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Increase in Indole-3-acetic Acid (IAA) level and Nitrilase Activity in Turnips Induced by *Plasmodiophora brassicae* Infection

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Abstract

Indole acetic acid (IAA) level and nitrilase activity were measured in infected and healthy turnips, *Brassica campestris* L., subsp. Rapa, over a time course to confirm increases in IAA levels upon infection by *Plasmodiophora brassicae* and to investigate underlying mechanisms. Healthy and infected seedlings were assayed from 20 to 90 days after sowing. IAA levels in both roots fluctuated similarly over days 20-35. By day 45, IAA in infected roots increased to five-fold over healthy roots then decreased to the level of healthy roots by day 90. Nitrilase activity was negligible on days 20 and 25 but increased thereafter in infected and healthy roots. However, activity in healthy roots decreased substantially by day 40 while infected roots showed a continued increase to day 45 then decreased to a low level. These findings suggest that IAA concentration increases after *P. brassicae* infection, possibly due to IAA synthesis via pathways involving nitrilase.

Key words: *Brassica campestris* L., clubroot disease, indole-3-acetic acid, indole-3-acetonitrile nitrilase, *Plasmodiophora brassicae*, Turnip.

Abbreviations

IAA I, Indole-3-acetic Acid; nitrilase, indole-3-acetonitrile nitrilase; PAL, phenylalanine ammonia lyase.

Introduction

Clubroot is caused by the obligatory root pathogen *Plasmodiophora brassicae* and is one of the most critical infectious diseases of crucifers. However, little is known about the biochemical basis of the disease process due to technical limitations such as the inability to grow the pathogen on artificial media and the difficulty in controlling its sporulation. The resting spores germinate in the root sphere of the host plants and release primary zoospores that infect root hairs (Aist *et al.*, 1971). Subsequently, secondary zoospores appear and infect cortical cells of roots (Ingram and Tommerup, 1972). The infected root hypertrophies in a phase during which there is an increase in sugar utilization (Keen and Williams, 1969a) and an increase in the levels of metabolites such as amino acids, sugars and lipids (Keen and Williams, 1969b; Williams *et al.*, 1969; Nomoto and Tamura, 1970; Chiang and Nip, 1973). The levels of phytohormone such as IAA and cytokinin have also been reported to elevate in hypertrophied tissue (Williams *et al.*, 1969; Dekhujizen and Overeem, 1971; Grsic-Rausch *et al.*, 2000). But these findings have not been fully established yet (Raa, 1971; Kavanagh and Williams, 1981), since the level has not been searched in whole life span of host plant. Thus, it must be important to evaluate the level in lengthy time course.

Some crucifers are resistant to clubroot, making them excellent targets to study the biochemical changes associated with the disease. Previously, we focused on the various biochemical changes in the early phases of *P. brassicae* infection and showed that PAL activity is induced upon contact with resting spores in clubroot-resistant turnip callus but not in the susceptible callus (Takahashi *et al.*, 2001). Furthermore, the induction of PAL is dependent on the influx of Ca^{2+} and inhibited by EGTA and Ca^{2+} channel blockers (Takahashi *et al.*, 2002). PAL activity is suggested to increase the synthesis

of IAA (Ludwig-Muller and Hilgenberg, 1988), thus providing a possible link between our findings and the previously reported changes in IAA during *P. brassicae* infection. To clarify the nature of these changes we investigated IAA levels in *P. brassicae* infected turnip roots over a 90-day period. We further examined the possibility that *de novo* IAA synthesis occurs within the roots by monitoring the activity of nitrilase, an intermediary enzyme in the synthesis of IAA from tryptophan.

Materials and Methods

Plasmodiophora brassicae

Resting spores of *P. brassicae* were isolated from Chinese cabbage and maintained on calluses of clubroot-susceptible turnip (*B. campestris*, L. subsp. Rapa cv. Natsumaki 13-go Kokabu) generously provided by Dr. Kiso, Musashino Shubyo-En (Minami Ikebukuro, Tokyo, Japan).

The calluses were induced and maintained in basal medium (Murashige and Skoog, 1962) supplemented with agar and plant hormones (MS-Agar medium) as previously described (Takahashi *et al.*, 2001). Every two weeks, the calluses were maintained with a fresh medium.

Preparation of resting spores from infected turnip callus

Calluses containing *P. brassicae* spores were homogenized with a mortar and pestle in sterilized distilled water. The homogenate was passed through eight layers of cheesecloth and the filtrate was centrifuged at 1,500*g* for 10 min at 4 ℃. The resting spores in the pellet were further purified by centrifugation at 500*g* for 10 min at 4 ℃ in a 36 to 12% (W/V) discontinuous Ficoll 400 gradient as described (Takahashi *et al.*, 2001). The resting spore preparations were then plated on MS-Agar medium for 48 h to test for microorganism contamination. Spores from clean preparations were counted under a microscope using Thoma's hemocytometer.

Cultivation of turnips and inoculation with *P. brassicae*

Seedlings of turnip cv. Natsumaki 13-go Kokabu were grown in 8x7 cm ots containing sterilized soil. The growth chamber was held at 25 ℃ with a 12 h dark/light cycle (10,000 Lux) and pots were kept in shallow water trays for hydration. Seven to eight days after sowing, ~10 g of soil containing resting spores of *P. brassicae* (10^9 spores per g dry soil) were added to each seedling as described by Ogawa *et al.* (2001) to initiate infection. Non-infected controls were grown as above without the addition of contaminated soil. Turnip growth was calculated by weighing the turnip root after removing leaves.

Extraction of IAA from turnip roots

Plant materials were harvested on the designated days and washed with distilled water. One gram samples from infected and non-infected turnip roots were homogenized with a pre-chilled mortar and pestle in 10 ml of 100% methanol containing 0.01% ascorbic acid (IAA extraction solvent). After 30 min, the homogenate was centrifuged for 5 min at 1,500*g* at 4 ℃ and the supernatant was collected. The precipitate was extracted second time as above in 10 ml IAA extraction solvent and the supernatants were pooled together. The extract was lyophilized at 30 ℃ and sequentially re-dissolved with 1 ml distilled water followed by 2 ml methanol and 1 ml distilled water. The resulting extract (~4 ml) was dried with a Speedvac centrifuge concentrator (Advantec Toyo, Japan) and re-dissolved in 0.5 ml of distilled water. The extract was then passed through a 4 ml Dowex 50W column (H^+ form) and non-adsorbed materials were applied to a 4 ml DEAE ToyoPearl 650M column equilibrated sequentially with 2 ml each of acetonitrile, H_2O and methanol. The column was washed with 2 ml 100% methanol and bound materials were eluted with 2 ml 100% methanol containing 2% acetic acid. The eluate was dried by vacuum centrifugation, re-dissolved with 1 ml of 100% methanol and finally adjusted to 50% total methanol by addition of distilled water. The extract was then passed through a 0.45 μm cellulose membrane filter for HPLC analysis.

HPLC analysis of IAA

The extracted IAA was analyzed by HPLC with a D-7000 System Manager (Hitachi, Japan) equipped with a 4.6x250 mm column of TSK-Gel ODS-80TS (C_{18}: 5 μm ϕ, Tosoh Corp., Japan). Briefly, 10 μl of each sample was fractionated at 25 ℃ for 60 min in 40% methanol containing 1% acetic acid at a flow rate of 0.5 ml per min. The elution was monitored with a fluorescence spectrophotometer (F-1000, Hitachi, Japan) at 280 nm (excitation) and 350 nm (emission). A known concentration of IAA was also chromatographed as above to serve as a positive control and quantitative comparison.

Assay of nitrilase activity

Hypertrophied or healthy turnip roots (1 g per sample) were homogenized with a pre-chilled mortar and pestle in 10 ml of 5 mM Tris-HCl (pH 7.5) containing 1 mM EDTA and 0.04 mM β-mercaptoethanol. The homogenate was centrifuged

at 1,300*g* for 10 min at 4 ℃, decanted, and the supernatant was further clarified by centrifugation at 100,000*g* for 60 min at 4 ℃. The recovered supernatant constituted the nitrilase fraction used in the assay described below.

A mixture of 100 μl of 1 mM indole-acetonitrile in methanol solution and 400 μl of Tris-HCl buffer was pre-incubated at 37 ℃ for 5 min. The reaction was initiated by the addition of 500 μl of the nitrilase fraction and allowed to proceed for 30 min before being terminated by the addition of 150 μl of 1N HCl and 500 μl ethyl acetate. The mixture was then mixed vigorously and the upper phase was recovered, evaporated, re-dissolved in methanol and passed through Dowex columns as described above. The concentration of IAA synthesized by the nitrilase was then determined by HPLC. In control, instead of nitrilase fraction, the same amount of Tris-HCl buffer was added.

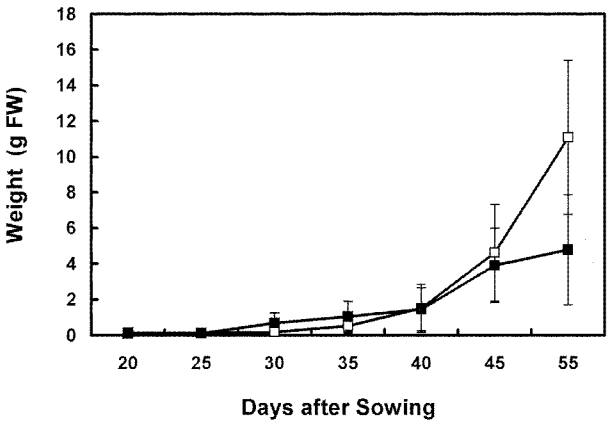

Fig. 1 Changes in root weight of healthy (□) and *P. brassicaee*-infected (■) turnips during clubroot development. The bar in each plot represents ± SD from five samples.

Results

Growth of turnip plants during clubroot development

The growth of turnips during clubroot development was measured by comparing root weight between infected and healthy turnips. From 20 to 40 days after sowing, the root weights of infected and healthy turnips were comparable (**Fig. 1**). However, from 40 to 55 days after sowing, the root weight of healthy seedlings increased dramatically while that of infected seedlings increased only moderately. The first clubroot symptoms appeared after ~30 days and became more apparent after 45 days (**Fig. 2A**).

Changes in IAA concentration in infected and healthy roots

IAA concentrations in infected and healthy turnip roots were monitored from 20 to 90 days after sowing and were found to fluctuate. In healthy roots

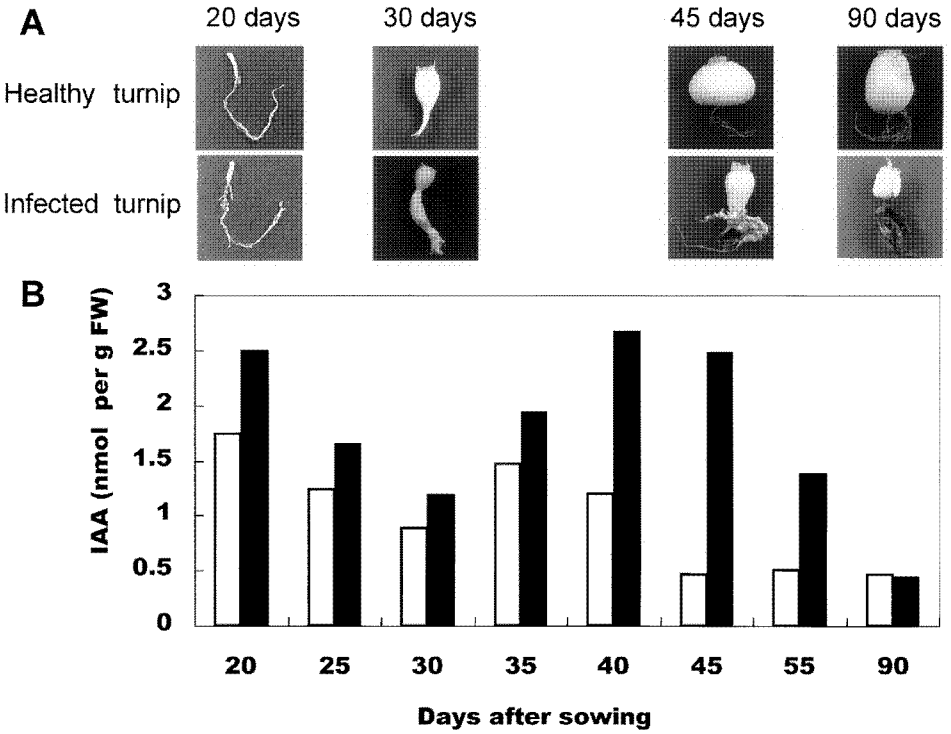

Fig. 2 Photographs of healthy (Panel A, upper picture) and clubroot diseased turnips (Panel A, lower picture) and changes in IAA concentration (Panel B) in healthy (□) and *P. brassicaee*-infected (■) turnip roots. Photographs were taken at 20, 30, 45 and 90 days after sowing.

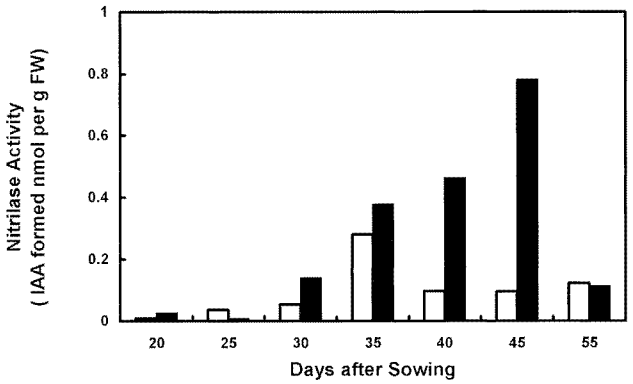

Fig. 3 Activity of indole-3-acetonitrile nitrilase in healthy (□) and *P. brassicaee*-infected (■) turnip roots. The activity is expressed as the amount of IAA produced per minute under our conditions.

20 days after sowing, IAA concentration was 1.7 nmol per g fresh weight (FW). IAA concentration decreased to 0.8 nmol per g FW by day 30, increased to 1.5 nmol per g by day 35, then decreased to 0.5 nmol per g by day 45 and maintained this level for the rest of the test period (**Fig. 2 B**).

During days 20-35 the IAA concentration in infected roots fluctuated in a manner similar to healthy roots though the concentration in infected roots was consistently higher by 0.5 nmol per g. However, on days 40 and 45 when clubroot symptoms became obvious, the levels of IAA in infected roots were up to five-fold higher than in healthy roots. This maximum level in infected roots was comparable to that in healthy roots on day 20. After 45 days the IAA concentration in infected roots decreased to that of healthy roots by day 90 (**Fig. 2 B**).

Nitrilase activity in infected and healthy roots

Nitrilase activity in infected and healthy turnip roots was monitored from 20 to 55 days after sowing. In healthy roots on day 20, nitrilase activity was very low (0.01 nmol per g) but increased gradually and peaked on day 35 (0.3 nmol per g). Subsequently, nitrilase activity decreased to 0.1 nmol per g where it held steady for the remainder of the test period (**Fig. 3**).

In infected turnip roots, nitrilase activity was similar to that of healthy roots on days 20 and 25. However, activity began to rise sharply thereafter and reached a maximum 0.8 nmol on day 45 when clubroot symptoms are evident. Following this peak, nitrilase activity decreased sharply to the level of healthy roots by day 55 (**Fig. 3**).

Discussion

Accumulated evidence suggests that infection with *P. brassicae* induces synthesis of IAA or cytokinin in host plant roots (Williams *et al.*, 1969; Dekhujizen and Overeem, 1971; Grsic *et al.*, 1998; Grsic *et al.*, 1999; Ludwig-Muller *et al.*, 1999; Grsic-Rausch *et al.*, 2000). However, fluctuation of IAA level has not been determined in whole developmental span of infected root. To address this behind, we examined IAA concentrations over a long time period that encompassed both early and late stages of root development. Thus, we examined healthy and infected turnips from 20 to 90 days after sowing and quantified both IAA concentration and activity of nitrilase, an intermediary enzyme in the synthesis of IAA from tryptophan.

During the first 90 days after sowing there was a clear change in IAA concentration (**Fig. 2**) and which seemed to correlate with the growth of turnip roots in lateral and/or apical directions (data not shown). Up to 35 days after sowing, IAA levels in infected and healthy roots fluctuated in a similar manner although infected roots showed consistently higher levels by ~0.5 nmol per g FW. However, by day 40 the IAA concentration in healthy roots began to decrease while that in infected roots increased sharply to a maximum (day 45) that was five-fold greater than the minimal level in healthy roots. Interestingly, the concentrations of IAA on day 20 and day 45 were almost identical even though the clubroot symptoms were absent on day 20 but present on day 45. Taken together, these results suggest that IAA does not increase simultaneously with *P. brassicae* infection as previously thought, but rather IAA reaches higher levels later in root development. Why and how these higher levels are achieved remains unknown, but there are at least two plausible hypotheses that address this issue. 1, There may be decreased IAA degradation in infected roots. 2, Host IAA synthesis may be higher in infected roots at later stages of infection. In addressing the second hypothesis, we questioned whether it might be possible to detect increased IAA synthesis within infected roots.

IAA is generally synthesized via tryptophan in individual three reactions that involve each intermediates indole-3-acetoaldehyde, indole-3-acetonitrile and indoleacetoamide catalyzed by aldehyde oxidase, nitrilase and indoleacetoamide hydrolylase, respectively (Ludwig-Muller and Hilgenberg, 1988). In *Arabidopsis*, IAA has been reported to be synthesized via indole-3-acetoaldoxim and indole-3-acetonitrile (Ludwig-Muller *et al.*, 1999; Lass-

well *et al.*, 2000). In *Arabidopsis*, two isoforms of nitrilase were found, while nitrilase 1 is expressed in early phase of the plant growth, isoform 2 in late stage (Bartling *et al.*, 1994). Infection of *Pseudomonas syringae* induces nitrilase 2 in *Arabidopsis* (Bartel and Fink, 1994). We focused on this induction of nitrilase activity as a marker of *de novo* IAA synthesis in infected roots. Nitrilase activity was maximized (0.8 nmol per g) in infected roots 45 days after sowing, coinciding with the maximum presentation of clubroot symptoms. In contrast, nitrilase activity was very low (0.1 nmol per g) in the healthy roots with the same age. Nitrilase activity did not mirror the observed fluctuation of IAA levels suggesting that change in nitrilase activity cannot solely explain these fluctuations. However, the high nitrilase activities in infected roots at late stages of growth suggest that IAA may be produced in the late stage from a pathway involving nitrilase. In contrast, the increase in IAA levels was not accompanied by enhanced nitrilase activity in early stages of both healthy and infected roots, suggesting that IAA may be synthesized via a different pathway in early development or perhaps be transported from aboveground tissues.

How does *P. brassicae* infection mediate this increase? Grsic-Rausch *et al.* (2000) found increased levels of the isoform nitrilase 2 in *Arabidopsis* roots infected by *P. brassicae*, but found no difference in nitrilase activity between healthy roots and infected cells harboring secondary plasmodia. These results suggest that only sporulating plasmodia induce nitrilase synthesis (Grsic-Rausch *et al.*, 2000). Therefore, to determine life span of *P. brassicae* in infected tissue is especially important to elucidate the IAA fluctuation.

Peroxidase has been also suggested to involve in IAA synthesis (Bennett *et al.*, 1995). Furthermore, the preoxidase is known to closely relate with defense reactions. Clubroot resistant turnip callus responded to *P. brassicae* but susceptible one did not (Takahashi *et al.*, 2001, 2002). Thus, PAL activity was transiently increased in the resistant callus on contact with *P. brassicae* spores, but no such defense reaction was observed in susceptible one. These suggest that in infected tissue of susceptible turnip, no preoxidase induction may occur, hence, the IAA synthesis in later stage is analogized to have no direct relationships with preoxidase induction at least in the susceptible turnip. Clearly, further investigation is warranted.

We showed that, contrary to common belief, IAA levels do not increase in response to primary infection. Rather, they spike at ~45 days after sowing, nearly concurrent with maximal clubroot symptoms. This spike is likely associated with IAA synthesis from tryptophan since levels of nitrilase showed a similar increase at that time. In total, these results suggest that increased IAA levels in later stages of *P. brassicae* infection are likely due to *de novo* synthesis of IAA and not to a decrease in degradation.

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