here could indicate its role in protein degradation during plant stress response. *AtCYP71B15* that also fell into this group encodes a step in the biosynthesis of camalexin. Thus, elevation of this phytoalexin could be part of a general stress response. The inclusion of *AtCYP72A8* and *AtCYP98A9* as well as of an ABC transporter *AtPDR8* within this group provided a first hint to their multiple involvements in plant stress responses.

Differential response to herbicides

As indicated by the PCA the responses to two sulfonylurea compounds, primisulfuron and prosulfuron, were clearly distinct from the reaction to bromoxynil. A number of genes comprising members of all families were specifically induced by either herbicide class (Tab. 1; Suppl. Tab. 5).

Several genes were induced by primisulfuron and prosulfuron but not responsive to bromoxynil. With the exception of the specifically induced *AtGSTU24*, *AtUGT74E2*, *AtUGT75B1*, and *AtUGT86A1*(see below), all other genes of this group were also responsive to other stressors. *AtGR2* was activated by sulfonylureas in contrast to its isoform *AtGR1* that is also expressed in leaves (Tab. 1, Fig. 1). Sulfonylureas enhanced the transcription of *AtCYP71B15*, involved in phytoalexin biosynthesis, and *AtCYP76C2* (Tab. 1). Both CYPs had been previously characterized as pathogen-inducible genes (Godiard *et al.*, 1998; Zhou *et al.*, 1999). In addition to *AtCYP81D8*, *AtUGT73C5*, and *AtUGT87A2*, which were not known to be stress-related, this group also contained the stress-responsive genes *AtGSTF2*, *AtGSTF6*, *AtGSTF7* and *AtPDR8* (Tab. 1; Fig. 4). PDR-type ABC transporters might be involved in defense and detoxification, which had been indicated for three homologous members from other plant species (Jasinski *et al*., 2001; Sasabe *et al.*, 2002; Smart and Fleming, 1996; van den Brûle *et al*., 2002). Similarly, the known induction of the ABC transporter *AtMRP3* by sulfonylureas (Tommasini *et al*., 1997) was confirmed by this analysis.

Only three genes were identified that are specifically induced by bromoxynil in comparison to sulfonylureas, the ABC transporter *AtTAP1*, the highly and ubiquitously expressed glutathione peroxidase *AtGPX3* and the glucosyltransferase *AtUGT75D1*. Neither *AtGPX3* nor *AtUGT75D1* were responsive to treatment with the three stress hormones or pathogen infection (Suppl. Tab. 4). Interestingly, recombinant AtUGT75D1 was shown to glucosylate the xenobiotic compound 2,4,5-trichlorophenol (Messner *et al.*, 2003). *AtGPX3* could be specifically involved in antagonizing oxidative damage that occurred as a result of inhibition of photosystem electron transport by BXN. Distinct members of this family were up-regulated by PSavir in both SA-dependent (*AtGPX7*) and SA-independent manners (*AtGPX1*, *AtGPX4*; Tab. 2, see below). No function has yet been assigned to *AtTAP1* that shares homology with a mammalian ATP-dependent peptide translocator involved in antigen presentation (Sánchez-Fernández *et al*., 2001).

A single gene, *AtUGT74F2*, was found to be co-induced by both chemical classes of herbicides as well as by pathogen treatment and SA application (Tab. 1, 2, 4; Fig. 4). Furthermore, its wound-inducibility has been recently described by Cheong *et al*. (2002; gene AAB64024). *In vitro* studies using recombinant enzymes showed that AtUGT74F2 is able to glucosylate SA by preferentially forming its glucose ester (Lim *et al*., 2002). A highly related second enzyme, AtUGT74F1 that specifically catalyzes the formation of SA-2-O-glucoside *in vitro* was neither induced by BXN nor the sulfonylureas (Suppl. Tab. 5). Thus, a specific pathway glucosylating SA might be induced by these herbicides. BXN is also an inhibitor of cinnamate 4-hydroxylase that may trigger the accumulation of SA (Schalk *et al*., 1997; Schoch *et al*., 2002). Thereby, the induction of *AtUGT74F2* could be part of an SA detoxification mechanism.

Genes directly involved in the detoxification of these xenobiotic compounds may be included among the induced transcripts. In particular, transferases genes that are not responsive to other treatments may be candidates. These include *AtGSTU24*, *AtUGT74E2*, *AtUGT75B1* and *AtUGT86A1* (Tab. 1; Suppl. Tab. 4). The role of the induced UGT genes deserves further investigation because glucosylation is known to be involved in the detoxification of both herbicide classes (Schaller *et al*., 1992; Klein *et al.*, 1996; Kreuz and Martinoia, 1999). Recombinantly expressed AtUGT75B1 catalyzed the glucosylation of benzoic acid and hydroxy derivatives thereof (Lim *et al*., 2002).

Finally, this analysis defined two groups of genes that are suitable to distinguish the response to sulfonylurea compounds and bromoxynil. Individual effector genes were associated with reactions of the plant towards different classes of exogenous chemicals.

Differential responses to plant stress hormones and bacterial infection

Inoculation by the avirulent pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*avrRpt2*) induces SA-dependent gene expression in *A. thaliana* (Maleck *et al.*, 2000). PCA showed that this correlation was also reflected at the level of effector gene families related to plant secondary metabolism. It was clearly distinct from the reaction to ETH and MeJA (Fig. 3). The latter hormones are known to activate overlapping signaling pathways (Kunkel and Brooks, 2002; Penninckx *et al*., 1998; Xu *et al*., 1994).

The transcriptional changes of several genes were responsible for these correlations. The ABC transporter *AtMRP3*, the putative salicylic acid glucosyltransferase *AtUGT74F2*, and two genes with unknown roles, *AtCYP71B28* and *AtGPX7*, were activated by PSavir and SA in contrast to ETH and MeJA, which could even repress the expression of the two latter genes (Tab. 2). On the other hand, the expression of three genes with unknown functions, *AtUGT71C3*, *AtUGT85A5* and *AtAOH1* was altered by ETH and MeJA but non-responsive to SA or pathogen infection (Tab. 2).

However, a number of these effector genes exhibited other co-induction patterns. This provided further evidence for alternative crosstalks among defense signaling pathways (for review: Kunkel and Brooks, 2002) and identified specific effector genes as targets. First, *AtCYP71B15*, *AtGSTF2*, *AtGSTF6*, and *AtGSTF7*, which had been already identified as comprehensively induced effector genes, were coordinately enhanced (Tab. 2; Fig. 4). Second, the *Pseudomonas*-responsive *AtCYP72A8*, *AtGSTZ1* and *AtGSTF10* were co-induced by either ETH or MeJA but not by SA (Tab. 2). Schenk *et al*. (2000) had previously described similar correlations among the different signaling pathways mostly based on known stress-responsive and signaling components. In contrast, the transcriptome of effector genes also reflected such responses and crosstalks in this study.

Interestingly, this compilation revealed a large group of genes from all families that was specifically induced in response to the PSavir. These genes indicated an induction or suppression by the bacterial pathogen that was independent from the response to the three stress hormones. Thara *et al*. (1999) had previously reported two tomato transcription factors that were induced by a virulent *Pseudomonas* strain independent of ETH, SA and jasmonate. Thus, independently mediated or obligatorily synergistic signaling pathways may exist. Further experiments including signaling mutants and kinetic analyses will be required to confirm these observations. More than half of the genes within this group had no known physiological functions, such as *e.g. AtCYP76C6*, *AtGSTU19*, *AtGPX4*, *AtUGT87A2*, or *AtPDR8*. *In vitro*, AtUGT80A2 glucosylated sterols (Warnecke *et al*., 1997) and AtMRP5 was implicated in the regulation of ion fluxes (Gaedecke *et al*., 2001). However, a few genes of this group had known functions in plant defense: *AtCYP73A5* encoding cinnamate 4-hydroxylase and two CYP genes, *AtCYP79B2* and *AtCYP83B1* involved in the biosynthesis of indole glucosinolates (Bak *et al*., 2001; Mikkelsen *et al.*, 2000). Using RT-PCR, Mikkelsen *et al*. (2003) could also demonstrate an induction of *AtCYP79B2* by MeJA that was not detected by the array analysis.

Correlation of responses to sulfonylurea herbicides and Pseudomonas

PCA revealed an interesting association of transcriptional responses to sulfonylurea herbicides with *Pseudomonas* inoculation and SA treatment (Fig. 3). Several comprehensively induced genes, *AtCYP71B15*, *AtCYP72A8, AtGSTF2*, *AtGSTF6, AtGSTF7*, *AtGSTZ1*, and *AtMRP3* were contributing to this correlation (Tab. 3). A number of additional genes including members of all effector gene families specifically linked the responses to sulfonylureas and to the bacterial pathogen (Tab. 3). Interestingly, the majority of these genes had been already identified as PSavir-enhanced genes that were not induced by individual stress hormones (Tab. 2, 3).

Their co-induction suggested that they might exhibit overlapping roles in reactions to the biotic and abiotic stressors. *AtCYP73A5* encodes cinnamate 4-hydroxylase as a key enzyme in the phenylpropanoid biosynthesis that may be either co-regulated with or dissociated from the response of other genes of this pathway (Bell-Lelong *et al*., 1997; Mizutani *et al.*, 1997; Jin *et al.*, 2000). The highly and ubiquitously expressed *AtGPX1* (Fig. 1c) had been shown to be responsive to oxidative stress (Sugimoto and Sakamoto, 1997). The pathogen-responsive, putative salicylate-glucosyltransferase *AtUGT74F2* was co-induced by sulfonylurea herbicides and BXN (Tab. 1, 3). For all other genes combined in this group except for *AtMRP5* and *AtPDR8* (see above), no function or relation to plant stress response had been described so far.

A possible explanation for the correlated induction of effector genes by sulfonylurea and pathogen could be an impact on aromatic amino acid metabolism, which is evoked by the inhibition of the synthesis of branched amino acids by sulfonylurea herbicides. Consequently, plant responses involving compounds derived from aromatic amino acids, such as the reaction to pathogens, might be affected. Sulfonylurea application could therefore influence the susceptibility of plants towards pathogens and, *vice versa*, a prevailing infection could alter the sensitivity towards these herbicides.

Independent controls of transcriptional alterations

In most instances, the expression of GST, CYP, UGT, and ABC transporter genes previously reported in the literature was in agreement with the array expression analyses (see above for references and discussion). To further confirm the data obtained with MetArray, the transcript levels of selected genes were examined by semi-quantitative RT-PCR (Experimental procedures). These analyses

confirmed the co-induction of *AtCYP71B15*, *AtCYP72A8*, *AtGSTF2*, *AtGSTF6*, *AtGSTF7*, *AtUGT74F2*, and *AtMRP3* by primisulfuron application and *Pseudomonas* infection, thus linking the responses to the abiotic and biotic stimuli (Tab. 4). In addition, these genes differentiated the reaction to primisulfuron and bromoxynil in agreement with the array analyses; *AtUGT74F2* was confirmed to be induced by both herbicides (Tab. 1, 4). However, the quantitative induction values were markedly different for both types of analyses. In most cases, the array induction values were greater than RT-PCR data, which is coherent with the fact that several array induction values were calculated using estimated, low control levels as denominators. Consequently, we never discussed a biological meaning of different induction values.

The probes directed against the highly homologous pairs of phi GSTs, *AtGSTF2*/ *AtGSTF3* and *AtGSTF6*/ *AtGSTF7* were likely to cross-react with transcripts from both genes, respectively. Since the array analyses revealed their responsiveness to multiple stimuli, their induction was scrutinized by RT-PCR. The induction of *AtGSTF6*, *AtGSTF7* and *AtGSTF2* by PRI, PSavir, SA, MeJA, and ETH was confirmed by RT-PCR (Tab. 4; Suppl. Tab. 7). In contrast, *AtGSTF3* was not detected by RT-PCR, although the array induction values were similar due to cross-hybridizing *AtGSTF2* transcripts (Tab. 4).

Conclusions

A. thaliana effector genes involved in consecutive steps of plant secondary metabolism had been chosen for a combined analysis of transcriptional responses to abiotic and biotic stressors. Most of them are represented by large gene families. In order to distinguish between highly homologous members gene-specific probes derived from 3´-regions were designed. Both the organ-specific expression patterns and the differential stress-responsiveness among members of these gene families emphasize the functional importance of the enormous genetic diversity that has evolved in plants. Although there are highly homologous gene clusters at the sequence level, these differential transcriptional regulations argue against truly redundant functions. Nevertheless, this notion will need additional experimental approaches such as analyses of knock-out mutants. From an evolutionary point of view Pichersky and Gang (2000) linked the plasticity of plant responses to environmental challenges in particular to the diversification of plant secondary metabolism and the creation of an increasing number of genes with new properties and tasks by a genetic "snowball" effect. As one consequence of these non-redundant, diverse functions, the transcriptome of these effector gene families appears to monitor and differentiate plant responses to various environmental cues. This allowed distinguishing three different types of reactions towards the stimuli applied in this study.

Of particular interest was the differentiation of plant responses towards two different exogenous chemicals. This differentiation probably reflects the different modes of action and potential side effects of these compounds. Thus, it highlights the potential of transcriptome analysis based on a focused collection of effector genes for a fast and straightforward analysis of new active compounds. Furthermore, an important overlap between abiotic and biotic stress responses was indicated by the correlation of *Arabidopsis* transcriptional reaction towards sulfonylurea herbicides and a bacterial pathogen. Such a crosstalk points to potential mutual interactions of pathogen defense and response to (herbicidal) chemicals. The MetArray is a tool to detect such interactions.

Besides these general reactions of the effector gene transcriptome analyzed, this study generated plenty of information on the function of individual genes by associating them to specific or multiple plant responses. Thus, this analysis provides a framework for their further functional characterization and their regulation by different signaling pathways.

Experimental procedures

Plant growth conditions and treatments

Arabidopsis thaliana Col-0 were raised on soil with about ten plants per 5x5 cm^2 pot using 16 h light periods at 23 _ 2°C. For pathogen infections plants were grown at lower density under short day light conditions to obtain larger leaves. Leaves of control experiments and treatments were harvested in parallel to exclude any diurnal effects. All treatments were done at the rosette stage 2 - 3 h after the onset of the light-period and lasted 24 h except the UV-B irradiation experiment. Biologically independent experiments were repeated three times or four times in two separate settings [UV-B] (Suppl. Tab. 5, 6). The UV-B experiment was conducted as a long term exposure in a sun simulator. After a precultivation period of five days, plants were exposed 19 days using a 14 h light period (PAR 1030 µmol m^{-2} s^{-1}) supplemented with 10 h UV-B irradiation at 120 mW m^{-2} (biologically effective weighting the spectrum according to Caldwell, 1971 and normalization at 300 nm). Control plants were grown under glass filters, which cut off UV-B radiation (Ibdah *et al.*, 2002). For herbicide treatments three week-old *Arabidopsis* plants were sprayed with 750 µg m^{-2} primisulfuron-methyl, 1.5 mg m^{-2} prosulfuron, and 1.5 mg m^{-2} bromoxynil octanoate. The herbicide stock solutions had been diluted 1 : 1000 with 0.05% (w/v) Silwet L-77 (Lehle Seeds, TX, USA) and sonified to get an even aqueous suspension. Control plants were treated in the same way with 0.05% Silwet. There were no visible damages at the harvest. For bacterial infections half the surface of 5 leaves from 5 week old *Arabidopsis* plants were syringe-infiltrated with *P. syringae* pv. *tomato* DC3000 (*avrRpt2*) at a titre of 5.5 x 10^5 colony forming units ml^{-1} in water. Whole leaves of inoculated and water inoculated control plants were harvested after 24 h. Treatments with salicylic acid, methyl jasmonate and ethylene were done as described in Wagner *et al*. (2002).

For analysis of organ-specific expression patterns plants were grown hydroponically (Gibeaut *et al.*, 1997) with the addition of 0.5 g l^{-1} 2-[N-morpholino]ethanesulfonic acid, pH adjusted to 5.4 with phosphoric acid. Leaves and roots were harvested after 3 weeks, flowers and stems after 5 weeks, immature siliques after 6 weeks.

Nucleic acids

Total RNA was isolated according to the method described by Chang *et al*. (1993; primisulfuron, bromoxynil treatments/ organ expression/ UV-B), by Zimmerli *et al*. (2000; pathogen infection/ signal molecules), or by Reymond *et al.* (2000; prosulfuron treatment). All procedures included a LiCl precipitation step. After spectrophotometric analyses the amounts of RNA were equalized according to the ethidium bromide fluorescence encompassing the region of 25S to 18S rRNA bands after gel electrophoresis.

Gene-specific probes were designed and analyzed using ProbeDesign algorithm (Affenzeller *et al.*, in preparation). Detailed information is accessible at mips.gsf.de/proj/thal/primerDesign/index.html (Suppl. Tab. 1). Probes were PCR-amplified from genomic DNA and cloned into pGEM-Teasy vector (Promega, Madison, USA).

Array production, hybridization and data acquisition

Specific DNA probes were amplified using flanking vector DNA sequences (Suppl. Tab. 8). PCR products were concentrated using Multiscreen plates (Millipore, Bedford, MA, USA), resuspended in

water, and spotted in duplicate onto Hybond-N^+ nylon membranes (Pharmacia, Freiburg, Germany) using the MicroGrid robot (400 µm pins; BioRobotics, Cambridge, UK). After spotting the filters were crosslinked (UV-Stratalinker 240, Stratagene, La Jolla, USA), denaturated and reference hybridized with 5´-[^{33}P]-labeled T7 oligonucleotide as described by Hauser *et al*. (1998).

For complex hybridizations total RNA was labeled by reverse transcription according to Hauser *et al.* (1998) using 30 µg RNA and 2.6 MBq of [_-^{33}P]dATP (42.9 kBq pmol^{-1}) or according to Ambion Strip-EZ kit using 10 µg RNA (Ambion, Huntingdon, UK). DNA arrays were hybridized at 68^{o}C in 5x SSC [750 mM NaCl, 75 mM trisodium citrate, pH 7], 5x Denhardt´s solution, 0.5% SDS, 100 µg ml^{-1} denaturated salmon sperm DNA. After final washings at 0.2x SSC/ 0.1% SDS primary data were acquired using a FLA-3000 image reader (Fuji, Düsseldorf, Germany) at a resolution of 50 µm and ArrayVision software (Imaging Research Inc., Haverhill, UK). About 300 additional probes encompassing other gene-specific probes and EST clones (Suppl. Tab. 9) were co-hybridized for normalization based on total gene expression in each experiment (see below). All subsequent statistical analyses such as PCA were solely based on the MetArray probe set.

Data evaluation

Primary data from each hybridization were processed and normalized using the Haruspex algorithm (Thimm *et al.*, 2001). The ratio of the expression values of corresponding pairs of treatment *vs.* control were calculated and then averaged using the replicas comprising three biologically independent experiments. Ratios smaller than 0.5 and larger than 2.0 were regarded as significant changes (Tab. 1, 2, 3). Signals from an hybridization that were lower than the twofold, local background were regarded as not detectable. In order to approximate ratios of transcriptional responses for these experiments those values were replaced by the lowest signal measured on that filter. Thereby, changes could be approximated by division as above using these values. However, ratios were labelled *n.d.* (not detectable), if transcripts were not detected in both control and treatment. Any results, that showed contradicting changes in replicas, were eliminated and labeled "*exp*" (Suppl.Tab. 4, 5, 6). If transcription was detected only in one replication, a mean value was calculated using ratio 1.0 (no change) for the *not detectable* replicas unless the resulting mean values would indicate a significant change. In the latter case, values were not taken into consideration and labeled "*o.o.*" (only once; Suppl.Tab.4, 5, 6). As long as all three replicas clearly indicated induction albeit at very different levels, data were kept for further analysis and labeled "*ind*." (for statistical analysis replaced by 2.0 as a minimal induction level). Most of these higher variations included induction ratios that were based on approximated, low control values as denominators. Accordingly, the maximum induction values were generally limited to 16 (= 2^4) by assigning this value to all inductions higher than 16 and, importantly, no emphasis was placed on the level of inductions *per se*. For statistical analysis *n.d.*, *o.o.*, and *exp*. results were included as ratio 1.00 meaning no change; all values were log-transformed to the basis 2.

For analysis of organ-specific expressions, individual sets of data were normalized as above. The mean values, representing relative gene activities, were calculated from three biologically independent experiments (Fig. 1; Suppl. Tab. 3). If no transcription above the twofold local background was detected in a single experiment, zero (no detectable expression) was used for calculation of the mean value.

Statistical analysis

The analysis of MetArray expression data was designed to find a *partitioning* of genes to specific plant reactions and to explore their concomitant participation in *groups* of transcriptional responses. Principal component analysis (PCA) was used to analyze how microarray data are structured (Holter *et al.,* 2000, Raychauduri *et al.,* 2000, Landgrebe *et al.,* 2002). PCA was applied to the pre-processed data to identify subsets of genes with large variation between transcriptional responses and to extract groups of correlated responses. In order to eliminate non-informative genes, those genes were excluded from the analysis that did not show a 2-fold deviation in expression ratios after any treatment. The resulting n x p data matrix (n = 134, p = 9) was preprocessed in order to focus the analysis on the *differential* gene expression for gene g (g = 1, …, n) and transcriptional response j (j = 1, …, p). Therefore, data were standardized with respect to columns (response) and mean-centered with respect to rows (genes). This was done iteratively. Then PCA reduces the dimensionality of the multivariate data to represent the objects (genes) in a reduced space and groups of variables correlating with each other can be extracted. Such groups are represented by new variables, which are linear combinations of original variables. If there are non-zero correlations between the original data, PCA allows reducing dimensions while only slightly reducing information: a large proportion of the variance will be explained by a smaller number of new variables. In a two-dimensional reduced space used in this study the variance for each gene between responses is proportional to the distance to the origin of the plot. Since genes with great variances contribute more significantly to the differentiation of transcriptional responses the *angular distance* was used to select genes. It was arbitrarily set at 1.4 resulting in a selection of 60 out of 134 genes. For each gene the *angular distance* from the x-axis describes the relation of this gene to both new variables. Therefore, this parameter was used to rearrange and cluster the data. Subsequently, the original ratios were substituted to create a color-coded expression-profile tables of this set of most informative genes (Fig. 4).

Verification of MetArray results by RT-PCR

Total RNA samples from replica experiments were pooled and equal amounts (0.5 or 1 µg) were reverse transcribed. Subsequent semi-quantitative PCR was performed using a limited number of PCR cycles that was individually checked to remain in the exponential phase of amplification (Bovet *et al*., 2003; Weig *et al.,* 1997). The gene for ribosomal protein AtS16 was amplified in parallel and used for normalization. Quantifications were based on ethidium bromide fluorescence or [_-^{33}P]-dAMP incorporation and phosporimaging (Bovet et al., 2003; Weig *et al.,* 1997). Gene-specific primers and numbers of PCR cycles for *AtGSTF2*, *AtGSTF3*, *AtGSTF6*, *AtGSTF7*, *AtUGT74F2*, *AtMRP3*, and *AtS16* are accessible as Suppl. Tab. 8. The expression of *AtCYP71B15* and *AtCYP72A8* was examined by real-time PCR using GeneAmp 5700 sequence detection system (Applied Biosystems, Courtaboeuf, France) with SYBR Green I (Roche, Mannheim, Germany). Amplification consisted of 40 cycles (95°C/ 15 s, 60°C/ 60 s). The actin2 gene was used as an internal, constitutively expressed standard of each cDNA sample. Primers are listed in Suppl. Tab. 8. Quantification of gene expression was performed using the cycle threshold method relatively to a calibrator (defined for each target with cDNA dilutions). All analyses were run at least in duplicate. Mean values were calculated for relative expression ratios.

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Tables and figures

Table 1 Genes differentially responsive to sulfonylurea herbicides and bromoxynil in *A.thaliana* leaves. Numbers indicate expression ratios after treatments *vs*. controls (Experimental procedures). Significant inductions are displayed on white background, non-responsive situations are underlaid in dark-grey, unclear results in grey.

Gene	PRI24	PRO24	PRI36	BXN24
Responsive to sulfonylurea				
CYP71B15	16.0	ind.	16.0	0.69*
CYP81D8	5.2	7.8	9.1	1.2*
GSTF6[1]	16.0	11.2	2.4	0.74
GSTF7	5.2	16.0	2.2	0.67
UGT73C5	2.9	6.9	2.1	1.5
UGT74E2	12.5	7.4	4.1	n.d.
UGT87A2	4.0	2.9	2.8	1.5
GR2	3.4	12.6	2.1	1.0
MRP3	10.2	16.0	7.0	0.51
CYP76C2	6.3	2.9	n.c.	1.3*
GSTF2	6.6	ind.	1.7	0.59
GSTU24	3.7	9.8	n.d.	n.d.
UGT75B1	3.2	2.8	1.1	1.7
UGT86A1	3.9	2.1	n.d.	n.d.
PDR8	6.4	6.2	1.6	1.0*
Responsive to sulfonylurea and BXN				
UGT74F2	8.0	4.0	n.c.	4.3
Responsive to BXN				
GPX3	0.65	0.54	1.13	2.5
UGT75D1	0.86	1.0	0.94	3.7
TAP1	1.4*	n.d.	n.c.	8.2

[1] the highly homologous *AtGSTF6/AtGSTF7* may cross-hybridize/ independent analyses showed that both genes are responsive to sulfonylureas

* mean expression value includes replica where no expression was detected; n.d. not detected; n.c. not clear due to conflicting data in replica hybridizations; ind. unambiguous induction but strong difference in replica experiments, see Supplementary data and Experimental procedures.

Table 2 Genes responsive to ethylene, methyl jasmonate, salicylic acid and bacterial infection. Numbers indicate expression ratios after treatments *vs*. controls (Experimental procedures). Significant inductions are displayed on white background, non-responsive situations are underlaid in dark-grey, reductions in black, and unclear results in grey.

Gene	ETH	MeJA	SA	PSavir
Responsive to PSavir/ SA vs. ETH/ MeJA				
CYP71B28	0.20	0.26	5.4	3.6
GPX7	0.88	0.40	3.1	7.0
MRP3	1.6	0.69	2.4	5.4
UGT71C3/C4[1]	2.3	2.0	0.81	0.87*
UGT85A5	0.31	0.46	0.80	n.d.
AOH1	3.0	2.3	1.5	1.2*
Coordinate induction				
CYP71B15	11.4	13.6	6.7	16.0
GSTF2	4.9	4.5	2.7	15.1
GSTF6[2]	3.3	2.4	6.9	16.0
GSTF7[2]	2.3	1.6	3.3	9.7
Responsive to PSavir and ETH/ MeJA				
CYP72A8	4.9	1.7*	1.2*	4.2
GSTZ1	2.6	1.3	1.6	4.3
GSTF10	1.9	2.7	1.1	2.3
Responsive to PSavir, not to ETH, MeJA, SA				
CYP73A5	1.1	0.88	1.5	2.9
CYP76C6/C4[1]	0.58	0.85	1.6	4.3
CYP79B2	n.d.	n.d.	n.d.	16.0
CYP81D8	1.2	0.82	0.90	2.2
CYP83B1	1.5	1.9	1.5	2.4
GSTU16	1.6	0.55	1.3	3.5
GSTU19	1.8	1.1	1.3	9.2
GLX-II.3	1.1	0.92	0.73	4.6
GPX1	1.2	1.2	1.5	14.6
GPX4	1.4	0.91	1.4	3.9
UGT74F2	n.c.	1.6	1.9	4.2
UGT80A2	1.1	0.75	0.83	3.1
UGT85A1	1.6	0.82	0.78	8.1
UGT87A2	1.9	1.2	1.9	5.3
UGT88A1	1.3	0.58	1.7	0.49
UGT89B1	1.6	0.64	0.95	0.39
MRP5	n.d.	n.d.	n.d.	4.2
PDR8	1.3	1.5	1.4	8.7
TAP1	0.82*	0.75*	0.99*	2.1

[1] probe may cross-hybridize with homologous member, which showed similar reaction.
[2] the highly homologous *AtGSTF6/ AtGSTF7* may cross-hybridize.
* mean expression value includes replica where no expression was detected; n.d. not detected; n.c. not clear due to conflicting data in replica hybridizations.

Table 3 Correlated responses to sulfonylurea herbicides and bacterial infection. Numbers indicate expression ratios after treatments *vs*. controls (Experimental procedures). Significant inductions are displayed on white background, non-responsive situations are underlaid in dark-grey, and unclear results in grey.

Gene	PRI24	PRO24	PSavir	SA
Comprehensively induced genes				
CYP71B15	16.0	ind.	16.0	6.7
CYP72A8	5.9	2.0	4.2	1.2*
GSTF2	6.6	ind.	15.1	2.7
GSTF6[1]	16.0	11.2	16.0	6.9
GSTF7[1]	5.2	16.0	9.7	3.3
GSTZ1	9.9	n.d.	4.3	1.6
MRP3	10.2	16.0	5.4	2.4
Co-induction by sulfonylurea and PSavir				
CYP71B19[2]	8.2	0.79	ind.	1.8
CYP73A5	1.0	3.2	2.9	1.5
CYP81D8	5.2	7.8	2.2	0.90
GSTU4/U3[3]	4.3	n.d.	2.8	0.90
GSTU16	2.1	0.73*	3.5	1.3
GSTU19	2.0	10.9	9.2	1.3
GLX-II.3/.5[3]	2.9	n.d.	4.6	0.73
GPX1	16.0	n.c.	14.6	1.5
GPX4	2.0	3.1	3.9	1.4
GPX7	3.5	n.d.	7.0	3.1
UGT74F2	8.0	4.0	4.2	1.9
UGT76B1	6.4	n.d.	7.6	1.5
UGT87A2	4.0	2.9	5.3	1.9
MRP5	3.8	n.d.	4.2	n.d.
PDR8	6.4	6.2	8.7	1.4

[1] the highly homologous *AtGSTF6/ AtGSTF7* may cross-hybridize, however, see independent controls.

[2] probe may crossreact with closely related *AtCYP71B16*, *AtCYP71B17*, and *AtCYP71B20.*

[3] probe may also detect homologous member, which showed similar reaction.

* mean expression value includes replica where no expression was detected; n.d. not detected; n.c. not clear due to conflicting data in replica hybridizations; ind. unambiguous induction but strong difference in replica experiments, see Supplementary data, Experimental procedures.

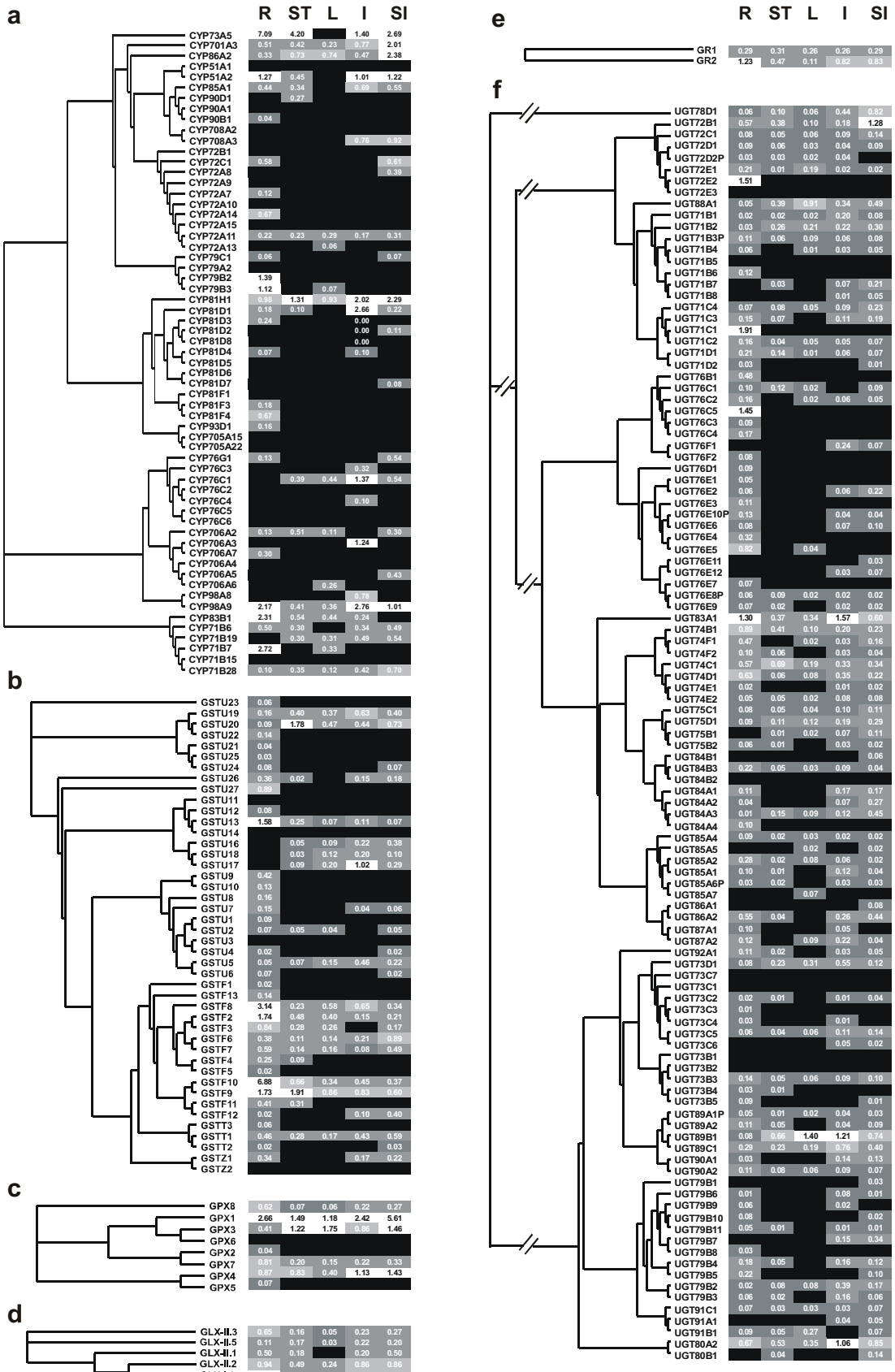
Figure legends

Fig. 1. Organ-specific transcription patterns. Expression in root (R), stem (ST), leaf (L), inflorescence (I) and immature (green) silique (SI) tissue was examined for CYP (a) GST (b), GPX (c), GLX (d), GR (e), UGT (f) and ABC transporter (g) gene families in *A. thaliana*. Normalized gene expression values (Experimental procedures) were underlayed by grey scales to visualize differences, black and white representing no detectable and highest expression levels, respectively. Due to size limitations the phylogenetic distances in the trees were shortened where indicated by double slashes; see Supplementary data for the correct distances based on ClustalW analyses.

Fig. 2. Herbicidal chemicals used for treatments. Herbicides were sprayed in sublethal doses onto three-week-old *Arabidopsis* plants: primisulfuron (a), prosulfuron (b), bromoxynil octanoate (c).

Fig. 3. Principal component analysis. The statistical analysis was based on a subset of 134 genes, which responded at least in one treatment with a 2-fold change in expression; the two components reflect 41.3 % of the total variability of the data set (Experimental procedures).

Fig. 4. Expression patterns in response to herbicides, endogenous signal molecules, UV-B irradiation and pathogen infection. The compilation is based on a principal component analysis. 60 out of 134 genes showing the highest variance in distingushing transcriptional responses are displayed (Experimental procedures). Color coding of the original, log2-transformed expression ratios ranges from intense red (larger than 2) to intense green (lower than -2), black meaning no change (ratio 0). Numerical data and individual expression values are available as Supplementary data.


a
R ST L I SI
CYP73A5 7.09 4.20 1.40 2.69
CYP701A3 0.51 0.42 0.23 0.77 2.01
CYP86A2 0.33 0.73 0.74 0.47 2.38
CYP51A1
CYP51A2 1.27 0.45 1.01 1.22
CYP85A1 0.44 0.34 0.69 0.55
CYP90D1 0.27
CYP90A1
CYP90B1 0.04
CYP708A2
CYP708A3 0.76 0.92
CYP72B1
CYP72C1 0.58 0.61
CYP72A8 0.39
CYP72A9
CYP72A7 0.12
CYP72A10
CYP72A14 0.67
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CYP79B2 1.39
CYP79B3 1.12 0.07
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CYP81D1 0.18 0.10 2.66 0.22
CYP81D3 0.24 0.00
CYP81D2 0.00 0.11
CYP81D8 0.00
CYP81D4 0.07 0.10
CYP81D5
CYP81D6
CYP81D7 0.08
CYP81F1
CYP81F3 0.18
CYP81F4 0.67
CYP93D1 0.16
CYP705A15
CYP705A22
CYP76G1 0.13 0.54
CYP76C3 0.32
CYP76C1 0.39 0.44 1.37 0.54
CYP76C2
CYP76C4 0.10
CYP76C5
CYP76C6
CYP706A2 0.13 0.51 0.11 0.30
CYP706A3 1.24
CYP706A7 0.30
CYP706A4
CYP706A5 0.43
CYP706A6 0.26
CYP98A8 0.78
CYP98A9 2.17 0.41 0.36 2.76 1.01
CYP83B1 2.31 0.54 0.44 0.24
CYP71B6 0.50 0.30 0.34 0.49
CYP71B19 0.30 0.31 0.49 0.54
CYP71B7 2.72 0.33
CYP71B15
CYP71B28 0.10 0.35 0.12 0.42 0.70
b
GSTU23 0.06
GSTU19 0.16 0.40 0.37 0.63 0.40
GSTU20 0.09 1.78 0.47 0.44 0.73
GSTU22 0.14
GSTU21 0.04
GSTU25 0.03
GSTU24 0.08 0.07
GSTU26 0.36 0.02 0.15 0.18
GSTU27 0.89
GSTU11
GSTU12 0.08
GSTU13 1.58 0.25 0.07 0.11 0.07
GSTU14
GSTU16 0.05 0.09 0.22 0.38
GSTU18 0.03 0.12 0.20 0.10
GSTU17 0.09 0.20 1.02 0.29
GSTU9 0.42
GSTU10 0.13
GSTU8 0.16
GSTU7 0.15 0.04 0.06
GSTU1 0.09
GSTU2 0.07 0.05 0.04 0.05
GSTU3
GSTU4 0.02 0.02
GSTU5 0.05 0.07 0.15 0.46 0.22
GSTU6 0.07 0.02
GSTF1 0.02
GSTF13 0.14
GSTF8 3.14 0.23 0.58 0.65 0.34
GSTF2 1.74 0.48 0.40 0.15 0.21
GSTF3 0.21 0.28 0.26 0.17
GSTF6 0.38 0.11 0.14 0.21 0.89
GSTF7 0.59 0.14 0.16 0.08 0.49
GSTF4 0.25 0.09
GSTF5 0.02
GSTF10 6.88 0.66 0.34 0.45 0.37
GSTF9 1.73 1.91 0.86 0.83 0.60
GSTF11 0.41 0.31
GSTF12 0.02 0.10 0.40
GSTT3 0.06
GSTT1 0.46 0.28 0.17 0.43 0.59
GSTT2 0.02 0.03
GSTZ1 0.34 0.17 0.22
GSTZ2
c
GPX8 0.62 0.07 0.06 0.22 0.27
GPX1 2.66 1.49 1.18 2.42 5.61
GPX3 0.41 1.22 1.75 0.86 1.46
GPX6
GPX2 0.04
GPX7 0.81 0.20 0.15 0.22 0.33
GPX4 0.87 0.83 0.40 1.13 1.43
GPX5 0.07
d
GLX-II.3 0.65 0.16 0.05 0.23 0.27
GLX-II.5 0.11 0.17 0.03 0.22 0.20
GLX-II.1 0.50 0.18 0.20 0.50
GLX-II.2 0.94 0.49 0.24 0.86 0.86
GLX-I.1 1.06 0.50 0.29 0.67 0.95
GLX-II.4 1.83 0.65 0.12 0.54 0.90
e
R ST L I SI
GR1 0.29 0.31 0.26 0.26 0.29
GR2 1.23 0.47 0.11 0.82 0.83
f
UGT78D1 0.06 0.10 0.06 0.44 0.82
UGT72B1 0.57 0.38 0.10 0.18 1.28
UGT72C1 0.08 0.05 0.06 0.09 0.14
UGT72D1 0.09 0.06 0.03 0.04 0.09
UGT72D2P 0.03 0.03 0.02 0.04
UGT72E1 0.21 0.01 0.19 0.02 0.02
UGT72E2 1.51
UGT72E3
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Fig. 1a-f

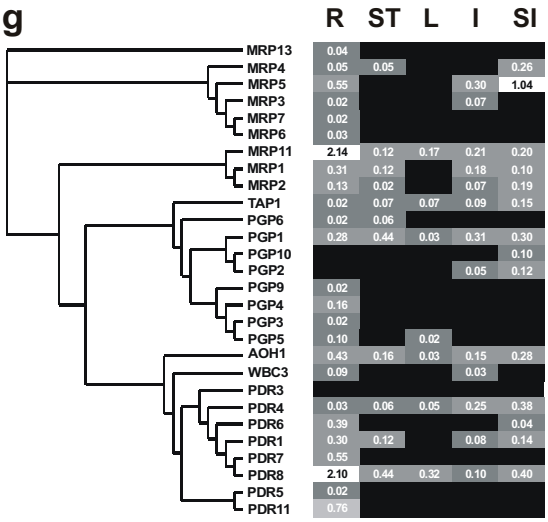
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Fig. 1g

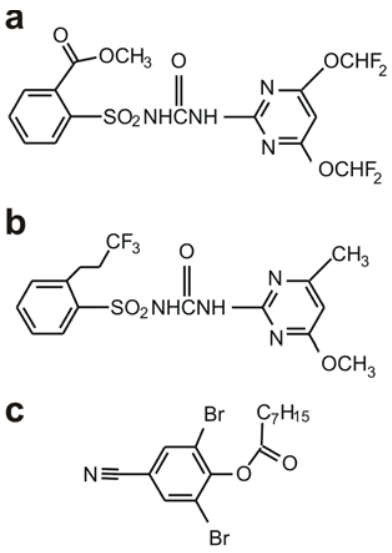
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Fig. 2

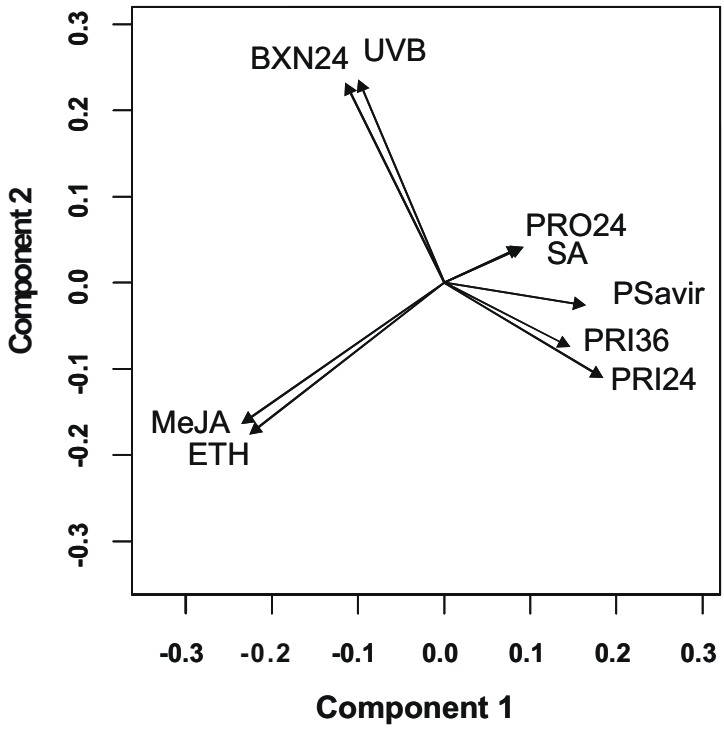
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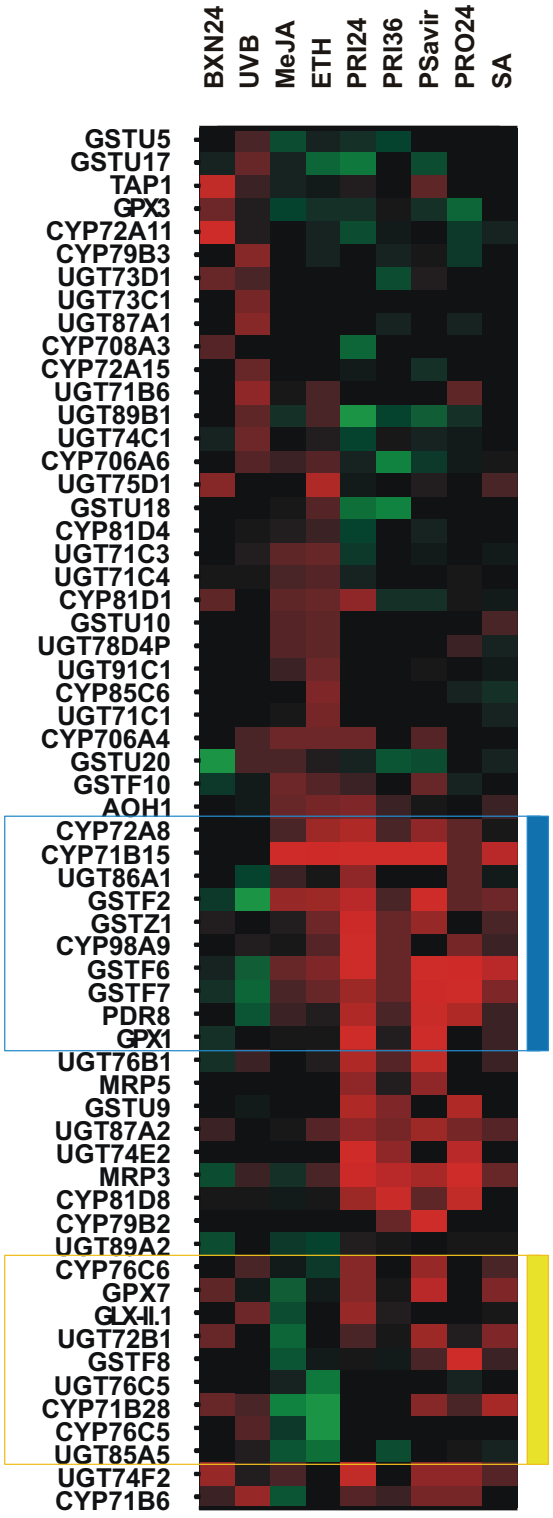
Fig.3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CYP76C5
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UGT74F2
CYP71B6

Fig. 4

Chapter 4

Subcellular localisation and functional analysis of the phi class glutathione *S*-transferase *At*GSTF8

Subcellular localisation and functional analysis of the phi class glutathione *S*-transferase *At*GSTF8

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Abstract

Plant glutathione S-transferases are multifunctional enzymes encoded by a large gene family containing 47 members in *Arabidopsis thaliana*. A member of the phi class GST, *At*GSTF8 (At2g47730), is upregulated by various treatments including oxidative stress and exhibits GSH-peroxidase activity. The chloroplastic localisation of GSTF8 was demonstrated by expressing a fusion protein consisting of the predicted GSTF8 signal pepetide and GFP in transgenic *Arabidopsis*. Analysis of the GST family indicated that GSTF8 is the only chlorplastic GST in *Arabidopsis*, making it a promissing candidate for functional analysis. To this end, GSTF8 over-expressing transgenic lines were produced and a T-DNA insertion knock out mutant was isolated from the SALK-collection. Phenotypic analysis of the transgenic plants revealed no observable change under normal growth condition and under conditions of oxidative stress like treatments with hydrogen peroxide and the herbicide paraquat. GSTF8 appears to be either not involved in protection from oxidative stress in chloroplasts or, alternatively, that in addition to GSTF8 other mechanisms contribute to this protection.

Keywords: Glutathione S-transferase, GSTF8, oxidative stress, chloroplast, Arabidposis thaliana.

Introduction

Glutathione S-transferases (GSTs; EC 2.5.1.18) constitute a family of soluble proteins with typical molecular masses of around 25 kDa. GSTs catalyse the transfer of the tripeptide glutathione (γ-glutamyl-cysteinyl-glycine, GSH) to a substrate (R-X) containing a reactive electrophilic centre to form a S-glutathionylated reaction product (R-SG) (Dixon et al., 2002). GSTs play critical roles in the detoxification of xenobiotics and the protection of tissues against oxidative damage. GSTs are important enzymes in plant responses to a number of environmental stresses including herbicides and pathogen attack. The 47 Arabidopsis GSTs are divided on the basis of sequence similarity into phi, tau, theta and zeta classes (Edwards et al., 2000; McGonigle et al., 2000; Dixon et al., 2002; Wagner et al., 2002). The most plant GSTs are members of the two plant specific phi and tau classes. In addition to the four classes of GSTs, *A. thaliana* contains two GST-like protein groups which contain the GSH binding domain, but have a cysteine in place of a serine at the active site (Dixon et al., 2002). These two groups are the GSH dependent dehydroascorbate reductase (DHARs) with 4 genes and the Lambda GSTs (GSTL) with 2 genes. However, DHARs and GSTLs do not possess glutathione transferase activity (Dixon et al., 2002).

The idea that GSTs have additional functions not directly linked to their ability to catalyze the formation of GSH conjugates has gained attention with studies demonstrating that several stress-inducible GSTs protect plants from oxidative damage by functioning as glutathione peroxidases (Roxas et al., 1997; Cummins et al., 1999). Certain theta, phi and tau GSTs have been shown to have glutathione peroxidase activity, with the GSTs using glutathione to catalyse the reduction of organic hydroxyperoxides of fatty acids and nucleic acids to the corresponding monohydroxyalcohols (Bartling et al., 1993; Cummins et al., 1999; Edwards et al., 2000). This reduction plays a central role in preventing the degradation of organic hydroxyperoxides to cytotoxic aldehyde derivatives. Interestingly, a further link between GSTs and oxidative-stress tolerance has been established by the finding that when expressed in yeast, a tau GST from tomato can suppress apoptosis induced by the Bax protein (Kampranis et al., 2000). Another catalytic role that does not involved GSH conjugation has been demonstrated for the *Arabidopsis* zeta GSTs which catalyse the GSH-dependent isomerisation of maleylacetoacetate to fumarylacetoacteate, the second to last step in tyrosine catabolism (Dixon et al., 2000). GSTs may also function in stress tolerance through a role in cell signaling. Induction of genes encoding enzymes of flavonoid biosynthesis in parsley by ultraviolet light requires GSH and the expression of a specific tau GST (Loyall et al., 2000). Biochemical and immunological investigations point to a largely cytosolic localisation for soluble GSTs in plants (Edwards et al., 2000). Genomic analysis of the Arabidopsis GSTs reveals that only one phi GST (GSTF8) appears to contain a clear putative plastid targeting sequence. However, experimental data confirming this prediction is missing.

GSTF8 (previously GST6, At2g47730) was first identified as a 215 amino acid protein (Chen et al., 1996). Further studies showed that a 48 aa putative signal peptide was missing and that the protein was indeed 263 aa long (Wagner et al., 2002). GSTF8 expression is under tissue specific control and is induced by treatments with auxin, salicylic acid, ethylene, methyl-jasmonate, pathogens and H_2O_2 (Chen et al., 1996; Chen and Singh, 1999; Wagner et al., 2002; Glombitza et al., 2004). The GSTF8 promoter contains a stress induced 20 bp *ocs* element (Chen et al., 1996; Chen and Singh, 1999). The induction of GSTF8 expression by SA and H_2O_2 may suggest a role for GSTF8 in protection against oxidative stress and possibly in plant-pathogen interactions. Substrate specificity profiling showed that GSTF8 has a strong GSH-peroxidase activity and only poor GSH transferase activity (Wagner et al., 2002).

In this study a fusion protein between the signal peptide of GSTF8 and GFP was used to demonstrate that GSTF8 is targeted to the chloroplast. The expression of this gene is induced by oxidative stress and the encoded protein has GSH-peroxidase activity. We therefore hypothesized that GSTF8 functions in the detoxification of products generated during oxidative stress in the chloroplast. Because GSTF8 was the only GST present in the chloroplast, it appeared to be a promising candidate for functional analysis without running into the problems of redundancy that hinders functional analysis of the large GST family. To this end transgenic *Arabidopsis* overexpressing GSTF8 and a T-DNA insertion knock out mutant in GSTF8 were isolated and tested for phenotypic changes in response to oxidative stress.

Materials and Methods.

Plant and bacterial growth condition

Arabidopsis thaliana accession Columbia (Col-0) plants were grown in commercial potting soil under a 16h light cycle (18°C night and 22°C day temperature) and 60-70% relative humidity. *Pseudomonas syringae* pv *tomato* DC3000 was grown in Luria Broth at 28°C with 25 µg/ml rifampicin. For bacterial infection half the surface of leaves was syringe-infiltrated with at a titre of 7 x 10^5 colony forming units per ml in water.

Transformation vectors and transgenic lines

The DNA sequence encoding the first 59 amino acids corresponding to the putative 48 aa signal peptide and the beginning of the predicted mature GSTF8 was amplified by PCR using the following primers containing an additional 5'-*NcoI* site: F-GSTF8S, 5'- gaaccatgggagcaattcaagctcg-3' and R-GSTF8S, 5'-gatccatggtgggaactccgtgaacc-3'. The resulting PCR product was cloned into pGEM®-T Easy (Promega, Madison, USA). The construct was sequenced for verification. The signal sequence was then excised as a *NcoI* fragment and inserted in the *NcoI* site of pMON30060 (Pang et al., 1996). The cassette including signal peptide and GFP was amplified by PCR using Expand High Fidelity PCR system (Roche, Basel, Switzerland) with primers containing the recombination sites *att*B1/B2 of the Gateway cloning system (Invitrogen, Carlsbad, USA) F-sGFP-attB1, 5'-ggggacaagtttgtacaaaaaagcaggctttccatgggagcaattcaagctcg-3' and R-sGFP-attB2, 5'-ggggaccactttgtacaagaaagctgggttcagatcttcacttgtagagttcat-3'. The purified PCR product was cloned by recombination into the binary vector pBENDER (http://www.mpiz-koeln.mpg.de/~weisshaa/BW-research/Vectors.html) between the CaM35S promoter and the Nos terminator (35S::signalGSTF8-GFP::nosT). For over-expression, the GSTF8 cDNA (AF288176) was amplified by PCR using Expand High Fidelity PCR system (Roche, Basel, Switzerland) with primers containing the recombination sites *att*B1/B2 of the Gateway cloning system (Invitrogen, Carlsbad, USA): F-GSTF8-OE-attB1, 5'-ggggacaagtttgtacaaaaaagcaggctttatgggagcaattcaagctcg-3' and R-GSTF8-OE-attB2, 5'-ggggaccactttgtacaagaaagctgggtgtcactactgcttctggaggtc-3'. The purified PCR product was cloned by recombination in the binary vector pBENDER creating a 35S::GSTF8::nosT cassette. PCR was performed on a Biometra TRIO-Thermoblock™ thermocycler (Biolabo, Châtel-St-Denis, Switzerland) using following conditions: 94°C for 4 min followed by 32 cycles of 94°C for 15s, 55°C [60°C for attB site containing primers] for 30s and 72°C for 1 min. Binary vectors were electroporated into the *Agrobacterium tumefaciens* strain GM3101 (pMP90RK) (Koncz and Schell, 1986).

Transformation of Arabidopsis was performed by the vacuum infiltration method (Bechtold et al., 1993). Transformants were selected on half strength Murashige and Skoog (MS) medium containing 50 µg/ml kanamycin. For the localisation of GSTF8, kanamycin-resistant plants were transferred on soil and leaves were observed under the microscope after 3 weeks. For GSTF8 over expression, kanamycin-resistant plants were transferred to soil for further cultivation. Screening of seeds for kanamycin-resistant progeny was carried out in the same medium as above. A homozygous line was used for the phenotypic tests (line 5).

A GSTF8 knock out mutant was identified in the SALK collection (SALK_039887) with the T-DNA inserted in the third exon. The seeds were obtained from the Arabidopsis Biological Resource Center (ABRC; www.arabidopsis.org/abrc/). T-DNA insertion location in GSTF8 was confirmed by PCR

according to the instructions of the SALK web site (http://signal.salk.edu/tdna_protocols.html) using gene specific primers for GSTF8 and a primer in the left border of the T-DNA. Homozygous GSTF8 knock out plants were identified by PCR.

Localisation of GFP expression

For subcellular localisation, signalGSTF8-GFP transgenic plants were examined using a Leica DMR fluorescence microscope with following filter sets: 480/40nm illumination path, 527/30nm observation path for GFP fluorescence and 450-490nm illumination path, 515nm observation path for chloroplast autofluorescence and GFP fluorescence together. Pictures were acquired using a Zeiss Axiocam CCD camera and Axiovision 2.05 software. Confocal pictures were taken with a Leica DMR using Leica TCS 4D operating system with Scanware 5.0 software.

Expression studies and phenotypic tests

For expression studies three week old plants were sprayed with 5mM H_2O_2 or 100µM paraquat in water with 0.2% Tween 20. For high light stress plants were acclimated at 25°C and 1600 lux in a SANYO growth cabinet (SANYO Electrics, Gunma, Japan) and after 4 days the conditions were switched to 10°C and 12000 lux to induce photooxidative stress. Total RNA isolation and RNA blot analysis were done as described in (Zimmerli et al., 2000). A gene specific probe was used 211 nucleotides containing 65 nuclotides of the end of the coding region and 146 nucleotides of the 3'-untranslated region. This specific probe was used for the expression analysis of GSTF8 in order to avoid crosshybridation between GSTF8 and its closest homologues (Glombitza et al., 2004). For tests of phenotype, *Arabidopsis* seeds from Col-0, GSTF8 over expression (GSTF8-OE) and knock out lines (GSTF8-KO) were grown on half strength Murashige and Skoog (MS) solid medium for 8 days and then transferred to liquid 0.5 MS medium containing the different chemicals to be tested. Hydrogen peroxide solution was obtained from a 30% stock solution (Sigma Chemicals, St Louis, USA) and paraquat was provided by Novartis (Basel, Switzerland).

Sequence analysis

In silico analysis were done using TargetP V1.0 (Emanuelsson et al., 2000) and PREDOTAR (www.inra.fr/predotar) for protein localisation and with Clustal_X (1.81) (Thompson et al., 1997) and Blockshade 3.21 for sequence alignment (www.molbiol.net). For sequence alignment the following genes were used GSTF2 (At4g02520), GSTF4 (At1g02950), GSTF5 (At1g02940) and GSTF8 (At2g47730).

Results

Alignment of the 47 members of the GST gene family shows that 3 GSTs, the GSTF4, GSTF5 and GSTF8 contain extended N-terminal sequences possibly corresponding to signal peptides. Figure 1 shows an alignment of these three GSTs together with GSTF2 as a representative of the cytosolic GSTs to indicate the position of the translational start site of most GSTs. *In silico* analysis of the putative signal sequences indicated that only GSTF8 contains a putative signal peptide for chloroplastic or mitochondrial localisation.

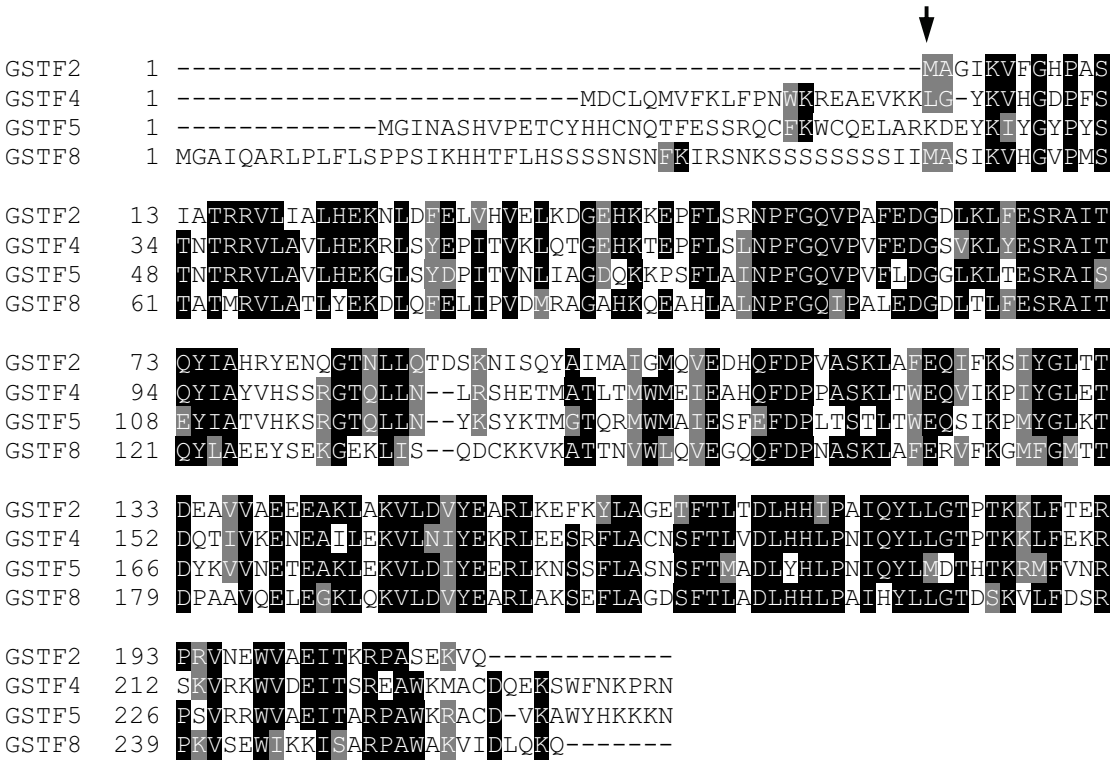

Figure 1. Alignment of GSTF2 (At4g02520), GSTF4 (At1g02950), GSTF5 (At1g02940) and GSTF8 (At2g47730). The black arrow indicates the translational start of most GSTs. The sequences of GSTF4, GSTF5 and GSTF8 exhibit a longer N-terminal sequence. Alignment was done with ClustalX (Thompson et al., 1997) and Blockshade 3.21 programs (www.molbiol.net). Residues identical and conserved between sequences are marked with black and grey, respectively.

To experimentally confirm the chloroplastic localisation of GSTF8, a fragment of 177bp corresponding to 59 N-terminal amino acids of the GSTF8 (At2g47730) was PCR amplified and used to produce a fusion protein construct with green fluorescence protein as visible marker (see Figure 2 and Material and Methods). Arabidopsis Col-0 transformed with pBender-GSTF8-signal-GFP were selected on Kanamycin and observed after 3 weeks. Figure 3 shows confocal microscopy pictures of transgenic plants. Comparison of GFP fluorescence (Figure 3A) and chloroplast autofluorescence (Figure 3B) demonstrated that GFP fluorescence is restricted to the chloroplasts.

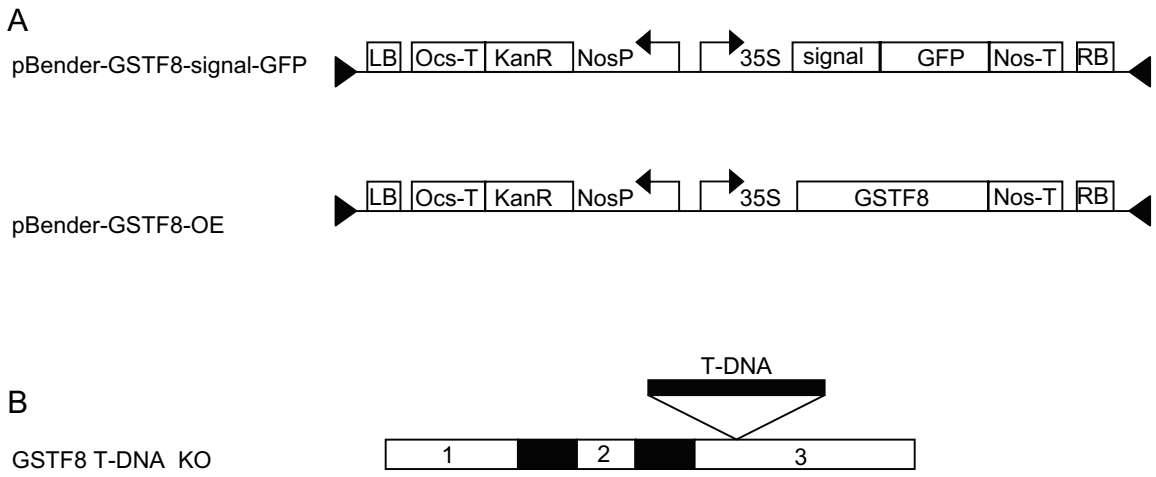

Figure 2. Map of transformation constructs (A) and T-DNA insertion in the GSTF8 KO line (B). pBENDER-GSTF8-signal-GFP shows the signal-GSTF8::GFP fusion protein construct used for the localisation of GSTF8. pBENDER-GSTF8-OE shows the construct used for over-expressing GSTF8 gene in Arabidopsis Col-0. GSTF8 T-DNA KO shows the approximate localisation of the T-DNA insertion in exon 3 of the GSTF8 gene in SALK_039887 line.

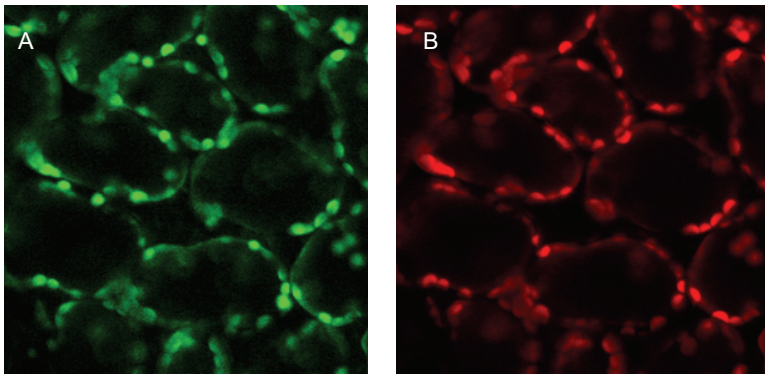

Figure 3. Subcellular localisation of GSTF8. A) GFP fluorescence in leaves of Arabidopsis expressing the fusion protein signalGSTF8::GFP and B) the corresponding autofluorescence of the chloroplasts. Pictures were taken with a Leica DMR confocal microscope using a Leica TCS 4D operating system and Scanware 5.0 software.

Figure 4A shows that the expression of GSTF8 was up regulated after treatment with 5mM H_2O_2 and 100µM paraquat, a herbicide interfering with photosystem I (Ye and Gressel, 2000). Hydrogen peroxide induced a transient increase of GSTF8 already 2 hours after treatment. After treatment with paraquat the expression of GSTF8 increased after 2 hours and was sustained over a longer period. These results confirm that GSTF8 is up regulated under conditions of oxidative stress. However, GSTF8 was not upregulated after high light treatment that is predicted to cause photooxidative stress in the chloroplast.

Figure 4B shows a RNA blot of GSTF8 expression in transgenic lines. Over-expressing GSTF8-OE plants showed diverse patterns. Some lines (lines 4, 5 and 7) show very strong expression and other only weak expression (lines 1, 2, 3 and 6). Lines such as line number 1 showing weaker expression than the control are indications for silencing of GSTF8 expression. For further studies we have used the homozygous line 5 which shows strong expression. For knock-out plants homozygous lines were isolated by PCR according to the instruction given on the SALK site (http://signal.salk.edu/tdna_protocols.html). All homozygous GSTF8-KO plants displayed in Figure 4B (lines 8, 9 and 10) show no expression of GSTF8 24 hours after infection with *Pseudomonas syringae*. Inoculation with *P. syringae* was previousely shown to induce a strong increase in GSTF8 expression (Lieberherr et al., 2003; Glombitza et al., 2004).

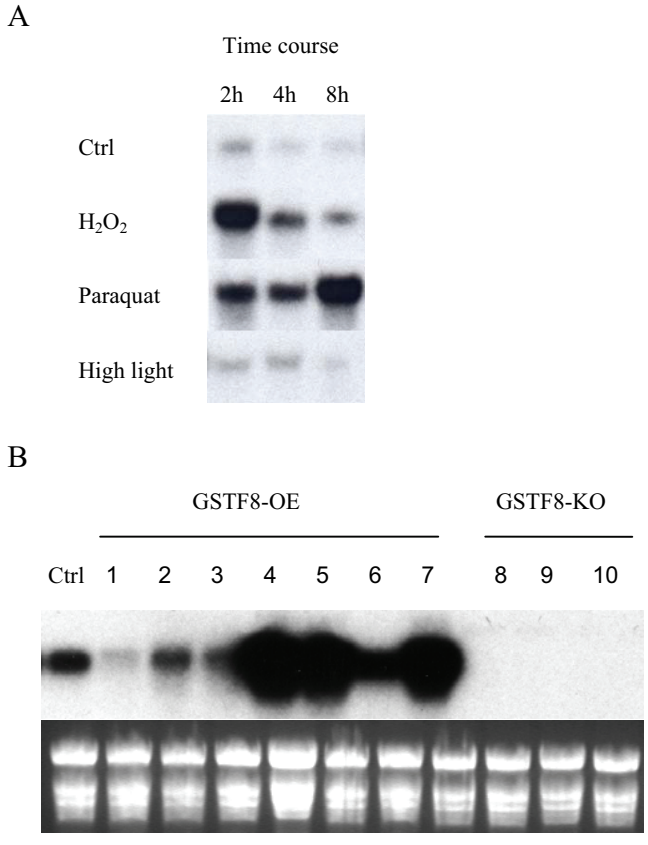

Figure 4. RNA blot analysis showing the expression of GSTF8 in stressed and in transgenic plants. A) Induction of GSTF8 after oxidative stress. Time course of expression of *At*GSTF8 after treatment with 5mM H_2O_2, 100 µM paraquat and photooxidative stress (high light) by switching light from 1600 lux 25°C to 12000 lux 10°C. B) Expression of GSTF8 in overexpressing and T-DNA knock out lines (GSTF8-KO, SALK_039887). Ctrl shows the level of

expression of Col-0 wild type plants. A gene specific probe was used in order to distinguish between GSTF8 expression and its closest homologues (Glombitza et al., 2004).

Compared to wild type Col-0 GSTF8-OE and GSTF8-KO plants grown in normal condition displayed no visible phenotype neither in growth, flowering time and root length. Considering that GSTF8 is the only chloroplastic GST with GSH-transferase activity in *Arabidopsis*, that its expression is upregulated after oxidative stress and that it has a GSH-peroxidase activity, we hypothesized that GSTF8 could play a role in the detoxification of reactive oxygen species produced in the chloroplast. To test this hypothesis phenotype tests were carried out by inducing oxidative stress using hydrogen peroxide and paraquat. Figure 5 shows 8 day old wild type, GSTF8-OE (line 5) and GSTF8-KO (line 8) plants treated with different concentration of hydrogen peroxide or paraquat. Hydrogen peroxide at a concentration of 4 mM induced the necrosis of cotyledons and leaves. Paraquat at 50µM induced wilting of the leaves and small necrosis. No differences could be seen between the wild type and the two transgenic lines.

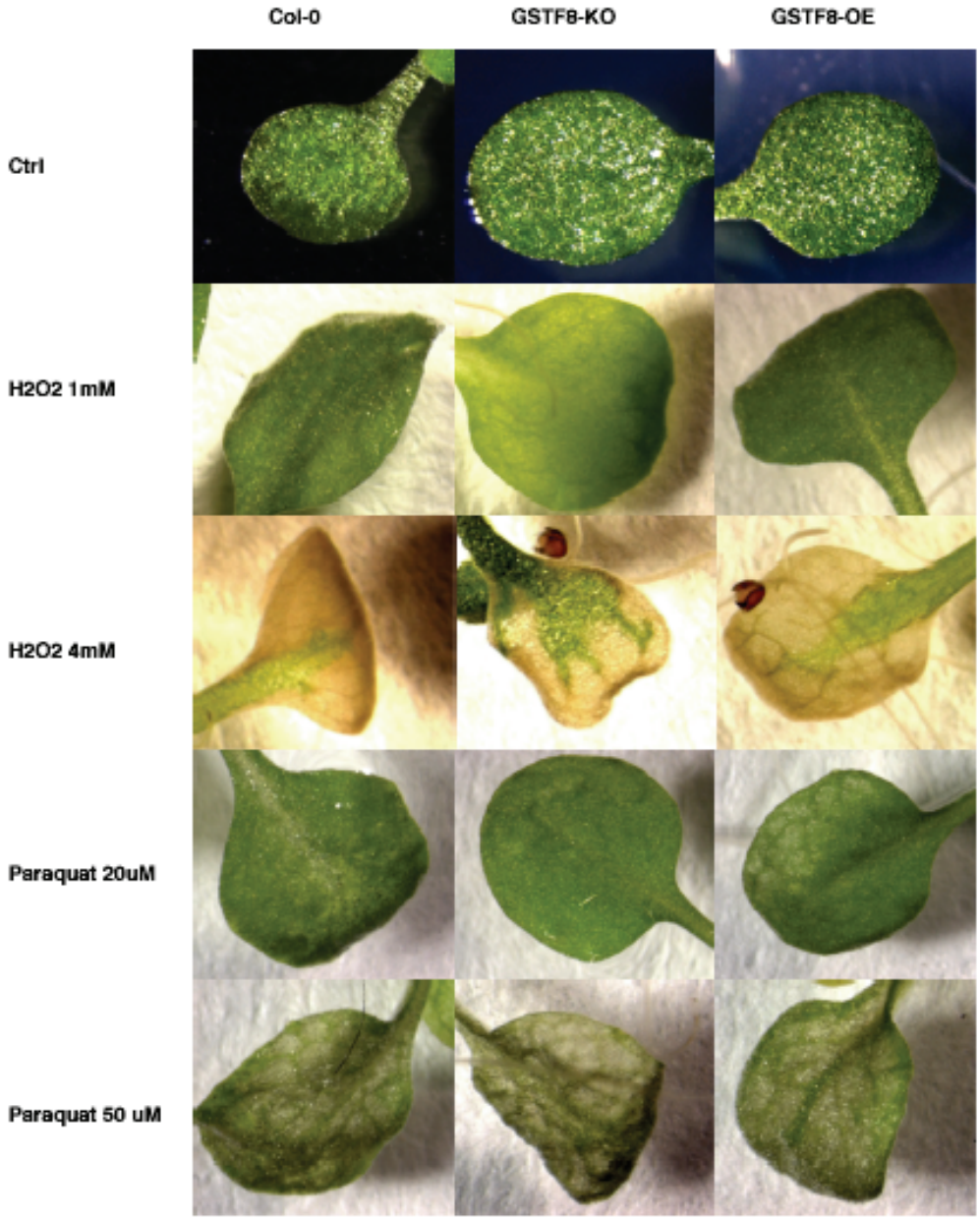

Figure 5: Phenotype tests of GSTF8 over expression and knock out plants. Eight day old Col-0 wild type, GSTF8-OE and GSTF8-KO plants grown on half strenght MS medium were treated with different concentration of H2O2 and paraquat. Pictures were taken 24 hours after treatment.

Discussion

Aerobic organisms and particularly plants have to deal with the production of reactive oxygen species not only in stress conditions but also under normal growth. Free radicals are natural by-products of biological redox reactions particularly in mitochondria and chloroplasts. Plants have evolved different mechanisms to neutralise free radicals before they can damage lipids, proteins or nucleic acids. To achieve this goal they use different antioxidant molecules such as ascorbate, glutathione (GSH), α-tocopherol, carotenoids, polyamines and flavonoids (Foyer et al., 1994; Noctor and Foyer, 1998; Ye and Gressel, 2000). They also use different enzymatic systems to scavenge directly free radials or to recycle antioxidant molecules. Ascorbate peroxidase detoxifies mainly hydrogen peroxides. Superoxide dismutase catalyses the transformation of O_2^- to H_2O_2. Glutathione reductase reduces oxidised glutathione to GSH and glutathione peroxidase detoxifies organic peroxides like lipid peroxides (Eshdat et al., 1997). Regeneration of oxidised ascorbate can be achieved by dehydroascorbat reductase, monodehydroascorbate reductase and ferredoxin. All these antioxidant molecules and enzymes are present in chloroplasts of higher plants

GSTF8 was first isolated as a 215 aa long protein that starts at Met^{49} (Chen et al., 1996). But a longer cDNA (AF288176, (Wagner et al., 2002) suggested a 48 aa longer N-terminal sequence resulting in a 263 aa protein. This N-terminal extension was predicted to be a putative signal peptide targeting the protein to the chloroplast. We demonstrated with the GSTF8 signal peptide fused to GFP that this signal peptide is indeed a chloroplast targeting sequence.

The strong up regulation of GSTF8 two hours after treatment with H_2O_2 is consistent with previous results (Chen et al., 1996; Chen and Singh, 1999; Desikan et al., 2001; Wagner et al., 2002). In contrast to previous study in our work the expression of GSTF8 was monitored using a gene specific probe. Paraquat treatment induces oxidative stress by interacting with photosystem I leading to the formation of reactive oxygen species. These ROS induced the increase of the expression of GSTF8 already after 2 hours and the expression increased further up to 8 hours post treatment. GSTF8 was shown to be induced by other stresses like SA, auxin, ethylene, MeJA, and pathogens (Chen et al., 1996; Wagner et al., 2002). In our study treatment with high light did not induce the expression of GSTF8. (Rossel et al., 2002)Rossel et al. (2002) reported the induction of the expression of GSTF8 already 10 minutes after high light treatment. They observed the highest expression one hour after treatment and two hours after treatment the expression decreased to a low level comparable to the level observed in our study.

Substrate specificity profiling had shown that GSTF8 has a GSH-peroxidase activity and only limited glutathione S-transferase activity (Wagner et al., 2002). Taken together literature data and our results showed that GSTF8 is the only GST present in the chloroplast, that it possesses a strong GSH-peroxidase activity and that it is up regulated by oxidative stresses including pathogen attack. Our hypothesis was that GSTF8 plays a role in the chloroplast by detoxifying reactive oxygen species mainly lipid peroxide by its GSH-peroxidase activity.

However, GSTF8 knock out and GSTF8 over expressing plants showed no phenotype under normal growth condition. When stressed either with hydrogen peroxide or paraquat no phenotypic difference could be observed. The GST family is a large gene family that contains 47 members and one explanation could be a redundant function for some othe GST. Another member could take over the function of GSTF8, but to do so this GST has to be present in the chloroplast. We can exclude the best candidate GSTF5 (At1g02940) as it is not expressed in leaf tissue, roots, stem, inflorescence and

silique.(Glombitza et al., 2004). Its expression was not detected under various stresses like herbicides, plant hormones, pathogen attack, UV-B. Furthermore no cDNA was cloned to date and no EST was isolated. All these data suggest that GSTF5 is not expressed. Moreover GSTF5 possesses no clear predicted chloroplast targeting signal peptide. In conclusion it is highly unlikely that another GST may replace GSTF8 in the chloroplast. However, some GST-like proteins, two DHARs and one GSTL, possess a chloroplast targeting peptide and their putative role as antioxidant enzymes was described (Dixon et al., 2002). And furthermore, Milla et al. (2003) described a chloroplastic GSH-peroxidase present in the chloroplast (GPX1, At2g25080). In addition, all the other antioxidant mechanisms present in the chloroplasts of higher plants described previously could make the antioxidative role of GSTF8. As an alternative GSTF8 could play another role not linked to protection against oxidative stress.

In conclusion we demonstrated that GSTF8 is localized in the chloroplast. GSTF8 expression is upregulated by oxidative stress like treatment with hydrogen peroxide and the herbicide paraquat. GSTF8-OE and GSTF8-KO transgenic plants showed no phenotypic differences to wild type neither under normal growth conditions nor under conditions of oxidative stress. This indicates that GSTF8 is either not involved in protection from oxidative stress in chloroplasts or, alternatively, that in addition to GSTF8 other mechanisms contribute to this protection. The presence of various other mechanisms of detoxification of reactive oxygen species in the chloroplast can explain the absence of visible phenotype.

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Chapter 5

Concluding remarks

Concluding remarks

In the first part we have investigated the role of sulfur nutrition for plants in relation to disease resistance. Sulfur-deficiency of oilseed rape had a dramatic effect on plant resistance against various pathogens such as the specific *Brassica* pathogen *Leptosphaeria maculans*, a generalist pathogen like *Botrytis cinerea* and the oomycete *Phytophthora brassicae* infecting a wide range of *Brassicas*. This was confirming observations from the field that suggested at a link between sulfur supply and plant disease resistance. We have to keep in mind that sulfur is also very important for the quality of crop production (Haneklaus et al., 1995; Schnug, 1996). This leads to the conclusion that sulfur is a very important component of the plant nutrition and that we have to pay more attention to its sufficient supply in crop production by early detection of putative deficient soil and by adding fertilizer containing sulfur. The enhanced susceptibility of S-deficient oilseed rape was linked to the loss of antifungal potential. The loss of antifungal potential correlated with a dramatic reduction in the glucosinolate content. Glucosinolate degradation products were shown to have antifungal activity (Osbourn, 1996). However all the isothiocyanate and degradation products of sinigrin tested in our study did not exhibit antifungal activity. To fulfil the study it could be very interesting to test the degradation products of all the glucosinolates present in oilseed rape leaves to find out if one or more glucosinolates are indeed responsible for the antifungal activity. An other interesting approach could be to fractionate plant extracts and in order to purify the compound(s) responsible for the antifungal activity.

In the second part, the analysis of the transcriptome of four gene families of secondary metabolism under various stress conditions showed that the genes were differently regulated. The majority of the genes were neither strongly expressed nor strongly regulated. The differentiating expression patterns provided a wide set of information regarding the individual genes and argued against widely redundant functions among these quite large gene families. In conclusion the MetArray constitutes a powerful tool to study the specific expression of particular members of large gene families. However, an improvement of the technique could be to spot the gene specific probes on glass slides in order to avoid problems of unequal cDNA spotting and radiolabelling differences.

In the third part, we could demonstrate that GSTF8 is the only GST localised in the chloroplast and that it expression is regulated by oxidative stress. However our hypothesis that it could play a role in defense against oxidative stress in chloroplasts turned out to be difficult to verify. Further interesting work would be to analyse if in GSTF8-KO and GSTF8-OE the expression levels of other enzymes involved in reactive oxygen species detoxification are modified in comparison to wild type. If this is the case this would be an indirect hint of a antioxidant role of GSTF8 in the chloroplast.

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