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Asian Soybean Rust – Meet a Prominent Challenge in Soybean Cultivation

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1. Introduction

The people inhabiting our planet are expected to touch the nine million mark within the next 40 years. Feeding the world population adequately is a problem which has been faced for a long time, and although considerable efforts have been made in tackling global food security, further solutions are needed to increase the supply of basic staples in the coming decades. Generally, there are two major strategies to approach this goal for food production: firstly, to grow more crop plants on newly reclaimed land or to increase yields on existing farm land by using improved cultivars; and secondly, to diminish the actual losses in crop yield arising due to pests, diseases or from the competition for nutrients with weeds. In view of the size of the challenge it is reasonable to assume that only a combination of different approaches can achieve global food security. However, implementation of the first strategy may have negative ecological impacts on existing ecosystems, and a higher input of fertilizer or water, both of which are expensive or limited, will be a consequence. The second strategy, by contrast, displays an attractive alternative because it deals with the conservation of already produced biomass. Thus, in principle, would not need any further input of resources for crop growth. It was estimated in 1994 that the actual losses due to insects, microbes and weeds reduced the theoretically achievable global crop yield by 42% (Oerke et al., 1994). Reducing this depletion would be a valuable contribution to ensure food security.
This chapter will deal with soybean as one of the world's major staple crops and strategies will be presented to combat yield losses caused by a prominent foliar pathogen known as Asian soybean rust caused by the fungus *Phakopsora pachyrhizi* (Fig. 1). Generally, all parts of soybean plants are targeted by different pathogens and pests during the growing season, e.g. roots by cyst nematodes, young seedling by different *Pythium*, *Rhizoctonia* or *Phytophthora* species and leaves e.g. by *Phakopsora pachyrhizi*. Asian soybean rust (ASR) affects yield because of premature leaf drop and reduced green leaf area which affects the photosynthetic capacity of the plant. Generally, agronomists try to keep plant diseases in check by breeding resistant cultivars or, more recently, by using biotechnological tools. However, it must be noted that the latter approach which includes the generation and release of genetically modified plants into nature are discussed controversially in public debates. Unlike for some other important plant diseases, neither breeding for resistant cultivars nor the biotechnological approach have so far led to novel soybean genotypes that could withstand all isolates of the ASR fungus.

Fig. 1. Disease symptoms caused by the Asian soybean rust fungus on soybean plants. Brownish spots become visible on the leaf surface of susceptible soybean plants eight to ten days after inoculation with the pathogen *Phakopsora pachyrhizi* (left picture). Disease symptoms, which are reminiscent of oxidised metal (rust), are referred to as 'rust pustules' and consist of an aggregation of individual fungal uredospores (right picture). These uredospores are dispersed by wind or water and each of them is able to generate a novel infection site on healthy plant tissue of the same plant or plants on distant field plots.

Here, we report on our research to get a deeper insight into the interaction of a soybean plant with the ASR fungus. In the short term, we aimed at targeting novel traits which might confer resistance to soybean against this disease and identifying crucial elements required by the pathogen to be virulent on soybean; and at the end of the day both approaches might channel into the development of a novel cultivar that effectively withstands *P. pachyrhizi*.

1.1 What is the problem?

The phenomenon of global warming has let to considerable changes in environmental conditions of ecosystems worldwide. Many plant pathogens and pests are extending their ranges and emerging in habitats where they had not been present before. While sporadic invasion by these species had been terminated in the past mostly due to freak, unfavorable weather conditions, nowadays they may survive more frequently and become endemic. The problem of pathogen dispersal is compounded by increased human mobility and long-distance travel. A recent example is the invasion of the Asian soybean rust fungus, *Phakopsora pachyrhizi*, into South and North America. Initially, a less aggressive relative of *P. pachyrhizi*, known as *Phakopsora meibomiae* and referred to as the Latin-American isolate, was indigenous in South-America where it was not considered a major problem in soybean cultivation. The situation changed dramatically with the appearance of the Asian-Australian isolate, *P. pachyrhizi*, which by now has reached top ranking among soybean diseases in South-America, e.g. in 2003 the disease spread through Brazil and caused losses estimated at US$ 2 billion (Yorinori et al., 2005). In 2004 ASR was reported for the first time in the

continental United States and since then it has attracted a lot of attention because of its potential to cause high yield losses within the corn belt in the Midwestern US (Schneider et al., 2005). In contrast to other soybean diseases no cultivars are available which can withstand all races of this pathogen, thus farmers rely on the costly use of fungicides to manage the threat (Miles et al., 2007). Because of its economical and ecological impact fungicidal treatments are seen critically. Furthermore, emerging resistance of the pathogen against certain fungicide classes has to be expected. Accordingly, new strategies are needed to combat this growing threat and below we present approaches currently pursued in our lab.

1.2 Our approach

Generally, research into fungal plant diseases at the academic level could be done by concentrating on the plant or fungal partner in the interaction. The plants can be investigated for traits conditioning susceptibility and trying to fortify the natural defense arsenal and the pathogen by analyzing the infection process, aiming at identifying an Achilles heel which could be used in subsequent approaches to avert infection of plants.

In our lab we follow a holoistic strategy and investigate both partners. As a source for traits for the potential improvement of plant defenses, we exploited a phenomenon known as nonhost resistance. The term "nonhost resistance" describes the resistance of plants against pathogens for which the plant species in question is not considered a host (Heath, 2001). For example, a pathogen of grasses would not necessarily be expected to infect other plants, e.g. soybean, to which it has not had the opportunity to adapt in the course of evolution. In natural ecosystems, as well as in agricultural habitats, nonhost resistance mediates a robust protection against pathogenic invaders and it was therefore often suggested that the mechanisms of nonhost resistance could be exploited to improve the resistance of crop plants (reviewed by Thordal-Christensen, 2003). The model plant *Arabidopsis thaliana* displays nonhost resistance against the Asian soybean rust and this enables us to take advantage of the extensive tool-box for genetic and molecular investigations which already exists. Thus, it has been shown that Arabidopsis mutants with defects in one or several of the so-called PEN (penetration) genes are compromised in nonhost resistance against another important pathogen, namely *Blumeria graminis* f. sp. *hordei*, which is the powdery mildew pathogen of barley (Stein et al., 2006; Schweizer, 2007). We tested the impact of mutations in these genes on infections with *P. pachyrhizi* on Arabidopsis in growth chamber experiments. Alterations in the infection process were monitored by macroscopic and microscopic evaluation. Testing of several other Arabidopsis mutants and analyses of defense gene activation were used to investigate the possible involvement of other resistance mechanisms in nonhost resistance of Arabidopsis against *P. pachyrhizi*.

In a second line of experiments we worked towards a better understanding of the infection process by *P. pachyrhizi* on soybean. By now it is widely accepted that phytopathogens actively create an accomodating environment in their cognate host by interfering with the plant's defense machinery. Such a scenario is well documented for bacterial pathogens which possess a dedicated secretory pathway to deliver so-called effector molecules into the host cells which then sabotage essential resistance mechanisms (Romer et al., 2007). It could be speculated that rust fungi, such as *P. pachyrhizi*, also recruit such molecules to avoid recognition or the initiation of defense, although no experimental proof for this hypothesis exists so far. Nevertheless, if this assumption were to be correct, and if identification of the

effector molecules were possible, these molecules or their targets inside the plant cell could be valuable targets in future plant protection programs. Testing this hypothesis in the soybean/*P. pachyrhizi* interaction is difficult because the pathogen has an obligate biothrophic life-style which means that it grows and multiplies solely on living plant tissue. This adds to the difficulty of purifying fungal-derived molecules because contamination with plant material must be expected. To minimize this drawback, we followed an approach of firstly isolating fungal infection structures from infected plant material and then trying to identify fungal genes expressed solely at this stage of infection. Purification of the desired infection structures was achieved using affinity chromatography. After sampling of sufficient material a snap-shot of active genes was taken by isolating RNA and sequencing the entire transcriptome. In this way, we obtained a large data set representing sequences of actively expressed genes. The challenging task is now the informed selection of interesting *P. pachyrhizi* genes which are crucial for successful infection amongst a group of genes which are not involved in pathogenicity.

2. The pathogen

The development of novel plant protection strategies is intrinsically tied to an in-depth understanding of the prerequisites of both interaction partners such that disease becomes established. This includes detailed knowledge of the life-style and the infection mechanism of a given pathogen as well as the defense reactions displayed by the host. The next paragraphs document the current status of knowledge in these areas.

2.1 Pathogen life-cycle and host range

Rust fungi belong to a group of fungi called basidiomycetes. They differ from other groups of fungi because the cells in most stages of the life-cycle contain two separate haploid nuclei, and the mycelium is referred to as dikaryotic. Rust fungi of different species cause disease on several host plants but all of them have a so-called obliagtely biotrophic life-style in common; which means that these pathogens can grow and multiply solely on living host tissue. However, it has to be mentioned that after intensive efforts it is now possible to grow some rust fungi in axenic culture apart from the host plant. However, this has so far not been achieved successfully for the Asian soybean rust fungus *P. pachyrhizi*.

Infection of soybean plants by *P. pachyrhizi* starts with the germination of a uredospore on the leaf surface (Fig. 2). Growth of the germ tube is terminated by the formation of a specialized, globose infection structure called an appressorium. The appressorium is separated from the germ tube by a septum. Since germination and appressorium formation also occur on water agar plates and artificial membranes it seems that both processes do not involve plant-derived signals (Koch and Hoppe, 1988). In these experiments a direct correlation was found between the frequency of appressorium formation and the pore size of the artificial membranes, which suggested the involvement of thigmo- rather than chemodifferentiation (Staples and Macko, 1994; Koch and Hoppe, 1988).

Penetration of *P. pachyrhizi* into host epidermal cells is initiated within the appressorium by building of an internal structure called the appressorial cone which then elongates into the penetration peg (Bromfield, 1984). Direct penetration of the host cuticle is a special feature of the Asian soybean rust, as opposed to stomatal penetration which is usual for the majority of other rusts. Epidermal cells of host plants undergo cell death after penetration by

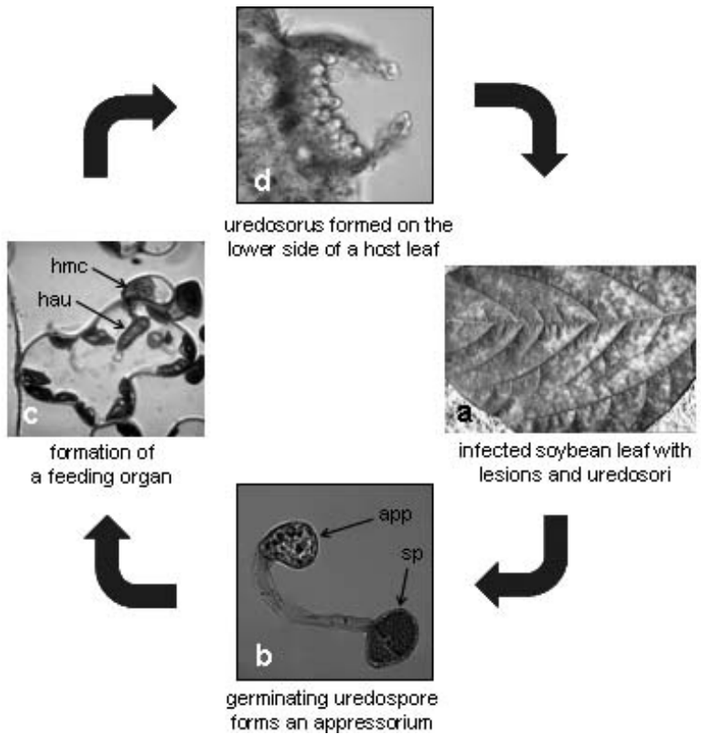

Fig. 2. Life-cycle of *P. pachyrhizi* on soybean. a) Uredospores of *P. pachyrhizi* are released from infected leaves and dispersed by wind. b) Upon contact with suitable surfaces, a uredospore germinates and forms an appressorium. c) Tissue of infected leaves is heavily colonized by fungal hyphae which give rise to haustorial mother cells (hmc) from which haustoria (hau) are inserted into host mesophyll cells. d) Asexual reproduction of *P. pachyrhizi* is completed by generating novel uredospores in uredosori. sp, uredospore; app, appressorium; hau, haustorium; hmc, haustorial mother cell; modified from (Goellner et al., 2010).

P. pachyrhizi; a feature which is generally uncommon among biotrophic plant pathogens in general and particularly in rust diseases (Keogh et al., 1980). However, penetration hyphae do not appear to be affected by this cell death event and grow on, branching after they reach the mesophyll and differentiating haustorial mother cells (Koch et al., 1983). The latter give rise to the formation of specialized infection structures called haustoria, which are the essential feeding organs of the pathogen. Haustoria are common among all obligate biotrophic fungal plant pathogens and failure to produce them is a knock-out criterion for infection by these pathogens. Once haustoria are successfully established the fungal mycelium intensively colonizes the host tissue and the production of new uredospores completes the pathogens life-cycle (Koch et al., 1983).

Worthy of special note is the broad host range of the Asian soybean rust fungus, a feature which is uncommon among the majority of other rusts. Actually, it has been documented that *P. pachyrhizi* is able to infect and sporulate on 31 different species in 17 different genera of leguminous plants (Ono et al., 1992). Using artificial inoculation methods under laboratory conditions this range of host plants can even be expanded. However the increased time span needed to sporulate on the latter plants in comparison to *P. pachyrhizi's* intrinsic soybean host indicates a lower degree of compatibility.

2.2 *P. pachyrhizi's* journey around the world

The degree of attention which was directed towards the Asian soybean rust after invasion of the continental US has been nearly unprecedented for an emerging plant healt problem (Stokstad, 2004). Cultivation of soybean is proposed to have started in the eastern half of northern China in the 11th century B.C. and soybean-associated diseases spread together with its propagation range throughout the eastern hemisphere. Hence, Asian soybean rust appeared in Australia in 1934 and in South America in 1976 (Vakili and Bromfield, 1976; Bromfield, 1984). It has to be noted that this first outbreak of the disease in the New World was due to the less aggressive Asian soybean rust isolate *Phakopsora meibomiae*, which is referred to as the Latin-American isolate (Bonde et al., 2006). Not until 2001 was the more aggressive Asian-Australian pathogen species *P. pachyrhizi* found on the American continent (Freire et al., 2008). After a first report from Paraguay, the pathogen spread to Bolivia, Brazil and Argentina. Possibly with tropical storms the pathogen spread north to the continental US but, due to its inability to overwinter, it was not able to cause great yield losses so far (Isard et al., 2005; Christiano and Scherm, 2007). Because uredospores are not frost-tolerant and the more robust teliospores have never been found for this pathogen, inoculum has to overwinter in the more temperate South during the winter. At this soybean-free time of the year alternative host plants such as the immigrant weed kudzu (*Pueraria montana*) can serve as a potential host. This alternation between domesticated and wild host species creates the source for initial infection at the beginning of the each soybean growing season (Fabiszewski et al., 2010).

3. The plant

Although plants are continuously exposed to pathogenic microorganisms disease seems to be an exception. It must be concluded, therefore, that plants have evolved efficient strategies to avoid disease. Since a constant activation of metabolic responses such as defense when they are not needed has high fitness-costs (Heil and Baldwin, 2002), most of the defense reactions rely on an inducible system (Jones and Dangl, 2006). On the other hand, pathogens have evolved mechanisms to circumvent recognition in order to successfully establish disease or, to actively suppress the execution of downstream signal networks involved in plant defense (Dodds and Rathjen, 2010). The following paragraphs will give a short introductory overview on the defense reactions displayed by soybean upon attack by the Asian soybean rust and on actual strategies pursued to combat the threat.

3.1 Breeding for disease resistance and other disease management strategies

Firstly, it has to be noted that none of today's soybean cultivars express resistance against all known races of *P. pachyrhizi*. Thus, it has to be kept in mind that at present, protection of planted soybean against the Asian soybean rust is incomplete and yield losses have to be expected in cases where virulent races of the pathogen encounter soybean communities.

So far five resistance genes, referred to as *Rpp1* to *Rpp5*, have been identified, each conferring resistance against a particular race of *P. pachyrhizi* (Bromfield and Hartwig, 1980; Hartwig and Bromfield, 1983; Bromfield, 1984; Morceli et al., 2008). These resistance genes have been identified genetically but not yet cloned. Screening for resistance against *P. pachyrhizi* is generally done using three different infection phenotypes: the highest degree of protection was observed in the so-called "immune" response where no disease symptoms were found on soybean leaves upon inoculation with the pathogen (Bromfield, 1984; Pham

et al., 2009). By contrast, susceptible interactions are characterized by tan-colored lesions, referred to as "TAN"-type, in which 2-5 uredia (syn. uredosori) are produced on average. An intermediate resistance phenotype occurred on plants showing reddish-brown lesion ("RB"-type) with little or no sporulation (Bromfield, 1984; Pham et al., 2009). Break-down of resistance against *P. pachyrhizi* conferred by single dominantly-inherited genes is well known due to the capacity of the fungus to form new races (Bromfield, 1984). A strategy to circumvent this problem could be the pyramiding of different resistance genes in a single cultivar, making it much more complicated for a pathogen to overcome resistance, or by incorporating novel sources of resistance. Thus, close relatives of *Glycine max*, e.g. *G. sojae* and other *Glycine spp.*, have been screened for resistance against *P. pachyrhizi* (Hartman, 1992; Hartman et al., 2005). Another approach has aimed at characterizing the spectrum of resistance in *P. pachyrizi's* alternate host kudzu (Jordan et al., 2010). So far, none of these approaches has led to the generation and field release of novel soybean cultivars.

The introgression of known resistance genes into the genetic background of other cultivars can be assisted by the availability of associated molecular markers (Babu et al., 2004). Efforts towards this goal have been made in soybean by mapping the genomic location of the *P. pachyrhizi* resistance genes (*Rpp1-Rpp5*) to molecular linkage groups (Hyten et al., 2007; Monteros et al., 2007; Garcia et al., 2008; Silva et al., 2008). These markers could e.g. be used to determine whether resistance genes of newly identified soybean accessions with resistance against *P. pachyrhizi* map to already known loci (Ray et al., 2009).

Asian soybean rust could also be kept in check using fungicide treatments. Yield losses could be avoided by prophylactic fungicide treatments between the early flowering (R1) and seed filling (R5) stages if the pathogen is around (Doerge and Trybom, 2008). The current practice for controlling the disease with chemicals in the U.S. includes a combination of timely observation and application of appropriate fungicides. This had kept soybean yield losses to a minimum even in 2007 which was a year of great expansion of Asian soybean rust occurrence in the U.S. (Doerge and Trybom, 2008). Past experience has told us that resistance against fungicides may develop (Steffens et al., 1996; van den Bosch and Gilligan, 2008) and the risk is minimized by using mixtures of chemicals belonging to different classes or chemicals with different modes of action. Presently, strobilurins and triazoles are used effectively to manage the disease. However, additional fungicides are needed since some had only emergency authorization for use in the U.S. (section 18 labels).

Besides chemical control of Asian soybean rust, it might also be possible to use biological control agents. Thus, it was reported that uredospores of *P. pachyrhizi* are colonized by fungal hyperparasites such as *Verticillium psalliote* (Saksirirat and Hoppe, 1990, 1991). However these data have been generated under laboratory conditions and field test have not been performed yet. It seems questionable, therefore, whether biological control could substitute chemical disease control or substantially contribute to its effectiveness (Goellner et al., 2010).

Since the source for the initial *P. pachyrhizi* inoculum at the start of the growing season comes from the immigrant weed kudzu, reducing the density of this weed host may help. However, due to the wide range of kudzu this seems not to be feasible. On the contrary, by using modelling of pathogen spillover between soybean and kudzu it turned out that applying efficient disease management for the soybean host will reduce infections on the wild host species (Fabiszewski et al., 2010). Currently, none of the discussed strategies are successful on their own in controlling Asian soybean rust and it seems that a combination of different approaches is most promising.

4. Results

In this section some results from our lab showing novel insights into plant defense mechanisms which are efficient in restriction of the Asian soybean rust disease are presented. Firstly, we employed the model plant species Arabidopsis *thaliana* which did not show any disease symptoms after inoculation with *P. pachyrhizi* and secondly, we isolated fungal haustoria from *P. pachyrhizi* to learn more about the cross-talk between pathogen and host.

4.1 Exploiting a model plant species reveals novel defense traits against *P. pachyrhizi*

Without doubt Arabidopsis is currently the best-understood plant species and consequently large tool boxes are available for forward and reverse genetic approaches. Therefore, it was not astonishing that plant pathologists also started to investigate Arabidopsis-pathogen interactions, including ASR. As described above, nonhost resistance is displayed by a plant species against pathogens from other host plants and it is believed that this type of resistance is conditioned multifactorially and emerged in the course of evolution.

We have tested a range of different Arabidopsis ecotypes collected all around the world for their response to inoculation with *P. pachyrhizi* and none of them showed disease symptoms or became infected (Loehrer et al., 2008). This result was taken as an indication that Arabidopsis and the Asian soybean rust indeed present a nonhost type of interaction. Obviously, Arabidopsis must have defense strategies which protect it effectively against this pathogen. To get further insights into the underlying mechanisms, we made use of a collection of Arabidopsis mutant plants which previously had been shown to be compromised against another nonhost pathogen of Arabidopsis, i.e. barley powdery mildew. These mutants are collectively called *pen*-mutants (penetration) and, so far, encompass three different genetic loci. While inoculation of *pen1-1* and *pen 2-1* mutants with *P. pachyrhizi* did not result in disease symptoms, pronounced necrotic spots were found on leaves of *pen3-1* mutant plants (Loehrer et al., 2008).

We further analysed the interaction of the *pen*-mutants with *P. pachyrhizi* by microscopy. Interestingly, we observed that *P. pachyrhizi* was able to invade epidermal cells on both Arabidopsis wild-type and mutant plants. But the striking difference was that the fungus was able to invade the mesophyll solely on Arabidopsis *pen* mutants (Fig. 3).

To further underpin this observation a quantitative assessment of *P. pachyrhizi* infection sites was done for each genotype. Therefore, cellular interaction phenotypes were grouped into five different categories each representing a typical fungal developmental stage and the absence or presence of a corresponding plant defence reaction (Fig. 4). At least 100 infection sites were inspected and categorized per infected leaf and for statistical reasons three leaves from different plants were analysed for each plant genotype. Importantly, we observed that in plants without a *pen*-mutation the fungus was not able to develop substantially behind the border between epidermal and mesophyll tissue. However, penetration of the epidermis was found regularly. In contrast, in *pen*-mutants the pathogen always invaded the mesophyll and the strongest impact on mesophyll cell collapse was found on Arabidopsis plants carrying the *pen3-1* mutation (Fig. 4).

We further investigated the requirements of nonhost resistance in Arabidopsis against *P. pachyrhizi* by analyzing Arabidopsis mutants carrying mutations not only in genes associated with penetration defense but also in post-penetration resistance mechanisms. Among other mutants, we identified *pen2-1pad4-1* which showed a previously unseen degree of compatibility towards *P. pachyrhizi* as indicated by the formation of haustoria-like

structures inside mesophyll cells. Hence, it must be concluded that the pathogen had overcome a further barrier of nonhost resistance, i.e. penetration resistance of mesophyll cells, in this mutant (Fig. 5). The haustoria of biotrophic fungi are essential feeding organs which are indispensable for continuation of their life-cycle beyond exhaustion of conidial resources. Successful formation of the haustorial stage is therefore thought to play a key function in establishment of biotrophy and is a prerequisite for the completion of the life-cycle by release of a new spore generation. However, it must be stated that in Arabidopsis *pen2-1pad4-1* mutants, irrespective of the initiation of haustorium formation, sporulation of *P. pachyrhizi* was not observed.

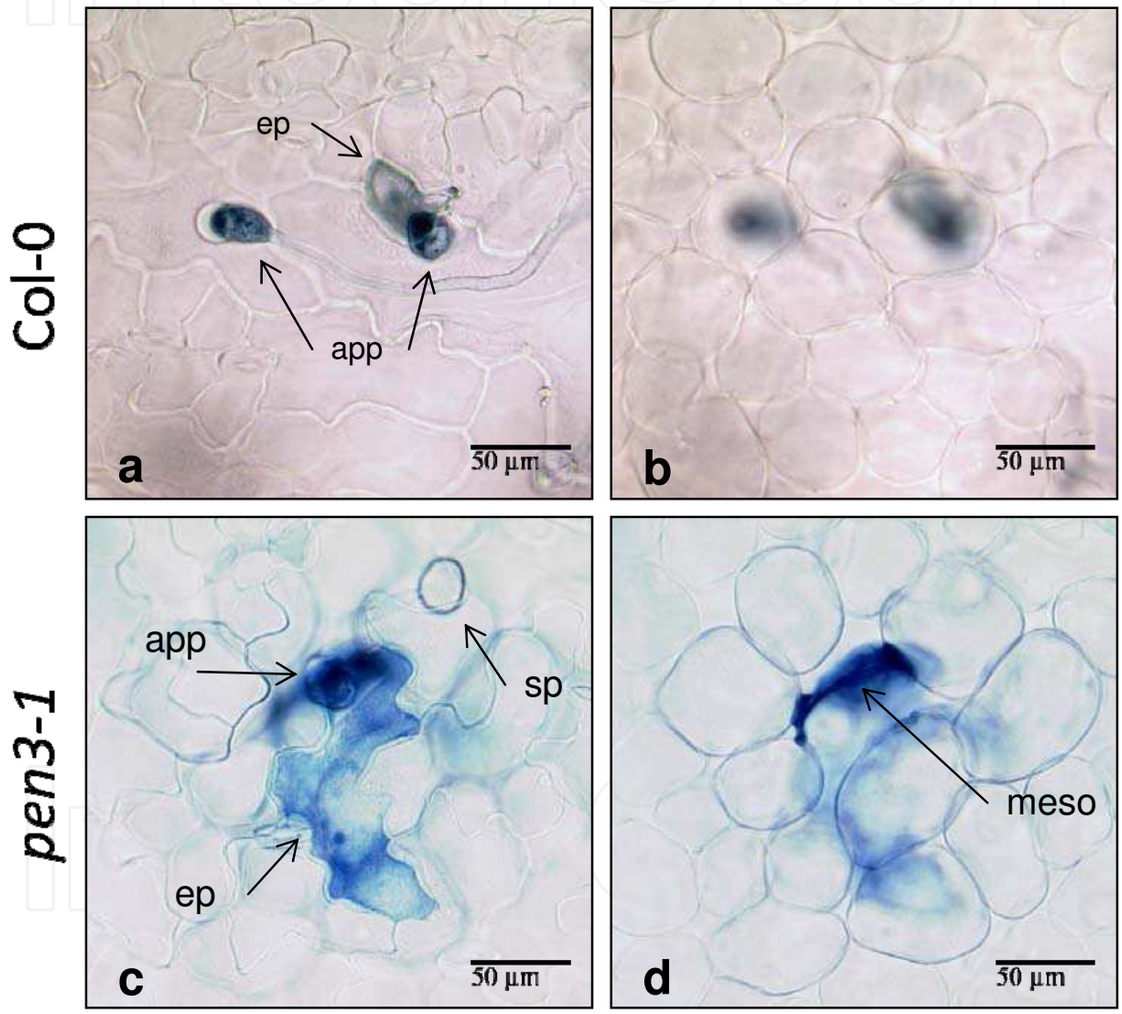

Fig. 3. Interaction sites between *P. pachyrhizi* and *Arabidopsis thaliana* at the cellular level. Leaves of Arabidopsis wild-type (Col-0) and mutant (*pen3-1*) plants were harvested 1 day after inoculation with uredospores of *P. pachyrhizi* and stained with trypan blue. Bright-field microscopy was done using a Leica DMR microscope. For each genotype, optical sections were recorded focussing on the epidermal cell layer (a and c) or on the mesophyll tissue (c and d). app, appressorium; ep, trypan blue-stained epidermal cell; meso, collapsed mesophyll cell stained with trypan blue; sp, uredospore

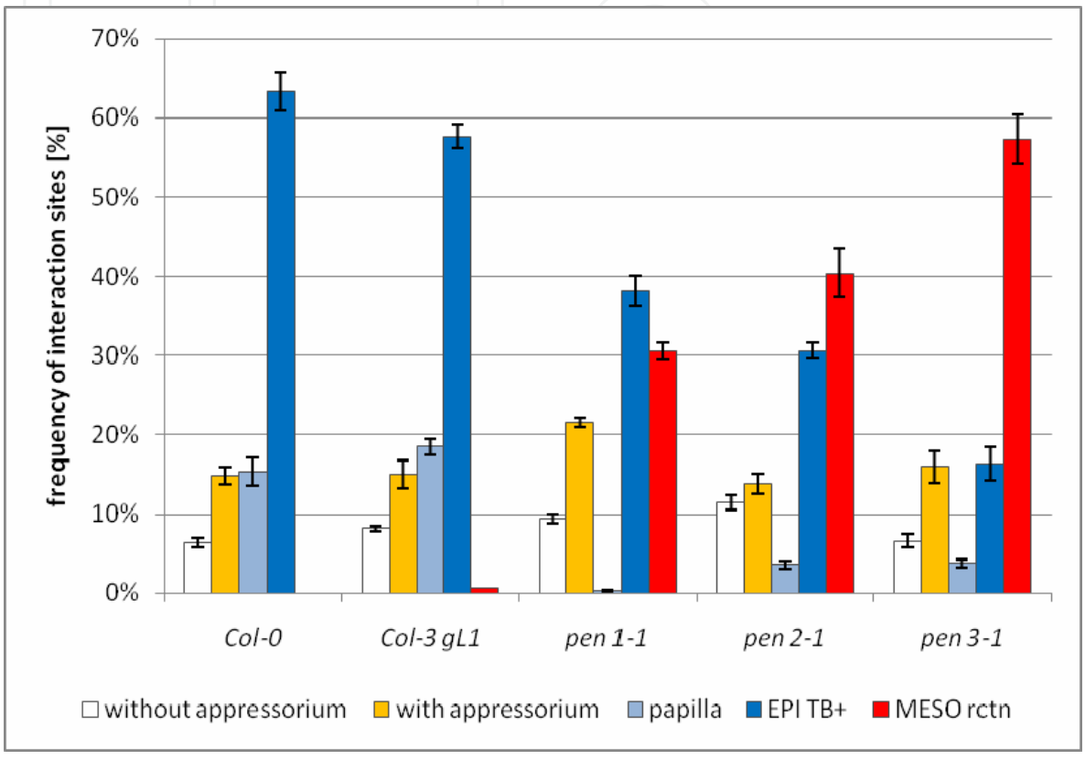

Fig. 4. Quantitative microscopic evaluation of infection sites of *P. pachyrhizi* on leaves of *Arabidopsis thaliana*. Different leaves of Arabidopsis wild-type and mutant plants were harvested 4 days post inoculation with uredospores of *P. pachyrhizi* and stained with trypan blue. Each interaction site was grouped into a particular category: the first comprises spores that had germinated but did not develop appressoria by the time of analysis (without appressorium); the second class encompasses germinated spores with an appressorium (with appressorium); the third category describes infection sites where fungal penetration is countered by the formation of a papilla (papilla); the fourth class represents infection sites with penetrated epidermal cells that retain the trypan blue stain (EPI TB+) and the fifth category comprises infection sites at which the fungus had proceed into the mesophyll tissue and caused cell collapse (MESO rctn). At least 4 leaves have been analysed per genotype and approximately 100 interaction sites were inspected per leaf. Frequencies are given as mean and standard error. Modified after (Loehrer et al., 2008).

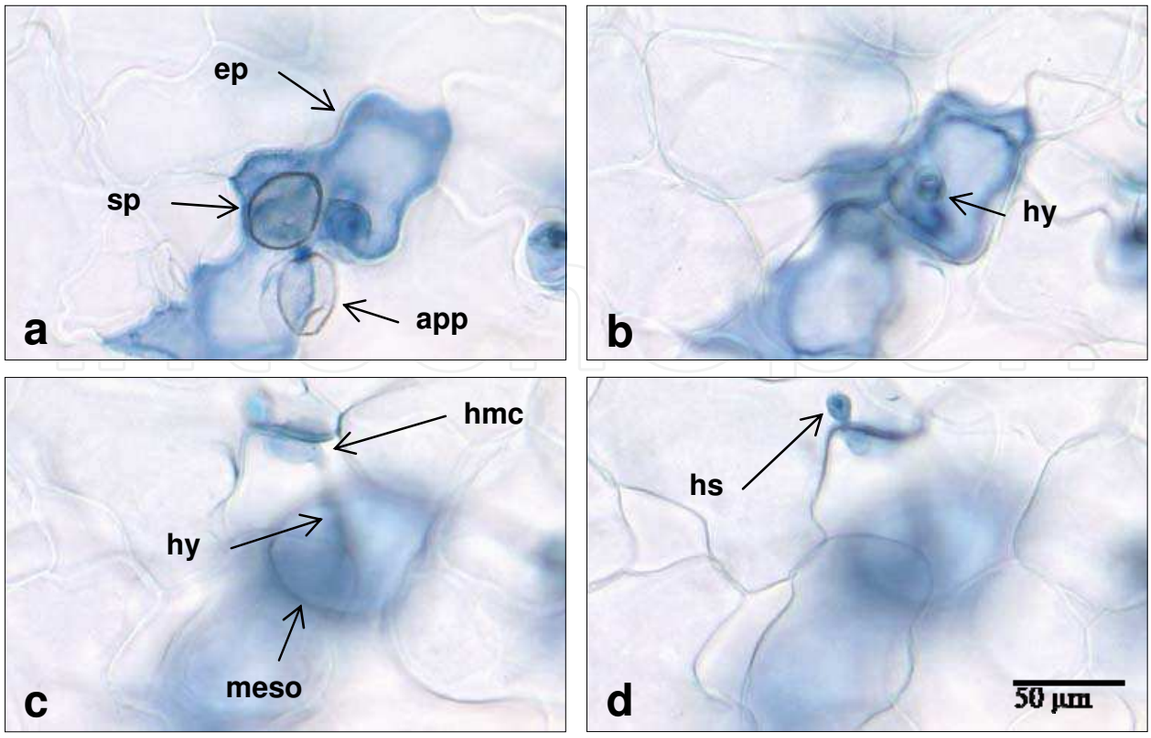

Fig. 5. Impaired penetration and post-penetration resistance mechanisms gave rise to the formation of a haustorium-like structure. Pictures a-d display optical sections at a particular infection site of *P. pachyrhizi* on an Arabidopsis *pen2-1pad4-1* mutant plant. Bright-field micrographs were taken by step-wise focusing on: (a) a fungal uredospore (sp) and the respective appressorium (app), (b) an epidermal cell (ep) penetrated by a hypha (hy), (c) a fungal infection hypha growing in the intercellular space between mesophyll cells and form a haustorium mother cell (hmc) and (d) a haustorium-like structure (hs) build inside a mesophyll cell.

4.2 Analysis of the fungal-plant interactome to unravel *P. pachyrhizi's* Achilles' heel

As discussed in the previous chapters, the successful initiation of the haustorial stage is a crucial event in the life-cycle of biotrophic fungal pathogens. During this process *P. pachyrhizi* penetrates cell walls of host mesophyll cells and invaginates their plasmamembrane without rupture. This gives rise to a double-layered membrane-bound compartment representing a venue of intimate contact between host and pathogen. Crosstalk, that is interchange of signal molecules across this compartment, is likely and knowledge of the interacting molecules might help to understand the basis of the disease.

Aiming at the identification of signal molecules which are released from *P. pachyrhizi's* haustoria, we first started to prepare intact haustoria from infected soybean leaves using a protocol established by Hahn and Mendgen (1992). This procedure exploits surface properties of rust haustoria which permits them to bind to the lectin Concanavalin A (Con A). The protocol depicted in Fig. 6 was established for *P. pachyrhizi* at the University of Konstanz in close cooperation with the group of Prof. R. Vögele. Each round of extraction ended with a small fraction containing the desired haustoria. These final fractions were rarely contaminated with chloroplasts whereas amyloplasts were found more frequently (Fig. 7).

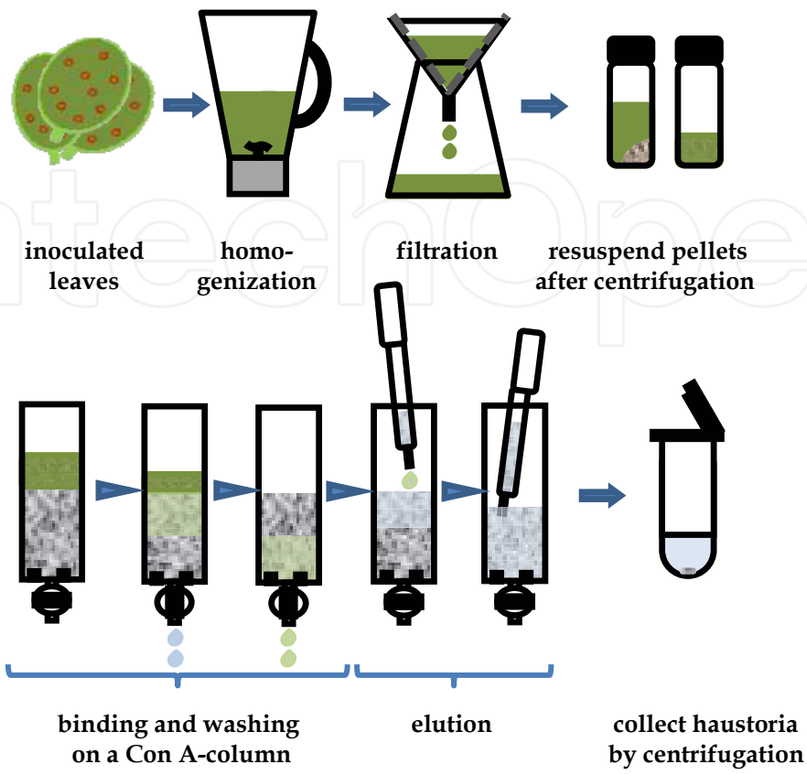

Fig. 6. Flow-chart of haustoria isolation using Con A-affinity chromatography.
Soybean leaves showing severe disease symptoms 8 to 9 days after inoculation were homogenized and filtrated through nylon gauze. After centrifugation the pellet containing the haustoria was resuspended in binding buffer and transferred onto an equilibrated Con A-sepharose column. After binding of the haustoria to Con A, the column was washed several times with fresh buffer to elute remaining cell debris. Afterwards, haustoria were mechanically eluted from the column by strongly agitating the matrix using a pipette. The supernatant containing the haustorial fraction was then transferred to a microcentrifuge tube for further processing.

Several rounds of chromatography were needed to collect sufficient haustorial tissue for down-stream processing. Predicting that genes encoding proteins necessary for the establishment of haustoria are under transcriptional control, we sequenced the whole haustorial transcriptome (Loehrer and Schaffrath, unpublished). Finally, we ended up with 111,440 *de-novo* assembled contigs from which 50 candidate genes were chosen based on structural features commonly found within known or predicted fungal effector genes (Ellis et al., 2007; Ellis et al., 2009). The functional testing of our candidate genes for an involvement in haustorium initiation requires the transformation of the pathogen. However, transformation of biotrophic fungi in general, and rust fungi in particular, is still a challenging task (Gregory et al., 2010). Efforts towards the implementation of a test system for gene function in *P. pachyrhizi* using different experimental set-up are in progress in our lab.

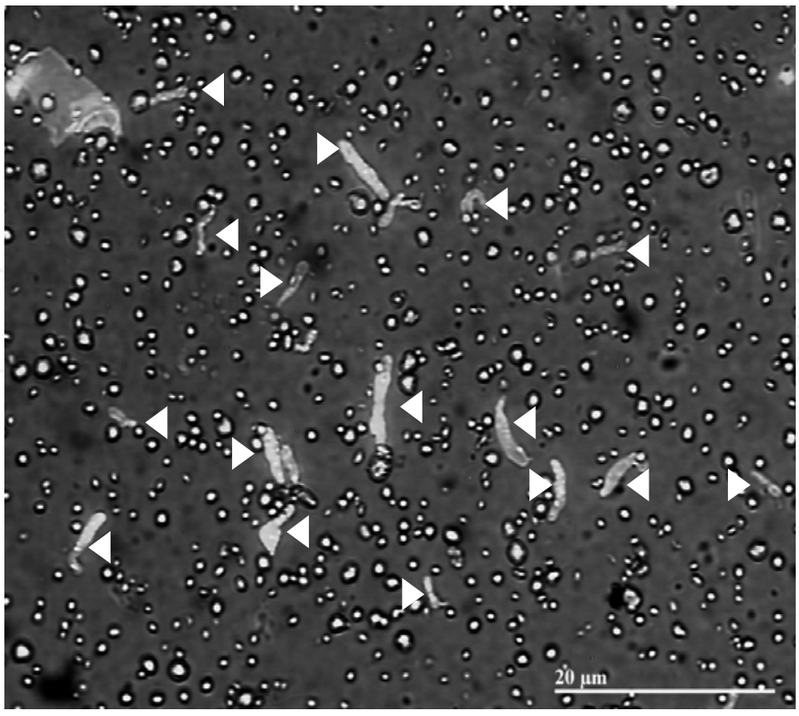

Fig. 7. Fraction enriched with haustoria of *P. pachyrhizi*
Haustoria were isolated form soybean leaves heavily infected with the Asian soybean rust pathogen *P. pachyrhizi* using Con A-affinity chromatography. Haustoria are labeled with white triangles. For details of purification please see text and Fig. 6.

4.3 Discussion

Undoubtedly, there is a constantly growing need for staple crops worldwide. Breeding for varieties with higher yield has contributed to solve this problem (Evenson and Gollin, 2003). Another promising perspective to meet this challenging task is the improved protection of plants against yield losses due to pests and diseases. Although considerable progress had been made towards this goal, the emergence of pathogens with novel virulence spectra or the invasion by previously inconspicuous pathogen species into new habitats bear novel problems which undermine these successes. A recent example for this scenario is the occurrence of a novel pathogen species causing Asian soybean rust (*Phakopsora pachyrhizi*, Asian-Australian type) across the American continent which is more aggressive than the endogenous Latin-American species *Phakopsora meibomiae*.

In an effort to identify novel sources for resistance against this pathogen we switched over to the plant model species *Arabidopsis thaliana* which shows complete resistance against the Asian soybean rust (Loehrer et al., 2008). This type of resistance is referred to as nonhost resistance and Arabidopsis is specifically suited to study this phenomenon because a whole range of different mutants is available which differ in their nonhost response to various pathogens (Thordal-Christensen, 2003). We investigated for the first time the interaction between Arabidopsis and *P. pachyrhizi* in our lab and we started, therefore, with a detailed cytological analysis of the fungal infection process on wild-type Arabidopsis plants. Doing so, we observed that the fungus was not only able to penetrate epidermal cell walls but also grew further to the border of the mesopyll tissue where the infection process came to an end

(Loehrer et al., 2008). Penetration of epidermal cells of nonhost plants was a known phenomenon for *P. pachyrhizi* (Hoppe and Koch, 1989). We further verified this observation using barley plants and, most importantly, we could show that the inhibition of cell death in epidermal tissue of barley limits penetration success of the pathogen (Hoefle et al., 2009). It must be concluded, therefore, that *P. pachyrhizi* did not attack plants like a blind battering ram but rather utilizing subtle methods to cause cell death which might contribute to successful infection by avoiding active defense reactions.

Looking at Arabidopsis mutant plants with impaired penetration resistance, we observed that after the breakdown of this first line of defense *P. pachyrhizi* was able to grow intercellularly into the mesophyll tissue and build haustorium mother cells (Loehrer et al., 2008). However, the pathogen was not able to invade mesophyll cells and establish haustorium-like structures until diminishing of a second line of defense in Arabidopsis *pen2-1pad4-1* double mutants. Conversely, it could be concluded that PEN2 and PAD4 proteins are important for keeping *P. pachyrhizi* in check, at least in Arabidopsis. Further experiments will reveal whether the same is true for homologous proteins in soybean. Our investigations with Arabidopsis and *P. pachyrhizi* led to a novel, rather unexpected, theory, namely that the pathogen 'hides' its bitrophic nature to force the ostensible host to invest in an inappropriate defense scenario. It appears as if *P. pachyrhizi* comes along as a 'wolf in sheep's clothing'. This hypothesis came from gene expression studies which showed that the biotrophic pathogen *P. pachyrhizi* triggers transcription of genes typically activated in Arabidopsis in response to necrotrophic pathogens (Loehrer et al., 2008). This activation, in turn, is associated with a shut-down in transcription of genes normally involved in defense against biotrophic pathogens due to a negative cross-talk of related signaling molecules (Spoel et al., 2003). Taken together, our experiments with Arabidopsis and *P. pachyrhizi* reveal the cell survival machinery and the artificial induction of genes associated with defense against biotropic fungi as potential targets to engineer durable resistance against this pathogen in soybean.

Having a closer look at the pathogen, the sequencing of *P. pachyrhizi's* haustorial transcriptom had provided us with a lot of necessary information to identify crucial steps for its pathogenicity. However a major drawback to further progresses is the current lack of whole genome information for *P. pachyrhizi* in public databases. This makes functional testing of candidate genes, despite the general problem with the set-up of transformation systems for biotrophic fungal plant pathogen, a very challenging task.

5. Conclusions and outlook

Our investigations have brought novel insights into our understanding of the interaction between soybean plants and the causal agent of Asian soybean rust, *P. pachyrhizi*. Using novel approaches, we have identified so far unnoticed plant traits which seemed to be crucial to resist the pathogen. The next critical and challenging task will be the implementation and incorporation of these features into elite soybean cultivars. It must be mentioned, however, that plant pathogen interactions are dynamic systems where modifications in one partner selects for a compensating reaction in the other partner, a scenario best documented in the evolutionary arms race between pathogen virulence factors and plant resistance genes. Therefore, we must be aware that the introduction of novel resistance traits into soybean using classical breeding programs or genetically modified plants might lead to counteraction by *P. pachyrhizi* to overcome these novel barriers. Thus, it

is also indispensable for a sustainable plant protection strategy to increase our knowledge on the requirements of *P. pachyrhizi* to act as a successful pathogen. The in depth understanding of its pathogenesis might help in anticipating potential future activities of the pathogen in response to the introduction of soybean varieties with novel traits. In sum, it is obvious that Asian soybean rust can only be conquered using a holistic approach including researchinto both the plant and the pathogen.

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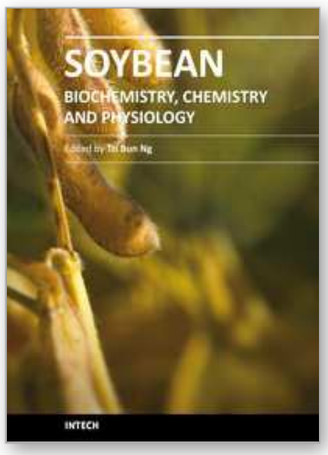

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