

PLANT HORMONES: A KEY IN CLUBROOT DEVELOPMENT

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ABSTRACT

Clubroot disease causes gall formation on Brassica roots. A holistic approach was undertaken to understand the role of plant hormones in early clubroot symptom development in Arabidopsis. We used the *CYCBI*;1::GUS, *DR5*::GUS and *ARR5*::GUS constructs to assess clubroot initiation. We compared plant hormone concentration between control and infected plants, screened different hormone mutants for resistance and evaluated auxin transport. Also, a differential proteome study was performed using two dimensional gel electrophoresis coupled to MALDI-TOF. Combining the hormone and proteome data, we postulate that at the first stages of clubroot disease, cytokinins are produced by the pathogen. This triggers a local re-initiation of cell division in the root cortex. Consequently, a *de novo* meristematic area is established that acts as a sink for host-derived IAA, carbohydrates, nitrogen and energy to maintain the pathogen and to trigger gall development.

INTRODUCTION

Clubroot disease is caused by *Plasmodiophora brassicae*. This obligate biotrophic protist forms galls on *Brassica* roots and can have devastating effects on crop yield world wide. The life cycle of *P. brassicae* can be divided into two phases: a primary phase in which events are confined to the root hairs and a secondary phase that occurs in the cortex and the stele of hypocotyl and roots of the infected plants. The morphological changes, like hyperplasia and hypertrophy in the host's root, suggest alterations in the plant hormone balance.

MATERIAL AND METHODS

The concentrations of different plant hormone compounds are screened during biological relevant stages of infection using gas and liquid chromatography coupled to tandem mass spectrometry. In both Chinese cabbage wild type plants and in susceptible and tolerant *Arabidopsis thaliana* mutants, hormone concentrations in infected roots are compared with concentrations in non-infected roots. Complementary, *in situ* immunolocalisation is performed to study the spatial distribution of indole-3-acetic acid (IAA) and zeatin in infected and non-infected Chinese cabbage. Concomitant with these studies, the auxin activated *DR5* promoter-GUS construct and the cytokinin activated *ARR5* promoter-GUS construct of *A. thaliana* are used to visualise patterns of phytohormone gene responsiveness during clubroot development. To obtain information on the input of auxin transport in clubroot development and to verify the origin of the plant hormone IAA present at the site of plasmodia formation, the IAA transport inhibitor *N*-1-naphthylphthalamic acid is added. To study proteins involved in symptom development, a proteome approach is applied to investigate differential protein expression during *P. brassicae* infection on *A. thaliana*.

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RESULTS AND DISCUSSION

We screened the endogenous concentration of plant hormones in the economically important Chinese cabbage host. Infection results in a transient increased auxin biosynthesis, leading to an increased total auxin pool at 6 DAI. The initial accumulation of the total IAA pool possibly triggers cell expansion and consequently the early growth promotion observed during the early infection stage. During the phase of growth promotion, a reduction in active cytokinins occurs. 13 DAI can be considered a transition between the primary and secondary infection stage, observable in a switch in almost all plant hormone concentrations and a clear control on the ACC concentration. At 21 DAI, a lower IAA content in infected plants was observed. At later stages, uncontrolled cell division and cell expansion proceeded, resulting in cell rupture and consequently the release of indole-3-methyl glucosinolate (IMG) (Butcher *et al.*, 1974). This substrate is a host specific precursor of indole-3-acetonitrile (IAN) and results in *de novo* IAA synthesis via the IAN pathway (Grsic-Rausch *et al.*, 2000). This conversion may be responsible for the steady IAA accumulation, mentioned in many reports. In intact cells this pathway is not active because IMG accumulates in the vacuole, whereas the enzyme resides in the cytoplasm.

Also in *Arabidopsis thaliana*, which is a member of the *Brassica* family and accordingly a host for *P. brassicae*, plant hormone changes upon infection were screened. The availability of *A. thaliana* mutants has been exploited to investigate a set of biochemical and morphological parameters, which might affect the interaction with *P. brassicae*. The ethylene mutants tested showed an altered degree of susceptibility towards clubroot, i.e. the ethylene receptor mutant *etr1-3* was more susceptible compared to the wild type and the ethylene insensitive mutant *ein5-1* showed a clubroot tolerant phenotype. Both mutants and the susceptible wild type (Col-0) were included in a screen for the endogenous phytohormone concentrations upon infection. A lower IAA content was found during secondary infection in the susceptible mutants and a higher concentration was detected in the tolerant mutant. This corroborates with the results obtained in the susceptible host Chinese cabbage, where the concentration of IAA was lower in the galls (Devos *et al.*, 2005). We can conclude that the tolerant mutant *ein5-1* maintains the endogenous IAA levels upon infection, whereas the susceptible plants do not.

The general idea that the parasite, the host or both produce hormones that promote cell division and cell expansion in clubroot is reconfirmed by this study. The plant hormones IAA and zeatin, together with gene responsiveness towards these hormones are labelled in the developing plasmodia. Furthermore, disease development is reduced and less IAA is observed in plasmodia if we block host auxin transport at the start of infection. However, if host IAA transport is allowed during secondary infection, normal galls are formed. Neither IAA nor zeatin is found in resting spores. At this stage the plasmodial life cycle was completed and presumably, plant hormones are no longer required. When we examine the endogenous concentration of IAA in control and infected roots, we observe an overall lower concentration in the infected tissue. However, we noticed that it is difficult to extract IAA from infected tissue. Therefore, we can raise the hypothesis that *P. brassicae* is able to retain host IAA in developing plasmodia.

We show proteins that are involved in ROS, ethylene or other plant defense, which are either induced or suppressed upon infection (Figure 1). Despite the plant's defense attempt, it is unable to prevent *P. brassicae* development (grey circle in figure 1). It is known from literature that cytokinins are produced by the plasmodia (Müller and Hilgenberg, 1986). Our study clearly

confirms these results as a higher cytokinin level (iP and iPA) and gene responsiveness (ARR5::GUS) was detected in infected tissue. Moreover, ADK was down-regulated, pointing towards an increased accumulation of active cytokinins. Since cytokinins have the ability to induce a metabolic sink in plant tissue (Mothes and Engelbrecht, 1963), the newly formed plasmodia can be considered to act as a sink and a new meristem is established. Supporting evidence for this hypothesis is that ARR5::GUS is up-regulated first, followed by the up-regulation of DR5::GUS upon infection. The enhanced gene responsiveness to auxin in infected Arabidopsis plants suggests that plasmodia also act as a sink for auxin. At the proteome level this is supported by the up-regulation of three proteins that regulate IAA biosynthesis; myrosinase, IAR4 and GST. From previous results it is known that ethylene inhibits IAA transport (Morgan and Gausman, 1966). We showed that three proteins involved in ethylene biosynthesis were differently regulated upon infection. This might level the concentration of ethylene (ADK, methionine synthase, SAH hydrolase; Weretilnyk et al. 2001). After blocking auxin transport with NPA, clubroot development is disturbed, suggesting a role for auxin transport in clubroot development. From a previous study it is known that carbohydrates are reallocated to the plasmodia (Evans and Scholes, 1995). This study also revealed proteins that point towards an increased biosynthesis of carbohydrates in infected roots. Additionally, a putative source for nitrogen during clubroot disease was detected with the up-regulation of GABA transaminase. A higher abundance of RuBisCo LSU was observed, indicating chlorophyll retention. The up-regulation of the ATP synthase protein that was noted in our study suggests that galls require higher energy levels. The susceptible reaction of Arabidopsis towards *P. brassicae* leads to cell division, cell expansion and eventual cell degradation. We noticed differentially regulated proteins that may be responsible for this altered cell morphology, including RAN1, tubulins and pectin methylesterase.

Taken all these observations into account, we can conclude that plant hormones are a key in the development of clubroot formation and plasmodia have the ability to produce cytokinins that create a sink for host IAA on behalf of their own propagation. This hypothesis is supported by the proteome study (Devos *et al.*, 2006) (Figure 1).

LITERATURE CITED

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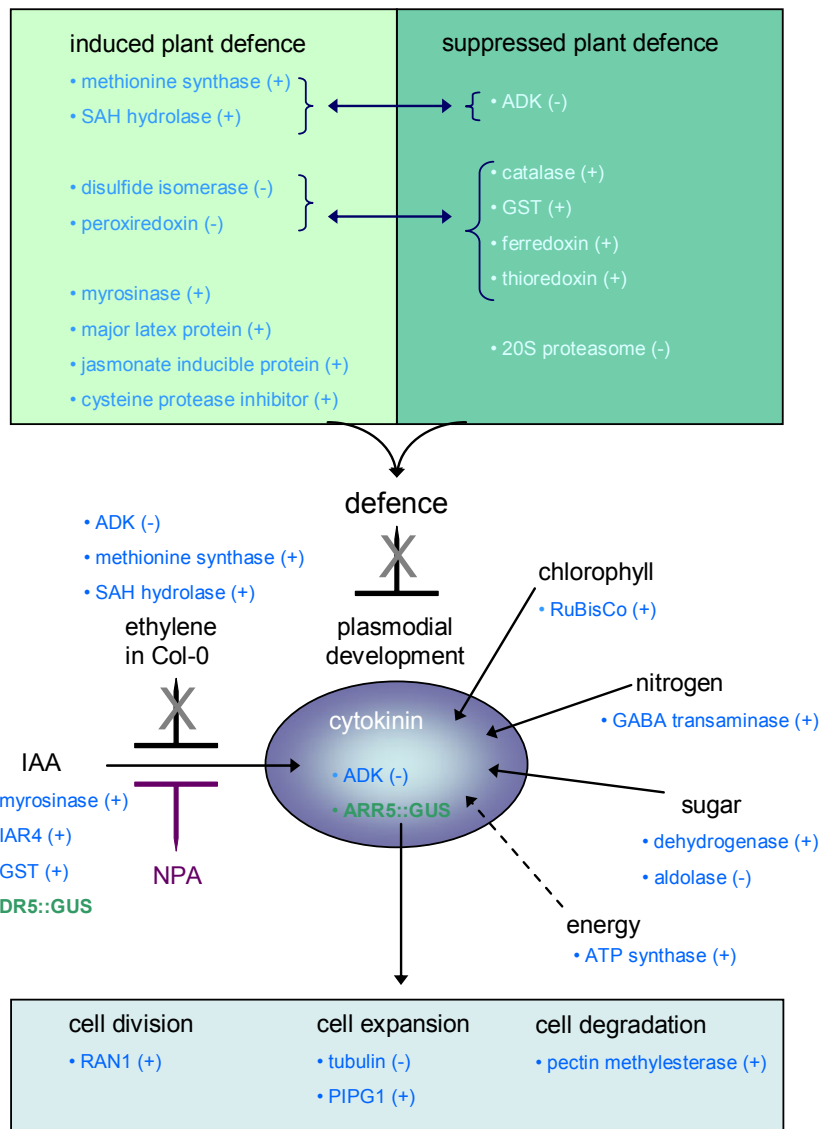


Figure 1. Hypothesis of plant processes upon *Plasmodiophora brassicae* infection of *Arabidopsis thaliana* Columbia-0. Proteins that indicate plant defence (see light green box) and proteins that point to a suppression of plant defence (see dark green box) control the defence against *P. brassicae*. The plant is unable to defend itself and plasmodial development proceeds (see blue circle). The plasmodia synthesise cytokinins that induce a sink for chlorophyll, nitrogen, sugar and IAA. This process requires energy (dashed arrow). IAA transport that is necessary for accurate plasmodial development is not blocked by ethylene, but when NPA is added (in purple), host IAA transport might be blocked and clubroot development is impaired. Upon a susceptible interaction, the newly formed plasmodial sink induces cell division, cell expansion and eventually cell degradation. The proteins that correspond to each process are presented in blue (up regulated (+) and down regulated (-) upon clubroot infection). In green are promoter-GUS constructs