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Metabolism and Plant Hormone Action During Clubroot Disease

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Abstract Infection of Brassicaceae with the obligate biotrophic pathogen *Plasmodiophora brassicae* results in the development of root galls (clubroots). During the transformation of a healthy root to a root gall a plethora of changes in primary and secondary metabolism occur. The upper part of an infected plant is retarded in growth due to redirection of assimilates from the shoot to the root. In addition, changes in the levels of plant growth regulators, especially auxins and cytokinins, contribute to the hypertrophy of infected roots. Also, defense reactions are manipulated after inoculation of suitable host plants with *P. brassicae*. This review summarizes our current knowledge on the changes in these parameters. A model is presented for how primary metabolism and secondary metabolism, including plant hormones, interact to induce clubroot formation.

Keywords *Arabidopsis thaliana* · Brassicaceae · Clubroot · Disease · Gall formation · Metabolism · Plant hormones · *Plasmodiophora brassicae*

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Introduction

Clubroot disease is caused by the obligate biotrophic pathogen *Plasmodiophora brassicae* in the family Brassicaceae, including the model species *Arabidopsis thaliana*. After entering the root cortex, the pathogen causes the root to swell and thereby induces gall growth. During this process the biotrophic pathogen is dependent on nutrients from the host such as carbohydrates, amino acids, and lipids. The gall constitutes a strong metabolic sink that leads to growth inhibition of the green plant parts because fewer nutrients are available for the leaves and shoots. In the growing root gall the pathogen alters host metabolism as well, especially plant hormones such as auxins and cytokinins that are important for induction of cell division and cell elongation. Other hormones might be signaling molecules to prevent drought stress or are involved in defense regulation. These changes occur during the so-called secondary infection cycle, whereas changes during primary infection, or root hair infection, are more subtle. During this phase an initial growth promotion and swelling of root hairs is observed. At the cellular level, cell division and cell elongation are induced in a strictly limited number of epidermis cell clusters. Consequently, *de novo* meristematic areas are established. Whether, for example, plant hormones play a role in these initial steps has yet to be investigated. However, a root hair defective mutant *rhd3-1* showed tolerance to clubroot (Siemens and others 2002).

It is also clear that that there are differences in the reaction of various host plants within the Brassicacea to *P. brassicae* infection. Resistance responses in *A. thaliana* seem to differ from those seen in *Brassica* species (Kobelt and others 2000). Also, in the various host plants different pathways are used for plant hormone biosynthesis.

Examples for these variations during gall development are presented and their significance discussed.

Milestones for Clubroot Research: Transcriptome and Proteome Analysis

Since the discovery of *Arabidopsis thaliana* as an effective model host for *Plasmodiophora brassicae* (Koch and others 1991; Mithen and Magrath 1992), this plant has been used in different ways to advance our understanding of the events leading to clubroot formation. These include resistance screens (Fuchs and Sacristan 1996), mutant analyses (Siemens and others 2002; Alix and others 2007), generation of transgenic plants to evaluate single-gene effects (Siemens and others 2006), and recently the so-called -omics approaches. Because in the following sections two key "omics" experiments are referred to frequently, they are described in more detail here.

Using the Affymetrix ATH1 full-genome chip, Siemens and others (2006) examined changes in gene expression in healthy and clubroot-infected *A. thaliana* root tissue at two different points in time. The first time point chosen (TP1) was 10 days after inoculation (dai) because at this time the pathogen had already fully colonized the tissue but no symptoms were yet visible. The second time point (TP2) was at 23 dai, where all developmental stages of the pathogen (young, secondary and sporulating plasmodia, resting spores) were present and root galls had developed. The host tissue showed pronounced cell divisions and also colonized cells were hypertrophied. Several general conclusions were drawn from this experiment. At TP1 only a few pathways seemed to be differentially regulated, including starch and sulfur (S) metabolism. At TP2 the photosynthetic apparatus was dramatically affected. This is due to the formation of galls in the hypocotyl that are above the soil and contain chlorophyll. In addition, starch, lipids, and secondary metabolism, especially flavonoids, seem to be upregulated. Second, major transport processes were differentially regulated between the two times. At TP1 few transport processes were differentially regulated, while at TP2 several were increased, including sugars, lipids, ions, and other nutrients (N, S, P). This indicates high reserve accumulation in infected roots during TP2. The genes induced more than 20-fold from infected roots at TP1 to TP2 included many genes associated with reserve accumulation (for example, lipid transfer proteins, starch synthesis), as well as dehydrins/LEA proteins which may indicate a stress response of the infected roots at later time points. In contrast, strongly downregulated genes in infected roots at TP2 compared with TP1 included several defense-related proteins. Consistent with the observation that *P. brassicae* can live inside its host for a long period without affecting vitality, the majority of annotated resistance and defense genes, however, were not differentially expressed in clubs. Finally, genes involved in cell division and elongation were strongly upregulated. Comparing the compatible interaction of *A. thaliana* ecotype Columbia and *P. brassicae* isolate 'e' with the resistant interaction of ecotype Tsu and the same isolate of the pathogen by microarray analysis revealed the upregulation of two families of transcription factors (Rehn and Siemens, personal communication).

Up to now only two proteomics articles on clubroot have been published; the first focused on changes in protein composition in *A. thaliana* 4 dai (Devos and others 2006), whereas the second analyzed changes in protein composition in *Brassica napus* during the very early events of the infection process: 0.5, 1, 2, and 3 dai (Cao and others 2008). Both studies performed a differential protein analysis of infected versus noninfected roots and hypocotyls using two-dimensional gel electrophoresis and quantitative image analysis, coupled to MALDI TOF-TOF (matrix-assisted laser desorption/ionization time-of-flight) mass spectrometry-based protein identification. In *A. thaliana*, 12% of the visualized proteins (corresponding to 46 proteins) showed altered abundance compared with the noninfected plants, including proteins involved in metabolism, energy metabolism, cell rescue and defense, cytokinin metabolism, cell differentiation, cytoskeleton, detoxification of reactive oxygen species (ROS), signaling, and cellular transport (Devos and others 2006). In *B. napus*, 20 differentially displayed proteins were characterized, including proteins involved in lignin biosynthesis, cytokinin metabolism, glycolysis, intracellular calcium homeostasis, and detoxification of ROS.

Primary Metabolism

During gall formation *P. brassicae* is dependent on the host for its nutrient requirements and several studies have demonstrated altered carbohydrate metabolism and partitioning in infected plants. Evans and Scholes (1995) showed that sucrose accumulated in uninfected leaves of *A. thaliana* plants during daylight but not in leaves of *P. brassicae*-infected plants, suggesting that in infected plants sucrose produced during the day was either metabolized rapidly or exported from the leaf. The amount of starch in leaves of infected plants was also reduced compared with control leaves, implying that the demand for carbohydrates exceeded their rate of production. These results are consistent with the fact that the rate of photosynthesis of clubroot-infected *A. thaliana* and *Brassica rapa* (syn. *campestris*) plants was similar to or, at later stages of infection, lower than that of control plants (Evans

and Scholes 1995) and that there was an increased rate of translocation of $^{14}CO_2$ photosynthates from the leaves to the galls/roots of infected *B. rapa* plants when compared with controls (Mitchell and Rice 1979). Both soluble sugars (hexoses and sucrose) and starch accumulated in the hypocotyls/galls of infected *A. thaliana* (Mithen and Magrath 1992; Evans and Scholes 1995; Brodmann and others 2002) and *B. rapa* plants (Keen and Williams 1969) which is consistent with an upregulation of the expression of sucrose (sucrose synthase) and starch (starch synthase) biosynthetic genes (Siemens and others 2006). Interestingly, the large subunit of ribulose bisphosphate carboxylase was upregulated during *P. brassicae* infection (Devos and others 2006). This upregulation confirmed previous transcriptome data where genes involved in photosynthesis were also found to be upregulated in galls (Siemens and others 2006). The authors attributed this upregulation to the greening of the hypocotyl part of galls; however, it also could be a result of the strong sink created by cytokinins (Devos and others 2006).

The nonreducing disaccharide sugar trehalose (α-D-glucopyranosyl-[1, 1]-α-D-glucopyranoside) is commonly found in bacteria and fungi (Elbein 1974; Müller and others 1995) and also accumulates in the galls and roots of *P. brassicae*-infected plants as disease develops (Keen and Williams 1969; Brodmann and others 2002). Trehalose accumulation in plants can lead to an increase in starch synthesis and other perturbations in sugar-sensing and -signaling pathways (Avonce and others 2006; Lunn and others 2006; Lunn 2007; Paul 2007). For example, when *A. thaliana* plants were grown on a medium containing trehalose, there was an inhibition in root growth and an accumulation of starch in the leaves. These changes were accompanied by increased expression of the gene encoding ADP glucose pyrophosphorylase (*ApL3*) and increased enzyme activity (Wingler and others 2000; Fritzius and others 2001) in the leaves. Accumulated trehalose in *P. brassicae*-infected tissue is likely to be located within plasmodia and resting spores as the pattern of accumulation correlated with the upregulation of a putative *P. brassicae* trehalose-6-phosphate synthase gene (*PbTPS1*) and not with that of *A. thaliana AtTPS1* gene, whose expression was constitutively low in both healthy and infected tissue (Brodmann and others 2002). To have an effect on the sugar metabolism of the plant, trehalose would need to be released from the pathogen at a concentration high enough to trigger the sugar-sensing pathways of the host. It is possible that trehalose is exported from *P. brassicae* or released as cells begin dying later in the disease cycle (Ludwig-Müller and others 1999b; Brodmann and others 2002). Current evidence suggests, however, that the accumulation of starch in roots and galls of clubroot-infected plants is not due to a trehalose-induced perturbation in host sugar-signaling pathways as there is little evidence for high accumulations of this molecule; plant trehalase activity (an enzyme that hydrolyzes trehalose to two molecules of glucose) was increased in *P. brassicae*-infected tissue compared with controls, whereas *ApL3* was not induced in the roots and hypocotyls of clubroot-infected plants (Brodmann and others 2002). The mechanism that underlies an accumulation of soluble sugars and starch in clubroot galls remains to be elucidated but may well be associated with increased cytokinin concentration in infected tissues (Devos and others 2005; Siemens and others 2006) as cytokinins enhance metabolic sinks and lead to increased starch synthesis (Roitsch and Ehneß 2000; Devos and others 2006; Siemens and others 2006).

Another energy-related protein that was downregulated during *P. brassicae* infection is fructose-bisphosphate aldolase (Devos and others 2006). The downregulation was interpreted as a shift toward the production of glucose. This correlates with the fact that galls of *P. brassicae* act as carbon sinks (Evans and Scholes 1995). A dehydrogenase (IAR4) was upregulated in the proteome analysis (Devos and others 2006) and also with *Agrobacterium tumefaciens* infection (Zimmermann and others 2004). The protein is possibly involved with the conversion of indole-3-pyruvate to indole-3-acetyl-coenzyme A, which is a potential precursor of IAA amino-conjugates (LeClere and others 2004). Therefore, it should be regarded as a protein with a dual function in hormone and energy metabolism (see subsection Regulation of Auxin Synthesis). Transcriptome data indicated, however, only weak upregulation of glycolysis and TCA cycle in general (Siemens and others 2006), whereas metabolism of sugar and starch was strongly upregulated when the galls were visible (see above).

Secondary Metabolism

Many different secondary metabolic pathways are upregulated following clubroot infection, as demonstrated by transcriptome analysis. In the following, selected pathways for the synthesis of secondary metabolites are described where additional experimental evidence for their relevance during club development is available.

Glucosinolates

Glucosinolates, a group of secondary plant products in the family Brassicaceae, have long been associated with clubroot disease symptoms. In general, glucosinolates (GSL) are regarded as defense compounds against generalist pathogens and as attractant for specialists, mostly insects (Halkier and Gershenzon 2006). The role of glucosinolates during clubroot disease has been reviewed

extensively by Ludwig-Müller (2009) and therefore only a short summary of the most important aspects is given here.

Glucosinolates can be divided into three groups according to their core structure (Halkier and Gershenzon 2006). The aliphatic GSLs are derived from methionine, the aromatic types from tyrosine and phenylalanine, and the indolic forms from tryptophan. The general pathways for GSL biosynthesis have been described elsewhere (Grubb and Abel 2006; Halkier and Gershenzon 2006). Aliphatic glucosinolates are regarded as defense compounds that act by releasing toxic thiocyanates and *iso*thiocyanates. Therefore, degradation of GSL in general might be an important feature during endogenous control of clubroot disease because myrosinase was differentially regulated when *A. thaliana* (Devos and others 2006; Siemens and others 2008) and *B. rapa* (Grsic and others 1999) roots were infected by the pathogen. Concomitant with the upregulation of myrosinases, a myrosinase-binding protein is upregulated during infection (Devos and others 2006). In addition, transcriptome and proteome experiments provide evidence for the involvement of genes from the glucosinolate pathway in gall formation in the model plant *A. thaliana* (Devos and others 2006; Siemens and others 2006; 2008; Ludwig-Müller 2009).

Glucosinolate levels were altered during clubroot disease in *B. rapa* and *A. thaliana*. For example, the total glucosinolate content in the roots of two susceptible cultivars was higher throughout the experimental period than in the roots of two resistant cultivars (Ludwig-Müller and others 1997). Although aliphatic GSLs were induced in the two susceptible cultivars compared with the resistant ones, the two resistant cultivars showed an increase in aromatic GSLs, possibly indicating a dual role for them. In *A. thaliana,* aliphatic and indole GSLs increased in galls compared with healthy roots (Ludwig-Müller and others 1999a). During an investigation of the host range of *P. brassicae* in various non-*Brassica* species, further evidence for the possible dual roles of aromatic GSLs during club formation was presented (Ludwig-Müller and others 1999b). In the glucosinolate-containing non-*Brassica* species *Tropaeolum majus* and *Carica papaya*, the concentrations of benzyl-GSL increased markedly in roots inoculated with *P. brassicae* compared with the controls. Small galls were observed in *T. majus* by Ludwig-Müller and others (1999b) who suggested that benzyl-GSL could act as a precursor for phenylacetic acid (PAA), which has auxin activity in *T. majus* (Ludwig-Müller and Cohen 2002). Also, *Lepidium sativum* (a member of Brassicaceae), which has a high benzyl-GSL content (Ludwig-Müller and others 1999b), formed clubs after *P. brassicae* infection (Butcher and others 1976) where phenylacetonitrile was detected, which would be the direct precursor of PAA. Analysis of *Brassica* cultivars as well as *A. thaliana* mutants provided correlative evidence between clubroot disease severity and indole glucosinolate content. The latter have been discussed as precursors for auxin biosynthesis. Because high auxin levels are associated with large root galls, indole glucosinolates could contribute directly or indirectly to disease development. Butcher and others (1974), Ockendon and Buczacki (1979), and Chong and others (1981, 1984) found correlations between resistance and low indole glucosinolate content in members of the Brassicaceae, whereas Mullin and others (1980) were not able to correlate the indole glucosinolate content and the resistance to clubroot. Ludwig-Müller and others (1997) found that two susceptible cultivars of *B. rapa* reacted with increased indole glucosinolates after infection with *P. brassicae*, whereas in two resistant cultivars no increase in indole glucosinolates was found.

Arabidopsis thaliana mutants that had been isolated on the basis of altered leaf GSL pattern (Haughn and others 1991) were used to investigate the relationship between aliphatic GSL content and *P. brassicae* infection (Ludwig-Müller and others 1999a). Two mutant lines with altered GSL content (Haughn and others 1991), *tu3* and *tu8*, were particularly useful for studying the involvement of glucosinolates and auxin in clubroot disease. Both showed reduced symptom development compared with the wild type, but in *tu8* indolic GSL decreased, whereas in *tu3* GSLs and auxin were unchanged compared with the wild type (Ludwig-Müller and others 1999a). Reduced symptom development was also accompanied by lower indole-3-acetonitrile (IAN) and indole-3-acetic acid (IAA) levels. The mutant *tu8* was identified as a heterochromatin-like protein 1 (Kim and others 2004; Bennett and others 2005) and, therefore, not directly connected to indole GSL metabolism. Mutants in the first steps of indole GSL biosynthesis are useful tools for investigating the direct role of these compounds in gall formation. The *cyp79b2/b3* double mutant is almost completely devoid of indole GSL (Zhao and others 2002). Surprisingly, the *cyp79b2/b3* double mutant also showed normal clubroot symptoms and the levels of free IAA in galls were comparable with wild types (Siemens and others 2008). It was concluded that a block in the early steps in indole GSL biosynthesis could be overcome in terms of IAA synthesis by other pathways such as the "YUCCA" pathway (Zhao and others 2001; see subsection Regulation of Auxin Synthesis) or by direct synthesis via indole-3-acetaldoxime (IAOx) and IAN (Nafisi and others 2007). Alterations in late steps cannot be compensated for, thus resulting in reduced gall size.

Camalexin

Other compounds related to the indole GSL pathway in *A. thaliana*, such as the phytoalexin camalexin (Glawischnig

2007), were induced during clubroot infection (Siemens and others 2008), possibly diverting the metabolic flow away from indole GSL. One protein in this pathway (CYP71A12/A13) could play a dual role in the formation of IAN, which is converted further either to IAA or to camalexin (Nafisi and others 2007). The gene encoding this protein was induced highly in root galls according to microarray analyses (Ludwig-Müller 2008). In contrast, the cytochrome P450 CYP71B15 (PAD3), which is involved in camalexin biosynthesis (Zhou and others 1999), is probably not directly involved in clubroot formation because the mutant *pad3* did not appear in tolerant phenotypes (Siemens and others 2002), even though the corresponding gene was upregulated during transcriptome analysis (Ludwig-Müller 2008; Siemens and others 2006, 2008).

Flavonoids

Flavonoids are a group of secondary products with many biological functions, including apparent roles in stress responses such as protection from UV radiation (Winkel-Shirley 2002). In addition, they are best known as the characteristic red, blue, and purple anthocyanin pigments of plant tissues that serve essential functions in plant reproduction by recruiting pollinators and seed dispersers (Winkel-Shirley 2001). Flavonoids are also thought to be involved in the establishment of plant-arbuscular mycorrhizal interactions (Vierheilig and Piché 2002; Scervino and others 2005). The phenylpropanoid pathway from which flavonoids are derived, however, has also been linked to plant defense (Dixon 2001; Ryder and others 1987). Some plant species synthesize 3-deoxyanthocyanins, which are involved in both defense (Snyder and Nicholson 1990) and pigmentation (Grotewold and others 1994).

The accumulation of three different flavonoids, naringenin, quercetin, and kaempferol, and their glycosides has been identified in developing root galls of *A. thaliana* after infection with *P. brassicae* (Päsold and Ludwig-Müller, unpublished results). Transcripts of selected genes involved in each step in the biosynthesis of flavonoids were also upregulated during disease development. However, transparent testa *tt3*, *tt4*, *tt5*, and *tt7* mutants did not show any tolerance to clubroot (Siemens and others 2002; Alix and others 2007). The possibility that flavonoids might influence auxin levels by regulating their efflux was investigated. In addition to synthesis, conjugation, and degradation, IAA levels are controlled by transport (Ljung and others 2002). Indole acetic acid is transported basipetally by a chemiosmotic polar transport mechanism that is regulated at the cellular level. The polarity of auxin transport is believed to be controlled by the localization of auxin transport proteins, with both putative efflux and influx carriers having asymmetric distributions (Geisler and Murphy 2006). Mutant analysis indicated an involvement of flavonoids in auxin transport. In a *transparent testa 4* (*tt4*) mutant, which is flavonoid deficient, IAA transport rates were increased and the pattern of IAA efflux carrier localization was altered (Buer and Muday 2004). The correct localization of IAA efflux carriers, and subsequently that of IAA distribution, was successfully restored by treatment with exogenous flavonoids, thereby proving their role in blocking polar auxin transport (Murphy and others 2000; Buer and Muday 2004). Some evidence has accumulated that flavonoids might act as auxin efflux modulators in clubroots, thereby contributing to increased auxin levels in galls (Päsold and Ludwig-Müller, unpublished results). Indeed, blocking IAA transport disturbs clubroot development (Devos and Prinsen 2006).

Polyamines

Polyamines are ubiquitous aliphatic cations that are involved in growth, cell proliferation, and differentiation (Thomas and Thomas 2001; Bais and Ravishankar 2002). It is interesting to note that polyamines and ethylene share a common intermediate during their biosynthesis (S-adenosyl-methionine). Whether they should also be considered plant hormones is a matter of debate because they are produced by almost all living organisms, including plants, animals, fungi, and bacteria.

It was suggested by Walters and Shuttleton (1985) that polyamines could be involved in club development. It was shown only recently, however, that polyamines may be involved in the regulation of gall formation in partially resistant interactions of the *A. thaliana* ecotype Bur-0 (Jubault and others 2008). At the transcriptional, enzymatic, and metabolic levels, polyamine metabolism and arginine catabolism were induced during the later stages of disease in compatible *A. thaliana-P. brassicae* interactions. Arginine catabolism, through arginase, which is strongly connected to polyamine metabolism, could play a role in responses to wound trauma and pathogen infection. Arginase induction could be a way of diverting nitrogen metabolism in favor of the pathogen. Susceptible and partially resistant plants showed strikingly different arginine metabolic patterns. Susceptible plants were characterized by transient agmatine production, massive induction of arginase, and strong accumulation of proline—with the latter metabolite possibly a protectant against drought stress. In contrast, partially resistant plants showed continuous agmatine production and weaker arginase pathway activity compared with susceptible genotypes. Results suggested that symptom severity was strongly associated with the differential regulation of root polyamine metabolism and arginine catabolism (Jubault

and others 2008). Walters and Shuttleton (1985) measured free polyamine levels in turnip (*Brassica rapa*) roots infected by *P. brassicae* and showed that putrescine, spermidine, and spermine concentrations were higher in regions of infection and gall formation than in noninfected roots or in regions of infected roots not exhibiting symptoms. In addition, in recent proteome approaches using early stages of infection of roots hairs of *B. rapa*, the upregulation of a protein involved in polyamine synthesis (spermidine synthase) has been reported (Cao and others 2008).

The Importance of the Plant Hormones Auxin and Cytokinin During Clubroot Disease

During primary infection by clubroot, initiation of cell division is observed from 4 dai and this continues during the secondary infection cycle. Later, large hypertrophied cells harboring secondary plasmodia and resting spores are observed. Similarly, cell elongation/expansion is induced at an early infection stage as indicated by xyloglucan endotransglucosylase/hydrolase (XTH) activity in epidermal cell clusters in root hair zones of infected *B. rapa* (Devos and others 2005). Plant hormones such as auxins and cytokinins have been associated with these phenomena (for example, Devos and others 2005, 2006; Siemens and others 2006). The availability of hormone-responsive reporter genes in *A. thaliana* permitted localization of enhanced auxin and cytokinin responsiveness, specifically in that part of the root where gall formation occurs (Devos and others 2006; Siemens and others 2006). The very first sign of an early cytokinin and auxin response started at 3 and 5 dai, respectively. The signals increased during subsequent gall development (Devos and others 2006). An increase in free and conjugated indole-3-acetic acid (IAA) levels was reported for *A. thaliana* and *B. rapa*, although at different times during infection, making comparisons difficult (Ludwig-Müller and others 1993, 1996, 1999a; Grsic and others 1999; Grsic-Rausch and others 2000; Devos and others 2005). It is not entirely clear, however, whether the pathogen or the host produces these 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).

Plasmodia Are a Sink for Auxins

Published data on the auxin content of roots during clubroot infection are often contradictory; both reduced and elevated IAA levels have been reported during clubroot formation (Raa 1971; Butcher and others 1974; Kavanagh and Williams 1981; Mousdale 1981; Ludwig-Müller and others 1993; Devos and Prinsen, unpublished data) irrespective of the phase in the disease cycle. Using plants containing the DR5::GUS reporter gene (Devos and others 2006) or in situ immunolocalization, *de novo* accumulation of IAA that occurred in specific epidermal cells and at a later stage of infection was localized in the galls of infected tissue. IAA accumulated gradually in the galls, with higher concentrations found at the borders of galls and lower concentrations near vascular tissue. The sites of immunoreactive response to IAA correlated with those of plasmodial development inside the gall (Devos and others, unpublished results), suggesting that plasmodia were accumulating IAA in a sink-dependent manner. Apparently, the IAA in plasmodia is difficult to extract from infected tissue. This together with the fact that the plasmodia act as a strong sink for auxins may explain some of the contradictory results present in the literature concerning concentrations of IAA extracted from clubroot tissue.

Regulation of Auxin Synthesis and Metabolism During Clubroot Development

For a recent review on auxin biosynthesis and metabolism, see, for example, Woodward and Bartel (2005). Microarray analysis (Siemens and others 2006) has revealed that several genes coding for proteins involved in the biosynthesis of indole GSL and IAA are differentially regulated during the development of the disease in *A. thaliana* (Ludwig-Müller 2008). It is noteworthy that only two early indole GSL biosynthetic genes (*CYP79B2* and *CYP79B3*; Hull and others 2000) that convert tryptophan to indole-3-acetaldoxime (IAOx) were upregulated at early stages, whereas genes operating later in the pathway, such as *SUR2* (Barlier and others 2000), *SUR1* (Mikkelsen and others 2004), and *UGT74B1* (Grubb and others 2004), were not differentially regulated or were downregulated (Ludwig-Müller 2008). Genes involved directly in IAA biosynthesis were upregulated later during the infection cycle, indicating that an increase in IAA via the indole GSL/nitrilase pathway is important only during the late stages of gall development.

The first enzyme required for the formation of indole-3-acetonitrile (IAN) from indole GSL is myrosinase. Although myrosinase genes were not upregulated in *A. thaliana* according to microarray analysis, proteome analysis found a myrosinase enzyme upregulated during early inoculation events (Devos and others 2006). In contrast, in *B. rapa*, the level of expression of myrosinase increased in infected roots compared with controls (Grsic and others 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 downregulation might not reflect reality *in planta*, where

maybe only a few infected cells contribute to IAN synthesis.

Indole acetonitrile can be converted to IAA by the enzyme nitrilase. In *A. thaliana* there is a family of four nitrilases; of these three are capable of converting IAN to IAA (Bartel and Fink 1994; Hillebrand and others 1998). Northern blot analysis, promoter-GUS studies (Grsic-Rausch and others 2000), and microarray analysis (Siemens and others 2006) showed that the expression of genes encoding nitrilases 1 and 2 increased in developing root galls during later stages of infection. Immunolocalization confirmed nitrilase protein in hypertrophied cells harboring sporulating plasmodia (Grsic-Rausch and others 2000), and this is concomitant with an immunolocalization of IAA in a similar location as reported above. Also, the mutant *nit1*, defective in the nitrilase 1 gene, produced smaller galls with fewer pathogen structures that were accompanied by reduced free IAA levels in clubroots (Grsic-Rausch and others 2000). Furthermore, a transgenic plant reduced in nitrilase 2 showed slower development of root galls, although overexpression of nitrilase did not result in altered symptoms after infection with *P. brassicae* (Neuhaus and others 2000). These data indicate that in *A. thaliana* nitrilase is associated with the late stages of clubroot development.

Early in vivo labeling studies showed that IAN is specifically converted to IAA from indole GSL in infected *B. rapa* roots, whereas wounding induced the formation of other metabolites (Rausch and others 1983). Also, increased nitrilase activity has been associated with increased IAA content and root gall development in several Brassicaceae (Rausch and others 1981; Grsic and others 1999; Ugajin and others 2003; Ishikawa and others 2007a). Northern blot analysis, however, did not show any differences in the mRNA levels of nitrilase in *B. rapa* (Chinese cabbage) during gall formation (Bischoff and others 1995), but the probes used in that study were not gene-specific and therefore might have missed subtle differences. This hypothesis is likely to be correct because Ishikawa and others (2007a) showed that in *B. rapa* (turnip) nitrilase was differentially expressed in clubroots. Recently, Ando and others (2008) reported that in *B. rapa* alternative nitrilase transcripts were observed only in clubbed roots, but the significance of this finding has yet to be elucidated. Both nitrilase 1 from turnip and Chinese cabbage convert IAN to IAA, but this is not the preferred substrate. Rather, phenylacetonitrile and phenylpropionitrile are mainly converted to the corresponding acid, indicating a possibly different or additional role for nitrilase in clubroot formation (Grsic and others 1998; Ishikawa and others 2007a).

Alternative pathways for the biosynthesis of IAA in root galls of *Brassica* species have been reported. For *B. rapa*, two different possibilities for the conversion of IAOx to IAA have been demonstrated at an enzymatic level. Ludwig-Müller and Hilgenberg (1990) found an activity capable of catalyzing the reaction from IAOx to IAN and Helmlinger and others (1987) described an activity converting IAOx to indole-3-acetaldehyde (IAAld). Both routes bypass indole GSL. The contribution of these pathways to IAA synthesis in clubroots, however, has not been investigated. Indole acetaldehyde can be further converted to IAA in *A. thaliana* by aldehyde oxidases (Seo and others 1998). Aldehyde oxidase was also induced in *B. rapa* clubroots (Ando and others 2006). By comparison, *A. thaliana* did not show such an induction according to microarray analysis. In *B. rapa*, in addition to nitrilase, the conversion of indole-3-acetamide to IAA, a pathway initially described for plant pathogenic microorganisms such as *Agrobacterium tumefaciens* (crown gall) (Inzé and others 1984), was increased in clubbed roots at an enzymatic level (Ishikawa and others 2007b). Amidase is also present in *A. thaliana* (Pollmann and others 2003) but does not contribute to IAA levels in root galls according to transcriptome data. Direct evidence for the contribution of other genes involved in auxin biosynthesis, such as the *YUCCA* family (Zhao and others 2001), has yet to be presented. Finally, the pathway via indole-3-pyruvic acid, which has been recently described in molecular terms (Stepanova and others 2008; Tao and others 2008), should be taken into account. The contribution of indole GSL to IAA biosynthesis and thus their (indole GSLs) contribution as possible links to gall size is only indirect, however, at least in *A. thaliana*. Indole acetonitrile as a precursor might be linked more directly to IAA, especially during the late stages of gall growth, whereas other pathways must be operating in earlier stages to contribute to an increase in IAA.

Another possible pathway for the biosynthesis of IAA would be the hydrolysis of free IAA from inactive auxin conjugates. First evidence for this was provided by Ludwig-Müller and others (1996) who described an activity capable of hydrolyzing several IAA amino acid conjugates from *B. rapa*. Interestingly, the substrate spectrum changed in infected roots compared with control roots and also seedlings. In the latter, mostly IAA-alanine was hydrolyzed by an extract from *B. rapa*, whereas the clubroots contained an activity that was capable of hydrolyzing IAA-aspartate, an IAA conjugate that has been attributed to degradation pathways in *A. thaliana*. Schuller and Ludwig-Müller (2006) cloned and characterized two auxin conjugate hydrolase genes from *B. rapa*. Although both hydrolases were slightly differentially regulated during gall formation, neither hydrolyzed IAA-aspartate. This leads to speculation that auxin conjugates with aspartate might be cleaved by the pathogen, although evidence has not been found so far. More evidence for possible involvement of auxin conjugates comes from proteome analysis where a

dehydrogenase (IAR4) is upregulated (Devos and others 2006). Besides its role in energy production, IAR4 is possibly active in converting indole-3-pyruvate to indole-3-acetyl-coenzyme A, which is potentially a precursor of IAA amino conjugates (LeClere and others 2004).

Finally, several members of the GH3 family of IAA amino acid conjugate synthetases from *A. thaliana* were upregulated during clubroot infection (Siemens and others 2006; Horn and Ludwig-Müller, unpublished results), particularly from 24 dai (Ludwig-Müller and others 1996). Devos and others (2006) found no altered levels of IAA conjugates at 4 dai but these metabolites start accumulating from 6 dai in *B. rapa* (Devos and others 2005) and *A. thaliana* (Devos and Prinsen, unpublished results). This high pool of IAA conjugates possibly results from an instant detoxification of potential excess of IAA in the infected roots. This might indicate an additional role for auxin conjugates during gall formation. Because some GH3 genes are auxin-inducible, they could additionally be involved in attempts by the host plant to regain regulation of auxin homeostasis. As mentioned above, the conjugates could be converted to free IAA by auxin conjugate hydrolases, either from the host plant or from the pathogen.

Auxin Transport

Developmental processes in the root clearly depend on auxin transport. In addition, IAA transport plays a crucial role in plant pathogenesis. In the presence of the auxin transport inhibitor 1-N-naphthylphthalamic acid (NPA), the number of *Picea abies* L. (Karst.) seedlings colonized by the fungus *Laccaria bicolor* S238 N (Maire) Orton was reduced. Also, lateral root formation, induced by *L. bicolor*, was partially reduced by NPA treatment (Rincón and others 2003). To verify the nature of the IAA that is present inside the galls, the IAA transport inhibitor NPA was added to *B. rapa* plants when moistening the soil immediately after infection (0 dai), after one single treatment at 6 dai, or after a single treatment at 13 dai. The disease indices at 21 dai were 78, 20, and 89%, respectively. Taking into account the resistance cutoff value of 20% or less (Toxopeus and others 1986), the plants treated with NPA at 6 dai may be considered resistant, whereas those plants transferred to a NPA solution at 0 and 13 dai show susceptibility to *P. brassicae* (Devos and Prinsen 2006; Devos and others, unpublished results). These experiments clearly show that polar auxin transport is crucial for early clubroot symptom development.

Proteins Involved in Signaling Pathways

During *P. brassicae* infection the 20S proteasome is downregulated (Devos and others 2006). This has consequences for protein degradation in signaling pathways. The GTP-binding nuclear protein from the *RAN1* gene of *A. thaliana* is expressed in all meristematic areas and RAN1 plays a key role in transporting the nuclear proteins responsible for suppression of auxin action and regulation in root tips (Kim and others 2001). Devos and others (2006) showed that the infection site of *P. brassicae* may be considered a new meristematic region and the upregulation of RAN1 during clubroot infection is consistent with this and indicates enhanced auxin response at early infection.

Cytokinins

In addition to auxin, increased concentrations of free and bound cytokinins were found (Dekhuijzen and Overeem 1971; Dekhuijzen 1981). Dekhuijzen (1981) also showed that the contents of bound and free cytokinins are different in host cytoplasm and in plasmodia of the pathogen. Cytokinin responsiveness (visualized by *ARR5::GUS* expression) was induced during the course of early clubroot disease (3 dai) in the cortex and vascular tissues of the infected root and hypocotyl. From 5 dai onward, this activity was seen in all cell layers of the infected root and hypocotyl (Devos and others 2006) and persisted throughout the developing gall (Siemens and others 2006). Also, immunoreactivity toward zeatin riboside is found in the infected tissue 13 and 21 dai at sites with the presence of sporogenic plasmodia but not with resting spores (Devos and others, unpublished results). This indicates the local accumulation of active cytokinins. In addition, Devos and others (2005) showed that amount of active cytokinins such as zeatin riboside was always greater in the infected *B. rapa* plants than in the healthy controls and *iso*pentenyladenine increased in clubroots at 21 dai. In *A. thaliana*, however, only the *iso*pentenyl-type cytokinins *iso*pentenyl adenine and *iso*pentenyladenosine accumulated during the onset of infection (Devos and others 2006). Müller and Hilgenberg (1986) isolated young secondary plasmodia and showed that they incorporated ^{14}C-adenine into *trans*-zeatin. Thus, it was assumed that the increase in cytokinins is at least partly the result of the active synthesis of zeatin by plasmodia of *P. brassicae.* Because of the small amount of detectable cytokinin production, it was unclear whether this constituted an important pathogenicity factor (Müller and Hilgenberg 1986). The morphologic description of the disease in *A. thaliana* identified two overlapping processes in the root cortex during the development of clubs. At the beginning of club growth, cell division is the predominant process, whereas cell enlargement dominates the later developmental stages (Kobelt and others 2000). This finding correlates with cytokinin production by the vegetative plasmodia of the pathogen during earlier disease

stages (Dekhuijzen 1981) and with increased levels of auxin during later disease stages when plasmodia were forming sporangia and resting spores (Grsic-Rausch and others 2000). Club growth might be an overall result of balances in the ratios of these two hormone types during the disease, whereby cytokinin dominated the earlier phase and auxin the later phase.

Strikingly, two of seven known putative cytokinin biosynthesis genes as well as cytokinin oxidases/dehydrogenases 1 and 6 (*AtCKX1*, *AtCKX6*) were downregulated in *A. thaliana* according to microarray analysis; this was confirmed for the latter by RT-PCR and promoter::GUS lines (Siemens and others 2006). A reduction of the degradation capacity of cytokinins by inhibition of specific *CKX* genes could in turn result in increased content of cytokinins, especially at the sites where plasmodia produce cytokinins. In *B. rapa*, an induction of *iso*pentenyl transferase genes involved in the *de novo* biosynthesis of cytokinins in clubroots has been described (Ando and others 2005).

The relevance of cytokinin for pathogenicity is highlighted by the resistance of *AtCKX*-overexpressing plants to *P. brassicae*. This might constitute a novel mechanism of disease resistance against *P. brassicae*. The resistance was independent of the isolate of *P. brassicae* used (Siemens and others 2006). Downregulation of cytokinins during gall formation was confirmed in *AtCKX* overexpressors that harbored a cytokinin-inducible promoter ARR5 (Siemens and others 2006). Proteome analysis of both *A. thaliana* and *B. napus* showed downregulation of adenosine kinase (ADK) (Devos and others 2006; Cao and others 2008). It was also suggested that ADK played a role in interconverting cytokinin ribosides to corresponding ribotides, which is possibly a key mechanism in regulating the level of active cytokinins (Chen and Eckert 1977; Laukens and others 2003; Kwade and others 2005). Downregulation of *AtADK* and *AtCKX* during *P. brassicae* infection helps maintain a high steady-state level of active cytokinins.

The response regulator genes *ARR10* and *ARR5*, which are involved in cytokinin signaling, and the cytokinin receptor gene *CRE1/AHK4* were upregulated by factors of 4.7, 2.3, and 2.8, respectively, in microarray analysis and ARR5 was confirmed by promoter::GUS experiments (see above). This indicated that active CK signaling is necessary for gall development. In addition, mutants with different cytokinin receptors have been shown to possess greater tolerance to clubroot (Siemens and others, unpublished results), indicating a specific role for cytokinins in gall development. It is noteworthy that recently cytokinins were also discovered to be key signaling molecules in other plant-microbe interactions such as nodule formation (Murray and others 2007) and the interaction of the growth-promoting fungus *Piriformospora indica* with *A. thaliana* (Vadassery and others 2008).

Stress- and Defense-Related Hormones

Abscisic Acid

One of the most visually obvious symptoms of the later stages of clubroot infection is foliar wilting. As the disorganized tissue of the gall develops in the root and hypocotyl, the vascular functions of the host are impaired and water supply to the aerial portions are restricted. Devos and others (2005) measured an accumulation of abscisic acid (ABA) in infected *B. rapa* plants at 21 dai, whereas Evans and Scholes (1995) observed reductions in stomatal conductance in *A. thaliana* leaves 5–6 weeks after infection. This loss of stomatal conductance is consistent with the accumulation of drought-responsive transcripts such as RAB18 (*responsive to ABA*), RD20 and RD22 (*responsive to dehydration*) observed by Siemens and others (2006) during the later stages of infection. As these responses are restricted to later stages, they could be a consequence of dehydration rather than signals involved in the development of galls.

Ethylene

Several studies have demonstrated that mutations in ethylene signaling can affect resistance or susceptibility to bacterial, fungal, or nematode pathogens. For example, in *A. thaliana* the *ein2* mutant is more susceptible to the fungal pathogen *Botrytis cinerea* (a cause of grey mold disease) (Thomma and others 1999), whereas ethylene-insensitive transgenic tobacco plants lose nonhost resistance to soilborne fungi (Knoester and others 1998). In other cases disease severity is decreased as plants tolerate infection due to a decrease in symptom development—the *never ripe* (*nr*) mutant of tomato shows fewer disease symptoms when infected by *Xanthomonas campestris*, *Pseudomonas syringae*, *Fusarium oxysporum*, and *Agrobacterium tumefaciens* (Aloni and others 1998; Lund and others 1998) and *etr1-1*, *ein2-1*, *ein3-1*, and *eir1-1* mutants of *A. thaliana* were less susceptible to the sugar beet cyst nematode *Heterodera schachtii* (Wubben and others 2001). The complexity of the role of ethylene-induced responses in disease development is amply demonstrated by the ethylene-insensitive soybean's disease symptoms caused by *Pseudomonas syringae* and *Phytophthora sojae* which were decreased, whereas those caused by *Septoria glycines* and *Rhizoctonia solani* were increased (Hoffman and others 1999).

No tolerance to clubroot infection was reported in the ethylene-signaling mutants (*etr1-1*, *etr1-3*, *ein3-1*, *ein4-1*)

examined by Siemens and others (2002) or those examined by Alix and others (2007) (*ein2-1*, *eir1-1*, *eto1, eto2*, *hls*). Because EIN2 is a central regulator of the ethylene signal transduction pathway (Li and Guo 2007), this signaling pathway is unlikely to play a central role in clubroot development but may be altered by stress responses within infected plants. Devos and others (2005) reported that a transient increase in the ACC content of uninfected roots of *B. rapa* was absent in infected plants at 14 dpi, whereas Siemens and others (2006) reported that there was downregulation of genes involved in ethylene biosynthesis at TP2 in clubroot-infected *A. thaliana*. This downregulation is in agreement with ethylene mutants being more susceptible to clubroot infection. Reevaluation of some of the ethylene receptor and signaling mutants at low infection pressure has shown that, for example, *etr1* and *ein2* mutants were more susceptible than wild types (Knaust and Ludwig-Müller, unpublished results).

It was found, however, that although the ethylene-signaling mutants described above do not show resistance to clubroot infection, gall formation is delayed in the weakly ethylene-insensitive mutant *ein5* (*ain1*) (Scholes and Rolfe, unpublished results). The recent cloning of the *EIN5* gene has shown it as ribonuclease targeting mRNAs involved in the regulation of components of the ethylene-signaling pathway (Olmedo and others 2006; Potuschak and others 2006). However, its targets are not limited to the ethylene signal transduction pathway as Dong and others (2004) demonstrated distinct roles for EIN2 and EIN5 in harpin-induced responses in *A. thaliana*. It is clear that a thorough understanding of the crosstalk between plant signal transduction pathways and the temporal and spatial patterns of gall formation is essential for understanding clubroot disease etiology.

The *alh1* mutant, which has a defect in the crosstalk between ethylene and auxins, probably at the site of auxin transport (Vandenbussche and others 2003), showed tolerance to clubroot infection. Although the infection ratio was three times less in the mutant, the plants that were infected had symptom progression similar to that of wild-type plants. The addition of NAA to the *alh1*-infected plants did not rescue the mutant phenotype. Therefore, it is likely that in this mutant IAA cannot reach the site of infection and consequently is unable to induce gall development. Therefore, it was speculated that the plants were more resistant to *P. brassicae* because host IAA transport was hampered (see the subsection Auxin Transport above; Devos and others 2006).

Jasmonic Acid

There is little evidence so far of involvement of jasmonic acid or its conjugates in clubroot development. Although for auxins conjugate formation is discussed as an inactivation reaction (Seidel and others 2006), it was recently shown that jasmonate-*iso*leucine is the active compound binding to the *A. thaliana* JA receptor COI1 (Thines and others 2007). Significant for this finding is the expression of JAR1, a member of the GH3 family involved in the adenylation of IAA or JA with subsequent synthesis of amino acid conjugates (Staswick and Tiryaki 2004; Staswick and others 2005). The microarray data obtained by Siemens and others (2006) indicated a strong downregulation of JAR1, which consequently led to increased susceptibility of the *A. thaliana jar1* mutant to *P. brassicae* infection (Siemens and others 2002). Particularly interesting is the finding that JA is upregulated in *B. rapa* roots during club formation because JA is able to induce indole glucosinolates and also nitrilase activity (Ludwig-Müller and others 1997; Grsic and others 1999), thereby possibly linking auxin and jasmonate metabolism in clubs (see subsections Glucosinolates and Regulation of Auxin Synthesis).

Defense-Related Genes and Salicylic Acid

As the identification of genes that lead to clubroot resistance in brassicas has often been hampered by complex inheritance patterns and the complexity of *Brassica* genomes, a number of studies have sought to identify resistance in *A. thaliana* which is ideally suited for molecular genetic analyses (Fuchs and Sacristan 1996; Kobelt and others 2000; Siemens and others 2002; Alix and others 2007). Although the majority of *A. thaliana* ecotypes are completely susceptible to clubroot infection, the dominant gene *RPB1* found in the ecotypes *Ze-0*, *Tsu-0*, and *Ta-0* leads to hypersensitive reactions with *P. brassica* isolate 'e,' thus restricting disease development. Thus, the hypersensitive mechanism has the potential to generate resistance to clubroot infection with specific ecotype-pathotype pairs.

As is common with many plant responses to infection by biotrophic pathogens, the induction of defense-related responses in susceptible plants infected by clubroot is of limited extent and duration. Ludwig-Müller and others (1994) found that there was no clear relationship between the patterns of induced peroxidase and chitinase activities in resistant and susceptible cultivars of *B. rapa*, although this study did identify a novel isoform of peroxidase induced in a susceptible cultivar at infection. Siemens and others (2006) found that the majority of defense-related genes were not differentially expressed in clubroot-infected *A. thaliana*. Of the 312 genes that did show differential expression, more were downregulated than were upregulated at each time examined and the magnitude of induced expression was modest.

Susceptible interactions may result either from the failure of the host plant to recognize and respond to the

pathogen appropriately or from the responses themselves being ineffective. Scholes and Rolfe (unpublished results) found that clubroot infection was only slightly impaired in the mutant *cpr1* and not impaired in the *cpr5* and *cpr6* (constitutive expressor of PR genes) mutants of *A. thaliana*, which accumulate salicylic acid, constitutively express defense-related proteins, and show enhanced disease resistance to a range of bacterial and oomycete pathogens (Bowling and others 1994, 1997; Clarke and others 1998). As *cpr5* and *cpr6* show constitutive expression of proteins whose expression is mediated by both the salicylic acid and JA/ET defense pathways, it is clear that activation of a diverse array of defense pathways and expression of multiple defense genes in advance of inoculation still does not provide resistance to clubroot infection. This is in accordance with the observation that treatment of infected *B. rapa* roots with SA did not result in tolerance against the clubroot pathogen (Ludwig-Müller and others 1995). Holtorf and others (1998), however, reported that transgenic *A. thaliana* plants expressing high levels of the mistletoe viscotoxin A3 (a thionin) showed a reduction in infection rate by *P. brassicae* although resting spores did eventually develop.

Modeling the Phytohormone Changes During Clubroot Development

A model is presented in Fig. 1 summarizing the hormonal changes and signals during the different stages of clubroot development. At the very early infection stages (from 12 h after infection on), there are reports of an upregulation of *iso*pentenyltransferases as well as enhanced cytokinin concentrations. The cytokinins produced are derived from both host and pathogen. These cytokinins are possibly the very earliest signal for reactivating the cell cycle (Devos and others 2006), which is the basis for the initial promotion of growth on infection reported by Devos and others (2005, 2006). In an infected root, IAA accumulates in the developing plasmodia, which acts as its sink. The plasmodia are recruiting IAA from the host at an early stage, channeling it toward them. Blocking IAA transport with NPA means that the IAA cannot reach the sites of infection. The increased amount of IAA may be synthesized by the host after the trigger of *P. brassicae* infection and is channeled toward the sites of infection in a sink-dependent manner. In addition, *de novo* IAA biosynthesis occurs via the upregulation of myrosinases, nitrilases, and glucosinolate metabolism. Indole acetic acid is further conjugated in the first stage, thus avoiding a toxic accumulation of IAA in the infected plant. This IAA induces XTH resulting in cell elongation. At a later stage (13 dai), *P. brassicae* is no longer dependent on host IAA for developing cortical

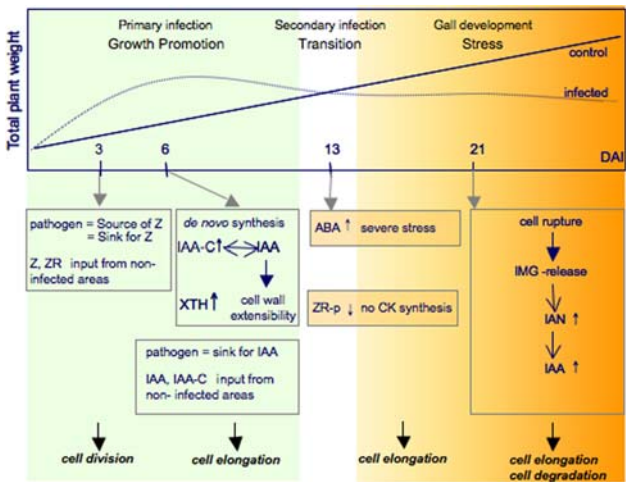

Fig. 1 Hypothesis of the roles of plant growth regulators during various stages of clubroot infection (modified after Devos and others 2005). The graph at the top represents the total plant weight (in arbitrary units) as a function of days in non-infected healthy plants (*solid line*) and *P. brassicae*-infected plants (*dotted line*). This graph is subdivided in three phases: Growth Promotion, Transition, and Stress marked with a colored background. The related plant growth regulator events occurring in these different phases are summarized. The *open arrows* show higher or lower regulator concentration and XTH action in infected plants compared with healthy control plants. The *black arrows* show metabolic and physiologic processes or events. ABA = abscisic acid, DAI = days after inoculation, IAA = indole-3-acetic acid, IAA-C = conjugated IAA, IAN = indole-3-acetonitrile, IMG = indole-3-methyl glucosinolate, XTH = xyloglucan endo-transglucosylase/hydrolase, Z = zeatin, ZR = zeatin riboside, ZR-p = zeatin riboside monophosphate

secondary plasmodia or symptom development. During the secondary stage, all IAA biosynthetic pathways are induced, thus boosting IAA, finally resulting in cell degradation. This in turn, induces IAA biosynthesis via glucosinolate metabolism. In addition, ABA is accumulating during the secondary stage, inducing stress responses. Finally, energy production is necessary to maintain this active sink.

Conclusions

Clearly, hormonal and metabolic control in developing galls is very complex. Figure 2 summarizes the results from the genomic and proteomic studies (Devos and others 2006; Siemens and others 2006; Cao and others 2008) described for *A. thaliana* and *B. rapa*. In the first 12 h after infection, the host defense is overwhelmed, resulting in downregulation of ROS metabolism (CuZn-superoxide dismutase, catalase, glutathione synthetase). Host defense recovers fully after 1-2 days. The ROS cell death is minimal, however, during the entire progression of gall formation. Cytokinin biosynthesis is upregulated after

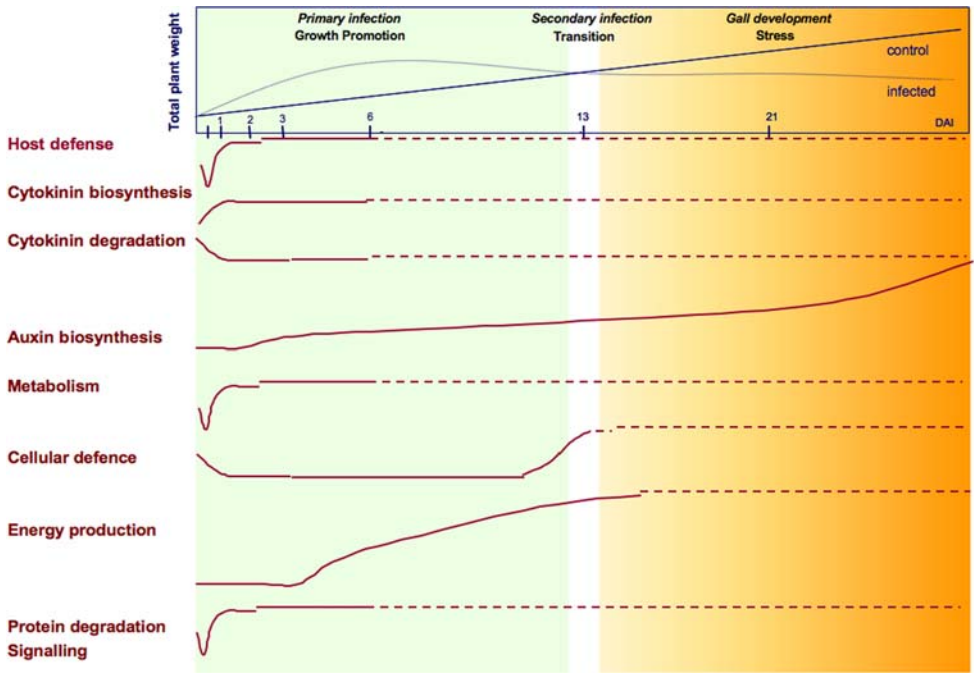

Fig. 2 Summary of the sequence of events during the stages of clubroot infection. The upper graph represents the total plant weight (in arbitrary units) as a function of days in non-infected healthy plants (*solid line*) and *P. brassicae*-infected plants (*dotted line*). This graph is subdivided into three phases: Growth Promotion, Transition, and Stress against a colored background. The brown lines represent the events summarized on an estimated basis for the corresponding up/downregulation of proteins or genes published earlier. Based on Devos and others (2006), Cao and others (2008), and Siemens and others (2006). *Dashed lines* correspond to a postulated progression of an event for which data for the corresponding developmental stages are not yet available. DAI = days after inoculation

3 days in combination with the parallel downregulation of adenosine kinase, catalyzing the conversion (inactivation) of active cytokinin ribosides to the corresponding ribotides. Auxin synthesis is gradually increased by inducing myrosinases and nitrilases. Along with the host defense, metabolism (S-adenosylmethionine [SAM] synthetase and glycolysis) and protein degradation/signaling mechanisms (more specific proteasome proteins and chaperonins) are overwhelmed in the first 12 h after infection and upregulated thereafter. Because SAM is also a precursor of ethylene and polyamines, it is possible to predict that these plant growth regulators might also have a similar pattern. Cellular defense (via chitinase, lignin biosynthesis, phenol synthesis, tubulins, peroxidases) is downregulated during the entire primary stage of the infection process. Finally, energy production is gradually increased from 4 to 5 days after infection. The latter corresponds with the functioning of the plasmodia as metabolic sinks.

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