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Glucosinolates and the clubroot disease: defense compounds or auxin precursors?

Jutta Ludwig-Müller

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Abstract The clubroot disease is caused by the obligate biotrophic protist *Plasmodiophora brassicae* and is one of the most damaging for the family of Brassicaceae. Since many economically important crops belong to this plant family, the understanding of mechanisms how the disease is developing, are of high importance. Glucosinolates, a group of secondary plant products in the family of Brassicaceae, have long been associated with clubroot disease symptoms. Measurements showed that several glucosinolates are induced in root galls. While aliphatic glucosinolates are regarded as defense compounds, analysis of *Brassica* cultivars as well as *Arabidopsis thaliana* mutants provided correlative evidence between disease severity and indole glucosinolate content. The latter have been discussed as precursors for auxin biosynthesis. Since high auxin levels are associated with large root galls, indole glucosinolates could contribute directly or indirectly to the extent of disease development. Transcriptome and proteome experiments have revealed evidence for the involvement of genes from the glucosinolate and auxin pathway in gall formation. These data have been complemented by expression and mutant analysis. It can be concluded that regulation of glucosinolate and IAA biosynthesis might differ in *Brassica* and *Arabidopsis*.

J. Ludwig-Müller (✉)
Institut für Botanik, Technische Universität Dresden,
01062 Dresden, Germany
e-mail: Jutta.Ludwig-Mueller@tu-dresden.de

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Abbreviations

ESP	Epithiospecifier protein
ESM	Epithiospecifier modifier
GSL	Glucosinolate
IAA	Indole-3-acetic acid
IAAld	Indole-3-acetaldehyde
IAM	Indole-3-acetamide
IAN	Indole-3-acetonitrile
IAOx	Indole-3-acetaldoxime
JA	Jasmonic acid
MBP	Myrosinase binding protein
MyAP	Myrosinase associated protein
PAA	Phenylacetic acid
QTL	Quantitative trait loci
SA	Salicylic acid

Development of the clubroot disease

Obligate biotrophic plant pathogens have evolved together with their host and therefore explore the host metabolism for their own needs. The clubroot disease of the Brassicaceae, caused by such an obligate biotrophic pathogen, is one of the most damaging within this family causing high yield losses in vegetable crops and oilseed rape (Voorrips 1995). Its control is only possible by complex methods

(Donald et al. 2006). The causal agent of the disease is the protist *Plasmodiophora brassicae*, a close relative of two other plant pathogenic protists of the genus *Polymyxa* and *Spongospora* (Archibald and Keeling 2004). The symptoms caused by *P. brassicae* are root galls which in the final stage of development lead to changes in water and nutrient supply due to destruction of vasculature and thus causing wilting in the green parts of the plant (Rausch et al. 1981a). In Fig. 1 healthy and infected *Arabidopsis* plants are shown with clear differences in growth (Fig. 1a, b). Also, the infected plants show accumulation of anthocyanins especially in the infected part of the rosette (Fig. 1c). In earlier stages of the infection, the pathogen is using plant signaling molecules such as cytokinins to re-distribute assimilates from the shoot to the root to guarantee its own nutrition (Evans and Scholes 1995; Siemens et al. 2006). Mono- as well as disaccharides and starch are increased in roots colonized by *P. brassicae* (Evans and Scholes 1995; Brodmann et al. 2002). In addition, the production of auxin is stimulated which in turn causes the root cells to enlarge (Grsic-Rausch et al. 2000; Ludwig-Müller and Schuller 2008). This intricate relationship is difficult to investigate because the pathogen can not be cultivated without its host. Although close to hundred genes from the pathogen have been identified up to now, for most of them no function could be assigned due to the low homology of sequences to other organisms found so far (Bulman et al. 2006).

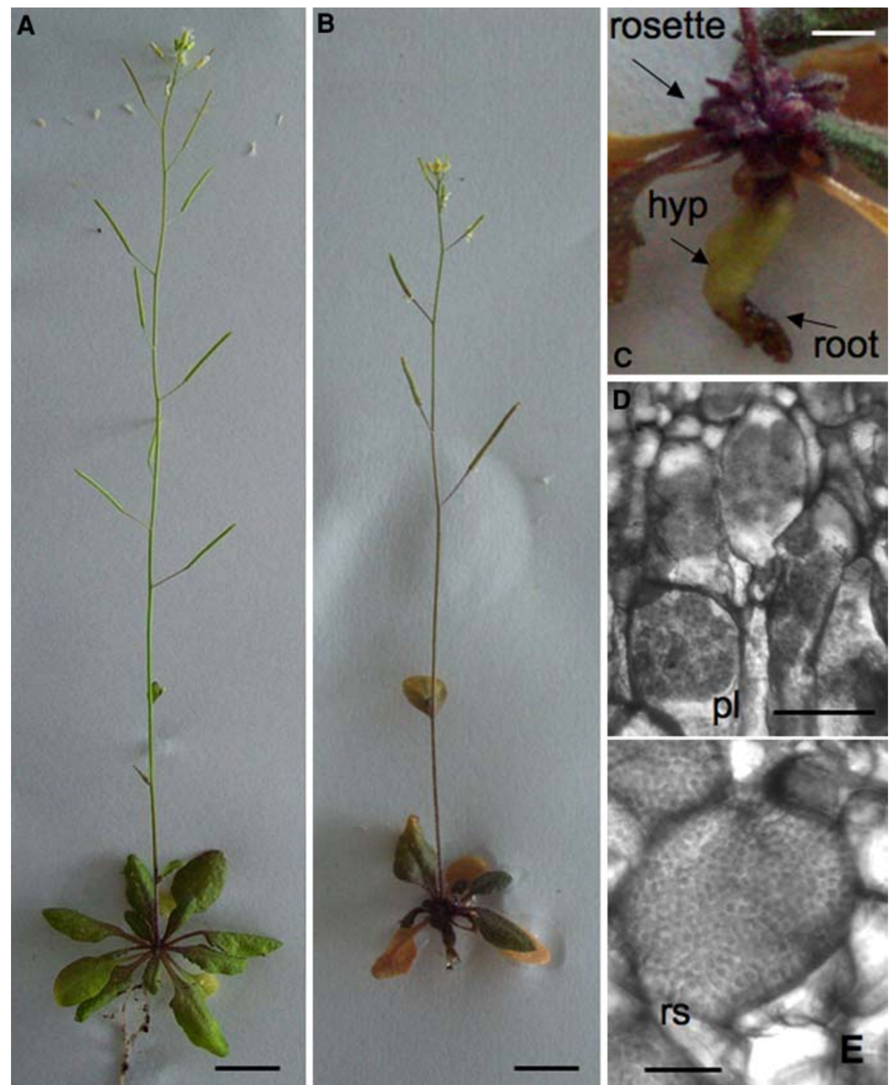

Fig. 1 Symptoms of the clubroot disease induced by *Plasmodiophora brassicae* on *Arabidopsis thaliana* as host. (**a**) Control and (**b**) infected *Arabidopsis* plant. (**c**) Close up of an infection where root, hypocotyl (hyp) and rosette infections are marked by arrows. Note also the anthocyan accumulation. (**d**) Young plasmodia (pl) inside the root. (**e**) Host cells filled with resting spores (rs). Bars in (**a**, **b**): 1 cm; (**c**): 5 mm; (**d**, **e**): 20 μm

Therefore, understanding of the mechanisms underlying the disease development is of high importance because the disease is difficult to control under field conditions. Breeding programs have yielded resistant cultivars, but on the other hand the pathogen itself has evolved more virulent pathotypes on these plants (Mattusch 1994). In *Brassica* species, QTL analysis have been carried out to find novel resistance mechanisms (Rocherieux et al. 2004; Piao et al. 2004; Hirai 2006; Saito et al. 2006). In the model plant *Arabidopsis thaliana*, also a suitable host for *P. brassicae*, a single resistance gene has been described in few ecotypes which causes resistance to a single spore isolate in a gene-for-gene manner (Fuchs and Sacristan 1996). However, the corresponding gene has yet to be identified (Rehn et al. 2006). Mutant analysis in *Arabidopsis* as well as exploring transgenic plants that are altered in gene expression which could be involved in gall formation show sometimes more tolerant phenotypes after infection with *P. brassicae*, but many others are not affected (Siemens et al. 2002). Recent transcriptome and proteome analysis on the interaction of *P. brassicae* with *Arabidopsis* (Devos et al. 2006; Siemens et al. 2006) and *Brassica* (Cao et al. 2007) could reveal novel targets for resistance breeding. One particular pathway has earned high interest since decades. Brassicaceae are one of the few plant families capable to produce glucosinolates (Rodman 1991a, b). Since these compounds have been linked to defense but also to plant hormone (i.e. auxin) biosynthesis (reviewed in Grubb and Abel 2006), it is feasible to believe that they could be involved in two aspects of clubroot development: (a) control of the extent of the disease and (b) control of gall size by auxin production. Here, I will summarize what has been proposed so far and provide novel experimental evidence to examine the hypothesis that gall formation is linked to auxin production from indole glucosinolates. Finally, a model on the roles of different groups of glucosinolates in clubroot formation will be presented.

Life cycle of *Plasmodiophora brassicae*

The life cycle of *P. brassicae* consists of two phases (Fig. 2): the primary phase, which is restricted to root hairs and epidermal cells of the host, and the secondary phase which occurs in the cortex and stele of roots and hypocotyl and leads to abnormal development (Ingram and Tommerup 1972). Not much is known about molecular events during the primary infection phase, although the mechanism of penetration has been well described (Buczacki 1983). Resting spores germinate and release zoospores. These penetrate the root hair by an apparatus consisting of a 'Stachel' and 'Rohr' (Braselton 1995), thereby releasing the complete protoplast of the pathogen into the root hair. There, a multinucleate primary plasmodium is growing until zoospores are formed which are again released into the soil (for recent review see Ludwig-Müller and Schuller 2008). These can again infect root hairs or they penetrate the root cortex by a yet unknown mechanism. Whether they have to fuse prior to penetration has not been unequivocally established, however, the observation of dikaryotic myxamoeba during this phase of infection would point to plasmogamy of two zoospores (Kobelt 2000). Since it is also possible to generate root galls from a single zoospore, it is assumed that different mating types are not necessary (Klewer et al. 2001). After the secondary infection has occurred the dikaryotic amoeba migrate, most likely actively, through the root cortex in direction of the central stele (Kobelt 2000; Mühlenberg et al. 2002) and in the early stages of the second infection cycle, young secondary plasmodia are formed (Fig. 1d). Later during gall development a pronounced increase in the number of secondary plasmodia can be observed and a passive distribution of the pathogen in the course of increasing host cell divisions occurs. Finally, multinucleate secondary plasmodia are formed which is accompanied by strong hypertrophy of infected cells (Ludwig-Müller et al. 1999a, b; Siemens et al. 2006). In this stage karyogamy has been observed followed by meiosis of the diploid nuclei (Ingram and Tommerup 1972). These processes lead to the cleavage of the plasmodium to yield numerous resting spores (Fig. 1e).

The importance of plant hormones during the clubroot disease

During the secondary infection cycle cell divisions occur and later large hypertrophied cells harbouring secondary plasmodia and resting spores can be observed (for more detailed review see Ludwig-

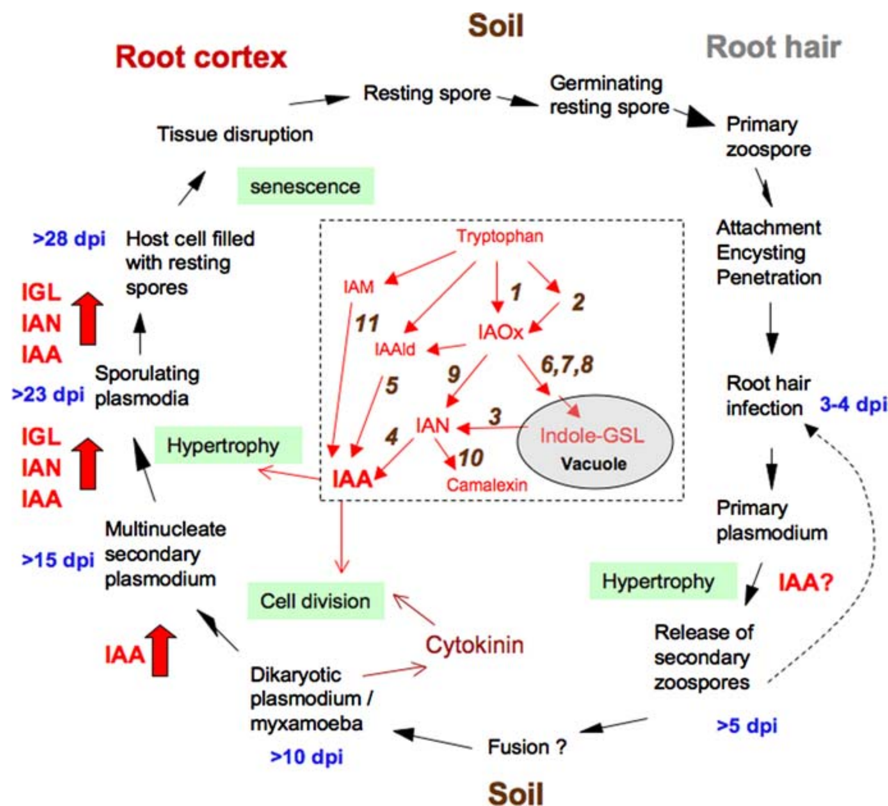

Fig. 2 The life cycle of *Plasmodiophora brassicae*. The life cycle starts with the germination of resting spores which infect root hairs of host plants during the primary infection cycle (right side). After root hair infection zoopsores are released which can again infect root hairs or which can invade the root cortex in the secondary infection cycle (left side) during which cell divisions and hypertrophy of host cells occur. When resting spores develop the host tissue undergoes senescence and the resting spores are released into the soil. In blue the approximate time frame after inoculation (days past inoculation—dpi) is given for better comparison of the different stages. In red the metabolites related to indole glucosinolate/IAA metabolims are shown which are up-regulated at the different infection stages. The inset shows biosynthetic pathways which are connected to the increase in indole glucosinolates/IAA during the disease development. Enzymes are given as numbers in brown. 1: cytochrome P450 (CYP79B2/B3), 2: YUCCA, 3: myrosinase (MYR), 4: nitrilase (NIT), 5: aldehyde oxidase (AAO), 6: CYP83B1 (SUR2), 7: C-S lyase (SUR1), 8: UDP-glucosyltransferase (UGT74B1), 9: CYP71A12/A13 (camalexin pathway), 10: CYP71B15 (PAD3, camalexin pathway), 11: amidase (AMI1). For further description on the possible role of these enzymes see text

Müller and Schuller 2008). Plant hormones, i.e. auxins and cytokinins have been associated with these phenomena (e.g. Devos et al. 2005; Siemens et al. 2006). The availability of hormone-responsive reporter genes in *Arabidopsis* enabled the localization of enhanced auxin and cytokinin responsiveness to the part of the root where gall formation occurs (Siemens et al. 2006). An increase in free and conjugated IAA levels was reported for *Arabidopsis* and *Brassica*, although at different time points during infection, making comparisons difficult (Ludwig-Müller et al. 1993, 1996, 1999a; Grsic et al. 1999; Grsic-Rausch et al. 2000, Devos et al. 2005). However, it is not entirely clear whether the pathogen or the host plant produces the hormones. Although evidence for auxin production by the pathogen is not available, it has been shown that plasmodia of *P. brassicae* are able to make small amounts of cytokinins (Müller and Hilgenberg 1986). In addition to the cytokinin production by the pathogen, the capacity of the host plant to degrade cytokinins is downregulated in root galls of *Arabidopsis* (Siemens et al. 2006). Transcriptome data have revealed that also other plant hormones might play a role as signals because genes involved in biosynthesis, metabolism, signalling or response were differentially regulated in gall tissue

compared to controls (Siemens et al. 2006). Particularly interesting is the finding that jasmonic acid (JA) is upregulated in *Brassica rapa* roots during club formation because JA is able to induce indole glucosinolates and also nitrilase (Grsic et al. 1999; Ludwig-Müller et al. 1997), thereby possibly linking auxin and jasmonate metabolism in clubs.

Role of aliphatic and aromatic GSL during clubroot

Glucosinolates (GSL) in general have been discussed as defense mechanisms by releasing toxic thiocyanates and isothiocyanates. On the other hand, the conversion to nitriles would yield less toxic products (Bones and Rossiter 1996). Only little information on aliphatic and aromatic glucosinolates during the development of the clubroot disease is available, because most research has focussed on indole glucosinolates (Agerbirk et al. 2008) as discussed below. *Brassica* sp. have been used as biofumigants to control clubroot in greenhouse as well as in field experiments, showing a reduction of symptoms on root systems of Chinese cabbage (Cheah et al. 2001). In these experiments, the green part of the plants (leaves and stems) as well as whole plants (left in the soil for decomposition for about 3 weeks) were used. Since *B. rapa* reduced the mean clubroot severity on cauliflower root systems better than *B. napus* and also had higher quantities of total isothiocyanates, the authors speculated that these GSL breakdown products were involved in clubroot control (Cheah et al. 2006). However, the effect was not attributed to a specific glucosinolate. Therefore, degradation of GSL in general might be an important feature also during endogenous control of the clubroot disease, because myrosinase, epithiospecifier protein (ESP), which is involved in nitrile formation (Lambrix et al. 2001), as well as myrosinase associated (MyAP) and myrosinase binding proteins (MBP) are differentially regulated during infection of *Arabidopsis* (Devos et al. 2006; Siemens et al. 2008) and *Brassica rapa* (Grsic et al. 1999) roots with the clubroot pathogen. The concept of a 'mustard oil bomb', which has been put forward by Matile (1975), would fit nicely with the model that during infection with *P. brassicae* the cellular compartimentation could be destroyed by growing plasmodia, thereby releasing GSL from the vacuole which could then in turn be degraded by myrosinase (see also Fig. 2). However, the discovery of „myrosin cells" which constitute specialized cells and are not present in all parts of the plants in similar numbers (Bones and Rossiter 1996) asks for a reconsideration of this theory. While in *B. rapa* the experimental evidence for the upregulation of myrosinase has been provided by Northern blot analysis (Grsic et al. 1999), in *Arabidopsis* the information came from transcriptome and proteome data. Devos et al. (2006) showed by comparing the protein patterns in control and infected roots that myrosinase was upregulated at a very early time point of infection, whereas Siemens et al. (2006) have found evidence for downregulation of myrosinase transcripts in *Arabidopsis* clubroots at later time points of gall development (see also Fig. 3). However, it has not yet been shown by functional analysis that myrosinase is a limiting factor in gall formation. Additional factors such as MBP, MyAP, ESP and ESM1 (Epithiospecifier modifier, a protein belonging to a known class of myrosinase-associated proteins, which inhibits ESP-mediated nitrile formation) would determine the outcome of metabolism and thus, among others, toxicity (Lambrix et al. 2001; Eriksson et al. 2002; Halkier and Gershenzon 2006; Burow et al. 2008). On the other hand, toxicity is encoded in the GSL core structure itself. Therefore, in resistant and susceptible *Brassica rapa* (Chinese cabbage) cultivars, the amount of aliphatic and aromatic GSL was analyzed (Ludwig-Müller et al. 1997). Overall, the observations did not entirely fit with the hypothesis that the group of aliphatic GSL are induced as defense response. The total glucosinolate content in roots of the two susceptible varieties was higher throughout the experimental period than in roots of the two resistant cultivars (Ludwig-Müller et al. 1997). While the aliphatic GSL were induced in the two susceptible cultivars compared to the resistant ones, the two resistant cultivars showed an increase in aromatic GSL, indicating maybe a dual role for these compounds (Fig. 4). In addition, in one of the resistant cultivars an induction of aliphatic and aromatic glucosinolates after jasmonate treatment was observed (Ludwig-Müller et al. 1997).

In an investigation on the host range of *P. brassicae* in various non-*Brassica* species further evidence for the possible dual roles of aromatic GSL during club formation was presented (Ludwig-Müller

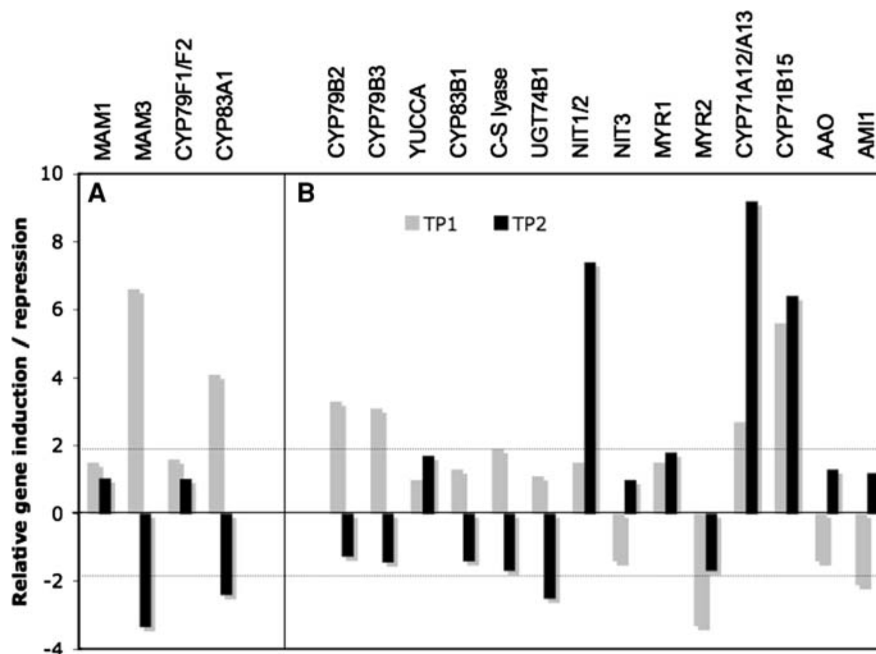

Fig. 3 Compilation of expression data from microarray analysis (Affymetrix ATH1) indicating up- or down-regulation of genes involved in indole glucosinolate/IAA metabolism. TP1 = time point 1, 10 days past inoculation (dpi) where no visible symptoms have been observed but the pathogen was already present in infected roots as plasmodium. TP2 = time point 2, 23 days past inoculation where root galls were clearly visible and all developmental stages of the pathogen were visible (see also Fig. 1). Only genes induced or repressed by about 2-fold should be considered as differentially regulated (dotted lines). For the detailed description of the experiment see Siemens et al. (2006). (**a**) Aliphatic glucosinolate synthesis: *MAM1* (At5g23010), *MAM3* (At5g23020), *CYP79F1/F2* (At1g16410; At1g16400*), *CYP83A1* (At4g13770). (**b**) Indole glucosinolate and IAA synthesis: *CYP79B2* (At4g39950), *CYP79B3* (At2g22330), *YUCCA* (At4g32540), *CYP83B1* (At4g31500), *C-S Lyase* (At2g20610); *UGT74B1* (At1g24100), *NIT1/2** (At3g44300; At3g44310), *NIT3* (At3g44320), *MYR1* (At1g47600; At1g51470**), *MYR2* (At5g25980), *CYP71A12/A13* (At2g30750; At2g30770*), *CYP71B15* (At3g26830), *AAO* (At5g20960), *AMI1* (At1g08980). For abbreviations see text and legend to Fig. 1. *Can not be distinguished on the microarray. **Two AGI numbers for one gene description

et al. 1999b). Severity of symptom development was correlated with certain glucosinolates in one species, while the increase in other GSL might be regarded as defense response. In the glucosinolate-containing non-Brassicas, *Tropaeolum majus* and *Carica papaya*, the concentrations of benzylglucosinolate were increased markedly in roots inoculated with *P. brassicae*, compared with the controls. Slight gall formation was observed in *T. majus* (Ludwig-Müller et al. 1999b) and it was hypothesized that benzyl-GSL could act as precursor for phenylacetic acid (PAA; see Fig. 4), which has auxin activity in *T. majus* (Ludwig-Müller and Cohen 2002). Also, *Lepidium sativum*, which has a high benzyl-GSL content (Ludwig-Müller et al. 1999b) was able to form clubs after *P. brassicae* infection (Butcher et al. 1976) and in root galls of *L. sativum* phenylacetonitrile was detected, which would be the direct precursor of PAA. However, in *Reseda alba* roots, the total glucosinolate content decreased after inoculation with *P. brassicae* compared with the controls whereas high root concentrations of 2-OH-2-phenylethylglucosinolate compared with low root indole glucosinolates in this species may limit *P. brassicae* infection and development.

In *Arabidopsis thaliana*, mutants which had been isolated for their altered leaf GSL pattern (Haughn et al. 1991), were used to investigate the relation of aliphatic GSL and *P. brassicae* infection. Two alkenyl-GSL were only detectable in infected roots of mutants and wild type and total aliphatic GSL were only slightly increased in clubroots. Consequently, a mutant in a gene encoding an enzyme involved in the biosynthesis of aliphatic GSL (*gsm1-1*; Kroymann

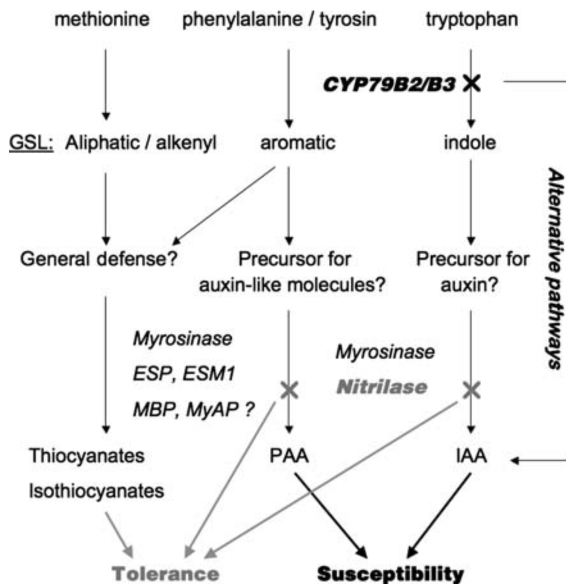

Fig. 4 A model explaining what might be happening during clubroot formation in terms of glucosinolate metabolism. The aliphatic glucosinolates derived from methionine might act as defense compounds thus being involved in tolerance. The aromatic glucosinolates could be also degraded to defense compounds but they might also serve as precursors for auxin-like molecules such as phenylacetic acid (PAA) in benzyl-glucosinolate-containing species. Finally, indole glucosinolates derived from tryptophan serve most likely as auxin precursors and are intermediates for indole-3-acetic acid (IAA). Increase of the auxin pathway would lead then to susceptibility. Inhibition of late enzymes in the pathway (i.e. nitrilase; in grey) would render the plant more tolerant because it can not be easily substituted. Blocking early steps in the pathway (i.e. CYP79B2/B3; in bold black) would not lead to tolerance because alternative pathways could operate. For further explanation see text. The role for myrosinase binding protein (MBP) and myrosinase associated protein (MyAP) has not been demonstrated. Epithiospecifier protein (ESP) and Epithiospecifier modifier 1 (EMS1) are involved in the control of glucosinolate hydrolysis and influence the outcome of the reaction, thereby also determining the synthesis of specific defense compounds

et al. 2001) did not show any tolerance to clubroot (Ludwig-Müller et al. 1999a). Such results could indicate that in host plants, even though the GSL metabolism was increased as a sign of defense response, the pathogen might be well adapted.

Since no direct experimental information is available on the control of aliphatic GSL synthesis during clubroot, transcriptome data from clubroot development in *Arabidopsis* were re-analyzed for the expression of genes involved in aliphatic GSL synthesis (Fig. 3a). In this experiment two distinct developmental stages of clubroot disease were analyzed (Siemens et al. 2006). At an early time point (10 dpi) no disease symptoms were visible and the pathogen was mostly at the stage of young vegetative plasmodia. A second time point was characterized by clearly visible clubs and in root sections plasmodia, sporangia and resting spores of the pathogen could be found (23 dpi). The genes selected included the methylthioalkymalate synthases MAM1 and MAM3 (=MAM-L) which are involved in chain elongation of methionine (Textor et al. 2007), as well as the cytochrome P450 enzymes CYP79F1/F2 involved in aldoxime formation (Chen et al. 2003) and CYP83A1 wich converts the oxime to further metabolites in the pathway (Naur et al. 2003). The *CYP79F* transcripts were not significantly deregulated in clubroots, which was also the case for *MAM1*. However, *MAM3* and *CYP83A1* were regulated in a similar manner. Both were upregulated at an early time point where symptoms were not yet visible, but the tissue already colonized by the pathogen (for more details see Siemens et al. 2006). At a time point where gall formation was clearly visible the two transcripts were then downregulated. It could be hypothesized that upregulation at the first time point is a transient defense reaction of the plant which is later downregulated by the pathogen by yet unkown signals to circumvent the production of toxic compounds.

The role of indole glucosinolates during clubroot

Variations in indole glucosinolates and disease development

A hypothesis which has been brought forward several decades ago indicated a role for indole GSL as precursors for IAA production during clubroot (Butcher et al. 1974). It was emphasized that if plants have large amounts of indole GSL, then after tissue disruption also large amounts of IAA would be produced, thus resulting in susceptible plants. This hypothesis has provoked the reverse assumption, namely that plants with low indole GSL levels and therefore a lower capacity for IAA synthesis by turnover of indole GSL, should be more tolerant to clubroot formation. However, conflicting results have

been published on this topic. For example Butcher et al. (1974) and Ockendon and Buczacki (1979) found clear correlations between resistance and low indole glucosinolate content in Brassicaceae. Chong et al. (1981, 1984) confirmed these results with cabbage breeding lines, whereas Mullin et al. (1980) were not able to correlate the indole glucosinolate content and the resistance to clubroot. Comparing two resistant and two susceptible *Brassica rapa* cultivars prominent differences in the indole glucosinolate pattern was found (Ludwig-Müller et al. 1997). Mainly the two susceptible cultivars reacted with an increase in the indole glucosinolates after infection with *Plasmodiophora brassicae* 14 and 20 dpi in roots, whereas there was no difference between infected and control roots of the two resistant cultivars. However, all cultivars tested showed increased glucosinolate levels after treatment with salicylic acid (SA) and jasmonic acid (JA). JA induced mainly the indole glucosinolates in the leaves, whereas SA induced the indole glucosinolates also in the roots of both cultivars. Also, the transcript for myrosinase and the activity of nitrilase with IAN as substrate were induced by JA in *Brassica* linking jasmonate and auxin synthesis (Grsic et al. 1999). It should be noted that both JA (Grsic et al. 1999) and SA (Ludwig-Müller et al. 1995) were increased in root galls compared to control roots during stages were galls were clearly visible making these two compounds good candidates for signals involved in the induction of GSL synthesis during the clubroot disease.

In *Arabidopsis thaliana* ecotype Columbia indole GSL were also increased during clubroot development (Ludwig-Müller et al. 1999a). Two mutant lines with altered GSL content (Haughn et al. 1991), named lines *tu3* and *tu8* proved to be particularly useful to study the involvement of glucosinolates and auxin in clubroot disease (see also below). Both showed reduced symptom development compared to the wild type, but only in *tu8* indolic GSL decreased while in *tu3* they were unchanged compared to wild type (Ludwig-Müller et al. 1999a). The role of indole glucosinolates, and more specifically their breakdown products, as potential precursors of IAA, therefore does not appear to be a simple relationship. Other major factors that need to be considered include the flux through the glucosinolate biosynthetic and degradative pathways (specifically the rate of indole GSL and 1-MeOH-indole-GL biosynthesis versus the rate of myrosinase-catalyzed degradation), and the rate of nitrilase-catalyzed conversion of IAN to IAA (see below).

Regulation of auxin and indole GSL biosynthesis and metabolism during clubroot

Work on the enzymes and genes involved in the biosynthesis has more concentrated on auxin synthesis/indole GSL metabolism than in the first steps of indole GSL biosynthesis. For a recent review on auxin biosynthesis and metabolism see e.g. Woodward and Bartel (2005). The general pathways for GSL biosynthesis have been described elsewhere in detail (Grubb and Abel 2006; Halkier and Gershenzon 2006). Microarray analysis (Siemens et al. 2006) has revealed that several genes coding for proteins involved in the biosynthesis of indole GSL and IAA are differentially regulated during the disease in *Arabidopsis thaliana* (Fig. 3b). It is noteworthy that only two early indole GSL biosynthetic genes (*CYP79B2/B3*; Hull et al. 2000) converting tryptophan to indole-3-acetaldoxime (IAOx) were upregulated at an early time point, whereas later genes in the pathway such as *SUR2* (Barlier et al. 2000), *SUR1* (Mikkelsen et al. 2004), and *UGT74B1* (Grubb et al. 2004) were not regulated or actually downregulated (Fig. 3b). Only those involved directly in IAA biosynthesis were upregulated later, indicating that the increase in IAA via the indole GSL/nitrilase pathway is important only during late stages of gall development. These stages are accompanied by hypertrophied cells harbouring plasmodia and resting spores of the pathogen (Figs. 1, 2).

The first enzyme necessary for the formation of indole-3-acetonitrile (IAN) from indole GSL is myrosinase. While myrosinase genes were not upregulated in *Arabidopsis* according to microarray analysis, proteome analysis found a myrosinase upregulated during early inoculation events (Devos et al. 2006). Contrary, in *Brassica rapa*, the expression level of myrosinase was increased in infected roots compared to controls (Grsic et al. 1999). It can be speculated that myrosinase expression and IAN formation are dependent on different endogenous and exogenous signals and that an apparent up- or down-regulation might not reflect the situation *in planta*, where maybe only few infected cells contribute to IAN synthesis. Up to now

there is no evidence for the involvement of other myrosinase associated proteins or for epithiospecifier protein in clubroot development.

IAN can further be converted to IAA by the enzyme nitrilase. In *Arabidopsis* there is a family of four nitrilases of which three are capable to convert IAN to IAA (Bartel and Fink 1994; Hillebrand et al. 1998). It was shown that nitrilase 1 and 2 are increased in developing root galls during later stages by Northern analysis, promoter-GUS studies (Grsic-Rausch et al. 2000) and microarray analysis (Siemens et al. 2006; see also Fig. 3b). Immunolocalization confirmed nitrilase protein in hypertrophied cells harbouring sporulating plasmodia (Grsic-Rausch et al. 2000). These data indicate that in *Arabidopsis* nitrilase is associated with late stages of clubroot development. Early in vivo labeling studies showed that IAN is specifically converted to IAA from indole GSL in infected *Brassica* roots, whereas wounding induced the formation of different metabolites (Rausch et al. 1983). Also, increased nitrilase activity has been associated with increased IAA content and root gall development in different Brassicaceae (Rausch et al. 1981b; Grsic et al. 1999; Ugajin et al. 2003; Ishikawa et al. 2007a). However, Northern blot analysis did not show any differences in the mRNA levels of nitrilase in *B. rapa* (Chinese cabbage) during gall formation (Bischoff et al. 1995) but the probes used in this study were not gene specific and might have therefore missed subtle differences. This hypothesis is likely, based on results from Ishikawa et al. (2007a) who showed that in *B. rapa* (turnip) nitrilase was differentially expressed in clubroots. Both, nitrilase 1 from turnip and Chinese cabbage convert IAN to IAA, but high K_m values indicate that IAN might not be the natural substrate. Instead, phenylacetonitrile and phenylpropionitrile are highly converted to the corresponding acid, indicating maybe a different or additional role for nitrilase in clubroot formation (Grsic et al. 1998; Ishikawa et al. 2007a). Whether this might be the conversion of nitriles to nontoxic compounds or whether phenylacetic acid is produced which might also act as auxin (see above) is not clear. However, alternatives for the biosynthesis of IAA in *Brassica* root galls have been reported. For *Brassica rapa* two different possibilities for the conversion of IAOx to IAA have been demonstrated on an enzymatic level. Ludwig-Müller and Hilgenberg (1990) described an enzymatic activity capable to catalyze the reaction from IAOx to IAN and Helmlinger et al. (1987) described an activity converting IAOx to indole-3-acetaldehyde (IAAld). Both routes bypass indole GSL. However, the contribution of these pathways to IAA synthesis in clubroots has not been investigated. IAAld can be further converted to IAA in *Arabidopsis* by aldehyde oxidases (Seo et al. 1998). Aldehyde oxidase was also induced in *Brassica* clubroots (Ando et al. 2006). In comparison, *Arabidopsis* did not show such an induction according to microarray data (Fig. 3b). In *B. rapa* (turnip) in addition to nitrilase the conversion of indole-3-acetamide to IAA, a pathway initially described for plant pathogenic microorganisms such as *Agrobacterium tumefaciens* (Inzé et al. 1984) was increased in clubroots on an enzymatic level (Ishikawa et al. 2007b). Amidase is also present in *Arabidopsis* (Pollmann et al. 2003), but does not contribute to IAA levels in root galls according to transcriptome data (Fig. 3b). Direct evidence for the contribution of other genes involved in auxin biosynthesis such as the *YUCCA* family (Zhao et al. 2001) has yet to be presented.

It should be noted that other compounds related to the IAOx/indole GSL pathway such as the phytoalexin camalexin were also induced during clubroot (Siemens et al. 2008), thus maybe diverting the metabolic flow away from indole GSL. The accumulation of IAN after clubroot infection could, for example, result in the competition of the two different pathways for one substrate (Nafisi et al. 2007). One protein in this pathway (CYP71A12/A13) could play a dual role in the formation of IAN which is converted either further to IAA or camalexin (Nafisi et al. 2007). The gene for this protein was also highly induced in root galls (Fig. 3b). On the other hand the cytochrome P450 CYP71B15 (PAD3), which is also involved in camalexin biosynthesis (Zhou et al. 1999), is probably not directly involved in clubroot formation, because the mutant *pad3* did not show tolerant phenotypes (Siemens et al. 2002), even though the corresponding gene was found to be upregulated in transcriptome analysis (Fig. 3b; Siemens et al. 2006, 2008). Alternatively, IAN could be channeled within each pathway or IAN could be only converted to IAA when excess IAN accumulates (Nafisi et al. 2007). The latter idea may explain why the expression of NIT1 and NIT2 is induced by exogenous IAN (Grsic et al. 1998). In *Arabidopsis* it

has not been shown whether the two pools of IAN are interchangeable. In *Brassica juncea* it was shown that IAN was most likely produced from trpytophan via IAOx after infection with *Phoma lingam*, but no evidence for the further metabolism of IAN was provided (Pedras et al. 2002).

Mutant analysis

The analysis of mutants and transgenic plants which have altered levels of genes involved in the indole GSL/IAA pathway will help to shed light on the role of these gene products for gall formation. In *Arabidopsis* many such mutants are available and have been studied. Mutants with altered indole GSL levels (Haughn et al. 1991; see above) showed reduced root galls with fewer pathogen structures (Ludwig-Müller et al. 1999a). This was accompanied by lower IAN and IAA levels, but amazingly, the indole GSL level was not so much reduced. This was a first indication that indole GSL are not the sole contributors to the pathway and thus IAA formation. The mutant *tu8* was shown to be not directly connected to indole GSL metabolism because the mutated gene was identified as heterochromatin like protein 1 (Kim et al. 2004; Bennett et al. 2005). Therefore, the phenotypes observed might be rather indirect effects of a chromatin modifying protein and the clubroot/indole GSL/auxin phenotypes observed in *tu8* have to remain correlative.

Focus was also placed on changes in nitrilase expression. For example the mutant *nit1*, defective in the nitrilase 1 gene, showed smaller galls with lesser pathogen structures and this was accompanied by a reduction in free IAA levels in clubroots (Grsic-Rausch et al. 2000). Furthermore, a transgenic plant reduced in nitrilase 2 showed slower development of the root galls (Neuhaus et al. 2000). However, overexpression of nitrilase did not result in altered symptoms after infection with *P. brassicae* (Neuhaus et al. 2000), implicating that wild type levels of nitrilase are sufficient for gall formation, but if there is a rate limiting step upstream in the pathway has not yet been determined.

Since nitrilase is situated late in the pathway after indole GSL (see Figs. 2, 4), mutants available in the first steps of indole GSL biosynthesis are useful tools to investigate the direct role of these compounds in gall formation. Single mutants of two cytochrome P450 enzymes involved in the synthesis of IAOx (*cyp79b2* or *cyp79b3*) did not result in any phenotype (Siemens et al. 2008). Since the two proteins could substitute for each other, double mutants were tested. The *cyp79b2/b3* double mutant is almost completely devoid of indole GSL (Zhao et al. 2002). Surprisingly, the *cyp79b2/b3* double mutant also showed normal clubroot symptoms and the levels of free IAA in galls were comparable to wild type (Siemens et al. 2008). These results show that indole GSL are not important in *Arabidopsis* for gall development. It was therefore concluded that a block in early steps in indole GSL biosynthesis could be overcome in terms of IAA synthesis by other pathways such as the YUCCA pathway (Zhao et al. 2001) or by direct synthesis via IAOx and IAN (Nafisi et al. 2007; see also Fig. 2). However, alterations in late steps can not be compensated, thus resulting in reduced gall size (Fig. 4). The situation could be different in *Brassica* as shown for other genes as well.

Other auxin related mutants which were investigated for clubroot formation included transport, signaling and homeostasis mutants such as *aux1*, *axr1*, *axr2*, *ilr1* (Siemens et al. 2002), *tir1*, *tir3*, *axr6* (Alix et al. 2007), but they did not show any tolerance to clubroot. However, *axr3*, also an auxin signaling mutant encoding the Aux/IAA protein IAA17 was partially resistant to clubroot (Alix et al. 2007).

Possible transcriptional control of glucosinolate synthesis during clubroot

Transcriptional control is an additional level to be considered for the regulation of metabolites from the indole glucosinolate pathway during clubroot formation. Since there are no experimental data available on transcriptional control of GSL synthesis during clubroot development, microarray data from *Arabidopsis* (Siemens et al. 2006) have been re-evaluated for expression levels of some transcription factors involved in aliphatic and indole GSL biosynthesis. A transcription factor (OBP2; At1g0640) involved in controlling a downstream step of IAOx to indole-GSL conversion via induction of *CYP83B1* (*SUR2*) was identified (Skirycz et al. 2006), but this zinc finger protein was slightly repressed according to transcriptome analysis at the second time point (−1.1/−2.0). Another transcription factor of the MYB family (ATR1; At5g60890) activates expression of a tryptophan

synthesis gene as well as the tryptophan-metabolizing genes CYP79B2, CYP79B3, and CYP83B1 (Celenza et al. 2005). This transcription factor was slightly increased according to transcriptome analysis at the first time point, but repressed at the second time point (1.9/−2.1). A close homolog to ATR1 was identified as another transcription factor involved in the control of indole glucosinolate biosynthesis (Gigolashvili et al. 2007a). The *MYB51/HIG1* (HIG = high indolic glucosinolates) gene (At1g18570) was downregulated at the first time point in the microarray and not regulated at the second time point (−2.9/−1.2). If the increase in indole GSL levels plays a role during clubroot development, the downregulation of transcription factors might indicate that transcriptional control of indole GSL synthesis is possibly not an important mechanism. Also the synthesis of aliphatic GSL is controlled by MYB transcription factors (Gigolashvili et al. 2007b, 2008, this issue). *MYB28/HAG1* (HAG = high aliphatic glucosinolates) transcripts (At5g61420) (Gigolashvili et al. 2007b) were upregulated at the early time point and slightly downregulated at the second time point (2.0/−1.6) according to transcriptome data. *MYB29/HAG3* (At5g07690) and *MYB76/HAG2* (At5g07700) transcription factors were also involved in controlling aliphatic GSL (Gigolashvili et al. 2008). Interestingly, it could be shown that the two latter transcription factors not only positively controlled aliphatic GSL content but negatively regulated indole GSL levels (Gigolashvili et al. 2008). Effects of aliphatic GSL on indole GSL have already been reported in the *bushy* mutant (*CYP79F1*) with lower aliphatic GSL levels, but the indole GSL were increased (Reintanz et al. 2001). However, trancript levels did not change dramatically according to microarray data for *MYB29/HAG3* (1.2/1.7) and for *MYB76/HAG2* (1.1/1.5). Either the pathway is not strictly under transcriptional control, or the transcription factors are regulated only in a few infected cells which would not show up in the analysis of the whole root tissue. This problem might be overcome in the future by using single cell analysis methods.

Conclusion

The question initially asked is still open to some extent. GSL and their metabolites may play a role as defense compounds also in the clubroot disease via degradation by myrosinase and associated proteins, but they are not essential factors responsible for resistance. Since transcripts for proteins involved in the biosynthesis of aliphatic GSL from methionine are partially upregulated, the role of aliphatic GSL as defense compounds is likely (see Fig. 4). Using *Arabidopsis* it could be feasible to introduce corresponding genes and investigate transgenic plants for altered responses to clubroot. On the other hand, the contribution of indole GSL to IAA biosynthesis and thus their contribution as possible link to gall size is only indirect, at least in *Arabidopsis*. IAN as precursor might be more directly linked to IAA, especially during late stages of gall growth, whereas other pathways must be operating in early stages to contribute to an increase in IAA. However, there are increasingly more mutants available in pathways for aliphatic and indolic glucosinolates which could be tested for tolerance or resistance against the clubroot pathogen. Also, in such mutant lines alternative auxin biosynthesis pathways could be investigated which would substitue for a missing pathway. Finally, the differences between the model plant *Arabidopsis* and *Brassica* species in terms of gene regulation or enzymatic activity have to be taken into consideration. Still, *Arabidopsis* is an important model for studying clubroot disease, because candidate genes possibly involved in gall formation can be much easier assessed than in *Brassica*. On the other hand, differences seen so far can be also studied involving for example *Brassica* microarrays which are now becoming available.

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