Evaluation of Roles of Amidase Which Converts Indole-3-Acetamide to Indole-3-Acetic Acid, in Formation of Clubroot in Turnip

Toshiki ISHIKAWA, Haruka KURODA, Keiichi OKAZAKI,
Kimiko ITOH, Toshiaki MITSUI and Hidetaka HORI*

(Received June 29, 2007)

Summary

Amidase was investigated if it was one of auxin-producing enzymes of *Brassica rapa*. We found amidase activity to convert indole-3-acetamide to indole-3-acetic acid in soluble protein extracts from *B. rapa*. Optimum condition for the enzyme activity was searched and two optimum temperatures were obtained at 45 and 55℃. Search for the heat stability of the enzyme strongly suggested that the two summits of the optimum temperatures were resulted from occurrence of variant amidases showing different temperature stabilities. The possibility of the presence of several amidase isoforms was supported by the following two observations, i.e., firstly, amidase activities at 45 and 55℃ had different pH optimums, 8.5 and 7.5, respectively. Secondly, the enzyme activities at the two temperatures were differentially fluctuated during the vegetative growth of turnip and clubroot development.

Fluctuation of the amidase activity and IAA contents observed in various turnip tissues such as healthy turnip leaf, hypocotyl and roots suggested that the enzyme had a constitutive role for keeping IAA homeostasis in those tissues. Interestingly, the amidase in turnip was shown to have high activity in hypocotyl and root rather than leaf, unlike Arabidopsis one. Changes of the enzyme activity during development of *B. rapa* was analyzed using the tissues of clubroot-diseased turnips. The activities fluctuated differently from each other in the temperature at 45 and 55℃ in infected tissues. In addition, activity at 45℃ was specifically enhanced in a later phase of clubroot development, whereas one at 55℃ increased only in an early phase in the infected root tissues. These results indicated that the amidase played an important role in turnip growth and clubroot development.

Bull.Facul.Agric.Niigata Univ., 60:53-60, 2007

Key words : amidase, *Brassica rapa*, indole-3-acetamide, indole-3-acetic acid, *Plasmodiophora brassicae*

Plasmodiophora brassicae is an obligate soil-borne pathogen causing clubroot disease in economically important crops of the Brassicaceae. Roots of infected plants exhibit abnormal division and expansion of cells due to pathogen proliferation, leading to the formation of galls. Severe disease development causes a delay of crop growth followed by a decay of the infected tissue. To control the disease, CR (clubroot-resistant) cultivars have been bred by conventional breeding, and recently several CR loci have been identified from *Brassica* plants (Suwabe *et al*., 2003; Hirai *et al*., 2004; Piao *et al*., 2004). However, *P. brassicae* can counter this resistance by generating genetic variation, since *P. brassicae* shows high genetic diversity in pathogenicity (Jones and Ingram, 1982a, 1982b; Kuginuki *et al*., 1999; Hatakeyama *et al*., 2004). To overcome this disadvantage, it is important to address the molecular mechanisms of gall formation that is a prerequisite for proliferation and maturation of the pathogen (Siemens *et al*., 2002).

Indole-3-acetic acid (IAA), a plant endogenous auxin, is an essential factor to induce clubroot development. In Arabidopsis, which is a cruciferous model plant and susceptible to *P. brassicae*, three enzymes catalyzing the final steps of *de novo* IAA synthesis pathway, i.e., nitrilase (Bartling *et al*., 1992), aldehyde oxidase (Seo *et al*., 1998) and amidase (Pollmann *et al*., 2003). Of the three enzymes, nitrilase has been well studied and demonstrated to be involved in clubroot of turnip (Ishikawa *et al*., in press; Ugajin *et al*., 2003), Chinese cabbage (Grsic *et al*., 1998) and Arabidopsis (Grsic-Rausch *et al*., 2000; Neuhaus *et al*., 2001). Aldehyde oxidase has been also investigated as an IAA synthetic enzyme of various plants and was recently reported to be upregulated in Chinese cabbage clubroot (Ando *et al*., 2006). In addition, hydrolysis of bound IAA pool is likely involved in clubroot development (Ludwig-Müller *et al*., 1997). All these insights indicate that multiple pathways for IAA synthesis may be involved in clubroot development and thus encompassing the pathways is necessary.

Amidase catalyzes the hydrolytic conversion of indole-3-acetamide (IAM) to IAA. This enzyme had been first identified from bacterial plant pathogens, i.e., *Pseudomonas savastanoi* (Magie *et al*., 1963), *Agrobacterium tumefaciens* (Inzé *et al*., 1984; Schröder *et al*., 1984; Thomashow *et al*., 1984) and *A. rhizogenes* (Camilleri and Jouanin, 1991). Subsequently, presence of the IAM amidase has been reported in rice (Arai *et al*., 2004; Kawaguchi *et al*., 1991), trifoliata orange (Kawaguchi *et al*., 1993), squash (Rajagopal *et al*., 1994) and

Graduate School of Science and Technology, Niigata University
*Corresponding author: hide-hri@gs.niigata-u.ac.jp

Arabidopsis (Pollmann *et al*., 2003). However, any specific their roles in plant growth as well as in clubroot development have not been elucidated. We report here brief characteristics and fluctuation of activity of turnip amidase in plant growth and clubroot development.

MATERIALS AND METHODS

Plant growth and inoculation with *Plasmodiophora brassicae*

Cultivation of turnip and inoculation with *P. brassicae* resting spores were performed as described elsewhere (Ishikawa *et al*., in press). Seeds of *Brassica rapa* L. cv. Natsumaki 13-gou kokabu, which is highly susceptible to clubroot disease, were sown on sterile soil and germinated at 24℃. One day after germination (dag), usually 4 days after sowing, each seedling was inoculated with 1 mL of *P. brassicae* resting spore suspension (10^{7-8} spores mL^{-1}, prepared as described below), and the plantlets were further cultivated at 24℃ under 9 h dark/15 h light at 130 μmol m^{-2} s^{-1} photon flux density. Control plants were not treated with spores. Plants were harvested at designated time points, weighed and stored at -80℃ until use for analyses.

Preparation of *P. brassicae* resting spores

Infected roots harvested on 40-45 dag were stored at -30℃ and homogenized in sterile distilled water with a mortar and pestle. The homogenate was passed through a four-layered cheese cloth. The filtrate was centrifuged at 1,000 × *g* for 10 min and the pellet containing spores was washed twice with sterile distilled water. The resulted precipitate was resuspended in sterile distilled water and spore concentration was adjusted to 10^{7-8} spores mL^{-1} using a hemocytometer.

Preparation of protein extract

Crude soluble protein extract was prepared as described previously (Ugajin *et al*., 2003). Frozen plant tissue was ground in liquid nitrogen with a mortar and pestle. The powder was homogenized in a 10-times volume of pre-chilled extraction buffer (5 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.04 mM β-mercaptoethanol). The homogenate was centrifuged at 1,300 × *g* for 10 min at 4℃ and the supernatant was further centrifuged at 100,000 × *g* for 60 min at 4℃. The supernatant recovered was used for enzyme assay described below.

Measurement of amidase activity

Prior to amidase enzyme assay, IAM purchased from Aldrich (St. Louis, MO, USA) was passed through DEAE TOYOPEARL 650M (Tosoh Co. Ltd., Tokyo, Japan) to remove very slight but not neglegible contamination of IAA.

Five hundred μL of crude protein extract was reacted with 0.1 mM IAM in 1 mL extraction buffer at designated temperature for 60 min. The reaction was stopped by acidifying the reaction mixture with HCl to pH 2 and reactants were extracted with ethyl acetate. The organic phase was evaporated to dryness. The residue was dissolved in 50% (v/v) methanol and passed through a Dowex 50W (Bio-Rad Laboratories Inc., Hercules, CA, USA), and the resin was washed with 50% methanol. The flow through and washings were collected and evaporated. The dried residue was then dissolved in methanol and applied onto a DEAE TOYOPEARL 650M column and after the washing the resin with methanol, bound IAA was eluted with methanol containing 2% (v/v) acetic acid. The elution was dried completely, dissolved in 40% (v/v) methanol containing 2% (v/v) acetic acid and analyzed by HPLC equipped with a Gemini 5μ C18 column (4.6 × 150 mm, Phenomenex, Inc., Torrance, CA, USA). The column was maintained at 25℃ and 40% (v/v) methanol containing 2% (v/v) acetic acid was used as an elution solvent at a flow rate of 0.5 mL min^{-1}. IAA was detected by fluorescence with excitation at 280 nm and emission at 350 nm, and quantified using a calibration curve.

IAA measurement

Free and total IAA content in turnip tissues was determined as described elsewhere (Ishikawa *et al*., in press). Five hundreds mg of plant tissues were ground in liquid N_2 and the resultant powder was supplemented with 50 pmol indole-3-propionic acid (IPA) and extracted twice with methanol containing 5 mM butylated hydroxytoluene. The extract was divided into two portions, evaporated to remove methanol and used for free and total IAA measurements.

For free IAA measurement, the evaporated residue was resuspended in 50 mM sodium phosphate, pH 7.5, and extracted twice with CE (cyclopentane/ethyl acetate, 1:1). The remaining aqueous phase was acidified with 1 M citric acid (pH 2.5) and extracted twice with CE. The acidic organic phase containing IAA was evaporated to dryness and used as free IAA fraction.

For total IAA preparation, the dried methanolic extract was resuspended in 7 N NaOH and the residue containing IAA conjugates was hydrolyzed at 100℃ for 3 h under N_2. After cooling, the suspension was extracted twice with CE. The aqueous phase was acidified with concentrated phosphoric acid and extracted twice with CE. The acidic CE extract was dried and used as total IAA fraction.

The free and total fraction was analyzed by HPLC equipped with C18 column (Inertsil ODS 4μm, 4.6 × 250 mm, GL Sciences Inc., Tokyo, Japan). The column was kept at 30℃ and samples were separated with 30% (v/v) acetonitrile containing 0.7% (v/v) acetic acid and detected fluorometrically as described above. IAA content obtained was adjusted using the internal standard of IPA. We confirmed that no peak appeared at the same retention time as that of IPA (data not shown).

RESULTS

IAM amidase activity in turnip tissues

We first examined IAM amidase activity in soluble protein extract from turnip leaves and hypocotyls at 20 to 40 days after germination (dag). Amidase activity was not detected in young leaves but increased slightly at 40 dag **(Fig. 1A)**. On the other hand, protein extracts from hypocotyls moderately converted IAM to IAA except for 20 dag, and the highest activity was observed at 35 dag **(Fig. 1B)**.

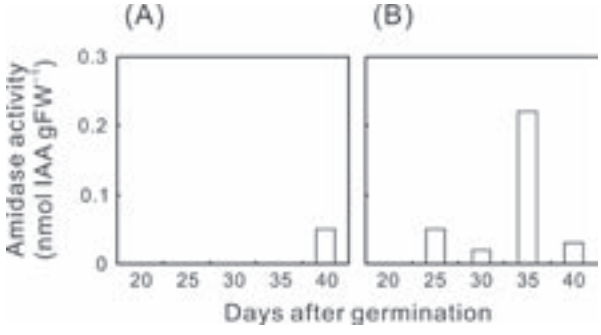

Fig.1. Amidase activity in the extract from turnip leaves (A) and hypocotyls (B). Crude enzyme fractions were prepared on the designated timing and 500 μL of crude protein extract was reacted with 0.1 mM IAM in 1 mL extraction buffer at 37℃ for 60 min. The reaction was stopped by acidifying the reaction mixture with HCl and reactants were extracted with ethyl acetate. IAA synthesized was separated by HPLC and concentration was measured by fluorescence with excitation at 280 nm and emission at 350 nm using a calibration curve. For the experimental detail see Materials and Methods.

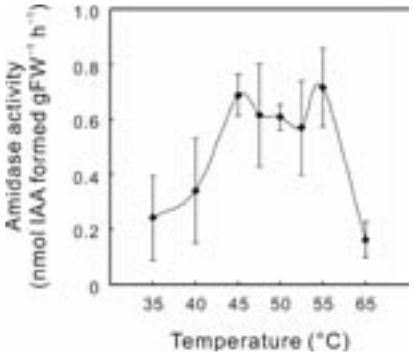

Fig. 2. Optimum temperature for turnip amidase. Crude enzyme was prepared from hypocotyls of 35-day-old plants and the activity was assayed as **Fig. 1** but at various reaction temperatures. Bars represent ± SD (n = 5).

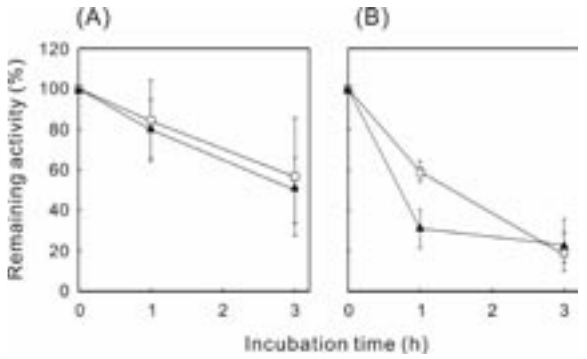

Fig. 3. Heat stability of turnip amidase in crude enzyme fraction. The crude enzyme amidase fraction was prepared as **Fig. 2**. The enzyme solution was incubated for 3 h either at 45℃ (Panel A) or 55℃ (Panel B). During the incubation, aliquots were taken at 1 or 3 h and immediately used for activity assay as Fig. 1 at 45℃ (closed triangles) or 55℃ (open squares). Bars represent ± SD (n = 5).

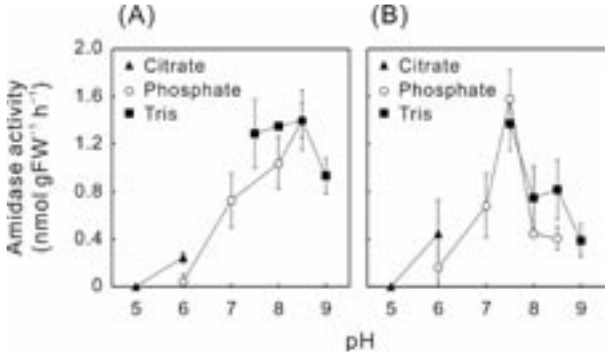

Fig. 4. pH optimum of turnip amidase at different temperatures. Amidase activity was measured as **Fig. 1** but at various pH at 45℃ (Panel A) or 55℃ (Panel B). All buffers shown in the figure were used as 200 mM solution in the final concentration. Bars represent ± SD (n = 3).

Optimum temperature of the activity of turnip IAM amidase

IAM amidase activity was clearly detected in turnip but the intensity was very low. Thus, we then tried to optimize conditions of the enzyme reaction using hypocotyls of the 35-day-old plants showing the highest activity as shown in **Fig. 1**.

Temperature dependency of the amidase activity was firstly examined. The activity was sharply increased till 45℃ and after the slight reduction, peaked again at 55℃ (**Fig. 2**). This result indicated the occurrence of several, at least two active forms of amidase having different optimum temperatures from each other in the crude extract. To elucidate above further, temperature stability of amidase was investigated. Protein extract was pre-incubated without IAM at either 45 or 55℃ for 1-3 h, and remaining activity was determined at 45 and 55℃. When the sample was pre-incubated at 45℃, remaining activities at 45 and 55℃ were comparable (**Fig. 3A**). On the other hand, in the pre-incubation at 55℃ for 1 h, activity at 55℃ was obviously higher than that at 45℃ (**Fig. 3B**), supporting the presence of multiple amidase isoforms showing different heat stability which would result in the two optimum temperature.

Based on these observations, amidase activity was determined at both 45 and 55℃ in the experiments below.

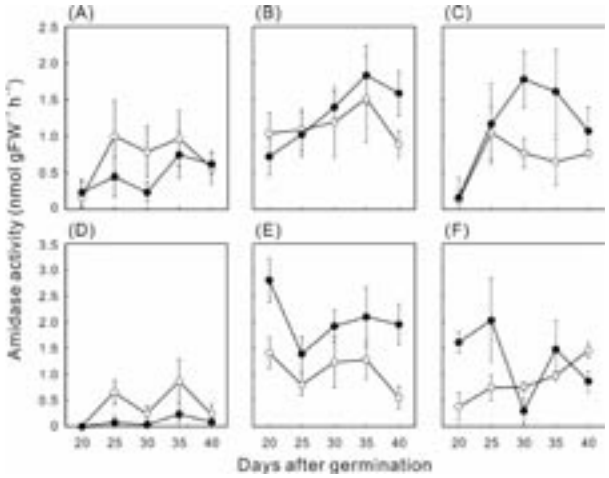

Fig. 5. Turnip amidase activity in healthy or clubroot-diseased tissues. Leaf, hypocotyl and root tissues were harvested from turnip plant on designated timing and crude enzyme solution was prepared. The enzyme activity was determined as **Fig. 1**. at 45℃ (Panels A-C) or 55℃ (Panels D-F). Panels A and D: leaves, B and E: hypocotyls and C and D: roots. Closed circle: tissue from *P. brassicae* infected plant. Open circle: tissue from healthy plants. Error bars represent ± SD from 3-7 replicates.

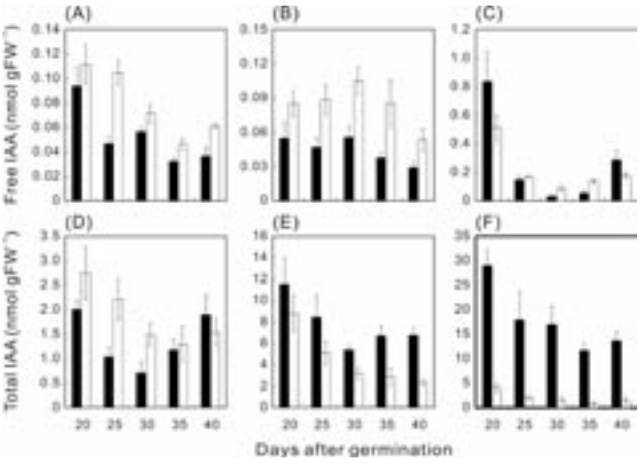

Fig. 6. Contents of free and total IAA in turnip leaf, hypocotyl and root. On designated timing, 500 mg of each plant tissue was prepared from the leaves (Panels A and D), hypocotyls (Panels B and E) and roots (Panels C and F) of healthy (open bars) and *P. brassicae* infected plants (closed bars). Using above various tissues, free (Panels A-C) and total IAA (Panels D-F) contents were measured with HPLC equipped with C18 column. For the experimental detail, see Materials and Methods. Error bars represent ± SD from six replicates.

pH optimum of the amidase activity

pH value of the reaction mixture for the amidase assay was then optimized. pH optimum was 8.5 in the assay at 45℃ **(Fig. 4A)**, whereas was 7.5 in that at 55℃. This significant difference strongly supported the presence of amidase isoforms.

Evaluation of amidase activity during vegetative growth and clubroot development

To elucidate biological functions of amidase in IAA *de novo* synthesis in turnip vegetative growth and clubroot development, its activity in leaf, hypocotyl and root was determined at 45 and 55℃ in vitro.

First, amidase activity in healthy plants was examined and clear significant activity was detected in all the tissues examined during 20 to 40 dag **(Fig. 5**, open symbols). In the reaction at 45℃, constant activity forming around 1.0-1.5 nmol IAA g-FW^{-1} h^{-1} was shown in all leaf and root tissues except 20-day-old leaves and roots **(Fig. 5A-C)**. Fluctuation pattern of the activity at 55℃, in healthy tissues, was exhibited to be resemble to that at 45℃ in each tissue **(Fig. 5D-F)**. At the both of reaction temperatures, it appeared that activity in hypocotyl was highest, one in root was the same or somewhat less, and leaf one was significantly lower than that of the others.

Amidase activity in tissues of *P. brassicae*-infected plants was determined to compare to that in healthy tissues **(Fig. 5**, closed symbols). Unlike in healthy tissues, fluctuations of amidase activity in infected tissues were varied not only between the two reaction temperatures but also in each tissue. In leaf of infected plants, amidase activity at both 45 and 55℃ reaction was lower than that of healthy tissue **(Fig. 5A, D)**. In infected hypocotyls, the activity at 45℃ was comparable to that in healthy one, whereas the activity at 55℃ was higher than that of healthy tissue at all the points examined **(Fig. 5B, E)**. The effect of disease infection on amidase activity in roots was complicated. In the reaction at 45℃, the level of activity was comparable at 20 and 25 dag but obviously overwhelmed the level in healthy tissue at 30 and 35 dag **(Fig. 5C)**. The increased activity, 1.78 nmol IAA formed g-FW^{-1} h^{-1}, reached up to 2.5-fold higher than that in healthy roots. In contrast, the activity at 55℃ was much higher in infected roots at 20 and 25 dag than that in healthy one, whereas during 30 to 40 dag, the activity was comparable or less **(Fig. 5F)**.

IAA content in turnip tissues

To evaluate the contribution of amidase activity to *de novo* IAA synthesis, IAA contents in the tissues were determined in the healthy and infected plants.

In leaves of healthy plants, free IAA was detected at 0.1 ng g-FW^{-1} during 20-25 dag but decreased to 0.05 ng g-FW^{-1} during 35-40 dag **(Fig. 6A**, open bars). Total IAA contents in healthy leaves reduced similarly as that of free IAA from 2.7 at 20 dag to 1.5 ng g-FW^{-1} at 40 dag. **(Fig. 6D**, open bars). In healthy hypocotyls, free IAA concentration was in a constant

level (around 0.1 ng g-FW^{-1}) during 20 to 35 dag (**Fig. 6B**, open bars). On the other hand, total IAA content in healthy hypocotyls was much higher than that in leaves, but the level was gradually reduced as the tissue became mature (**Fig. 6E**, open bars). In roots, very high amount of free IAA (0.5 ng g-FW^{-1}) was found at 20 dag, but the amount decreased to the levels comparable to that in leaves and hypocotyls (**Fig. 6C**, open bars). Total IAA in healthy roots was reduced in the later dag to that of healthy hypocotyls.

Infection with *P. brassicae* and thereafter the development of clubroot disease obviously affected IAA contents in a tissue-specific manner (**Fig. 6**, closed bars). In infected leaves, both free and total IAA levels were lower than those in healthy ones, which correlated to reduction of growth of leaves (**Fig. 6A, D**). In infected hypocotyls at 20 dag, free IAA level was significantly low compared to that of leaves, but total IAA contents significantly increased (**Fig. 6B, E**). On the other hand, in infected roots, considerable increase of free IAA content was observed at 20 dag, but the level was acutely reduced at during 25 to 35 dag (**Fig. 6C**). However, total IAA content in the root infected reached the 10-times higher level over whole range of the growth compared to that in healthy roots (**Fig. 6F**). The increased levels of total IAA observed in infected roots and hypocotyls was suggested to be resulted from inactivation of activity in conversion of free IAA to conjugated forms, which may end up reduction of free IAA content despite of up-regulation of its *de novo* synthesis.

DISCUSSION

Characteristics of turnip amidase

In temperature optimization study, two summits of amidase activity in turnip crude extract were found at 45 and 55℃ (**Fig. 2**). In addition, remaining activities measured at 45 and 55℃ after the heat treatment of the enzyme were significantly difference in 55℃ assay (**Fig. 3**). pH optimum also differed (**Fig. 4**). These results strongly suggested that more than two different amidases were present in the turnip. This was also supported by the evidence that the activities measured at 45 and 55℃ fluctuated differently in healthy and clubroot-diseased turnip tissues (**Fig. 5**).

Although amidase activity has been observed in various plants, this is the first case that plural numbers of amidases with different characteristics are present in a plant. Differential fluctuations of the activities at 45 and 55℃ indicated that each amidase must have specific roles in turnip tissues. It remains to be elucidated that the different features of turnip amidase activity is due either to occurrence of several isozymes encoded by different genes or several active forms derived from one gene. The latter will be resulted from different post-translational modifications. Isolation and cloning of amidases from turnip and further analyses of them will advance our understanding of biological roles and impacts of plant amidase on IAA homeostasis.

Involvement of amidase in vegetative growth and clubroot development of turnip

In Arabidopsis it was shown that mRNA and protein of amidase were abundant in leaf and inflorescence but very poor in root (Pollmann *et al*., 2006). In turnip, on the other hand, amidase activity was higher in hypocotyl and root than that in leaf (**Fig. 5**). This inconsistence in tissue localization indicates that biological functions of amidase are different between turnip and Arabidopsis. Growing turnip hypocotyl, corresponding to an edible tubercle, contains relatively high free IAA until 30-35 dag, and markedly high in total IAA compared to that in leaf (**Fig. 6**). Thus, turnip amidase may be involved in hypertrophy of the tubercle via IAA synthesis.

IAA is also a key factor of clubroot development. For the control of the disease, it is important to understand molecular mechanism of IAA synthesis pathway that is specifically up-regulated during clubroot development. Tryptophan dependent and independent pathways for IAA *de novo* synthesis are known in plants, and the former includes three IAA-synthesizing enzymes, i.e., nitrilase, aldehyde oxidase and amidase. In this study we focused on amidase, which has never been examined in association with clubroot disease. First of all, fluctuations of free and total IAA contents during clubroot development were determined. In infected roots, free IAA contents were significantly enhanced at 20 and 40 dag compared to those in healthy roots (**Fig. 6C**), indicating that IAA has important roles in early growth and maturation of clubroot. Unexpectedly, the free IAA level in infected roots was less than those in healthy roots during 25 to 35 dag. However, total IAA contents remained at a much higher level in infected roots during this period as well as at 20 and 40 dag. Thus, it is highly possible that IAA-conjugating activity was stronger than *de novo* IAA synthesizing activity in the period in infected roots, resulting in the apparent low free IAA level. Interestingly, in the above two phases in which free IAA level increased in infected roots, differential fluctuations of amidase activities were observed in the assay at 45 and 55℃ , i.e., activity at 45℃ was enhanced between the middle and later phase of the infected root development but not changed in the early phase, and activity at 55℃ contrarily increased especially in the early phase (**Fig. 5C, F**). We had found that patterns of gene expression of nitrilase isoforms were clearly different between the early and later phase (Ishikawa *et al*., in press). These absent observations suggest that different events inducing auxin overproduction may occur in the early and later phase. In addition, our results showed the possibility that amidase(s) is also involved in IAA synthesis induced by *P. brassicae* infection in an isoform-specific manner. In future studies, further molecular characterization as well as loss-of-function analyses of auxin-producing enzymes are necessary in order to identify the crucial factor(s) for clubroot development as a cue to control the disease.

REFERENCES

Ando, S., S. Tsushima, A. Tagiri, S. Kamachi, K. Konagaya, T.

Hagio and Y. Tabei. 2006. Increase in *BrAO1* gene expression and aldehyde oxidase activity during clubroot development in Chinese cabbage (*Brassica rapa* L.) *Mol. Plant Pathol.*, **7**: 223-234.

Arai, Y., M. Kawaguchi, K. Syono and A. Ikuta. 2004. Partial purification of an enzyme hydrolyzing indole-3-acetamide from rice cells. *J. Plant Res.*, **117**: 191-198.

Bartling, D., M. Seedorf, A. Mithöfer and E.W. Weiler. 1992. Cloning and expression of an Arabidopsis nitrilase which can convert indole-3-acetonitrile to the plant hormone, indole-3-acetic acid. *Eur. J. Biochem.*, **205**: 417-424.

Camilleri, C. and L. Jouanin. 1991. The TR-DNA region carrying the auxin synthesis genes of the *Agrobacterium rhizogenes* agropine-type plasmid pRiA4: nucleotide sequence analysis and introduction into tobacco plants. *Mol. Plant Microbe. Interact.*, **4**: 155-162.

Grsic, S., B. Kirchheim, K. Pieper, M. Fritsch, W. Hilgenberg and J. Ludwig-Müller. 1999. Induction of auxin biosynthetic enzymes by jasmonic acid and in clubroot diseased Chinese cabbage plants. *Physiol. Plant.*, **105**: 521-531.

Grsic-Rausch, S., P. Kobelt, J.M. Siemens, M. Bischoff and J. Ludwig-Müller. 2000. Expression and localization of nitrilase during symptom development of the clubroot disease in Arabidopsis. *Plant Physiol.*, **122**: 369-378.

Hatakeyama, K., M. Fujimura, M. Ishida and T. Suzuki. 2004. New classification method for *Plasmodiophora brassicae* field isolates in Japan based on resistance of F1 cultivars of Chinese cabbage (*Brassica rapa* L.) to clubroot. *Breed. Sci.*, **54**: 197-201.

Hirai, M., T. Harada, N. Kubo, M. Tsukada, K. Suwabe and S. Matsumoto. 2004. A novel locus for clubroot resistance in *Brassica rapa* and its linkage markers. *Theor. Appl. Genet.*, **108**: 639-643.

Inzé, D., A. Follin, M. Van Lijsebettens, C. Simoens, C. Genetello, M. Van Montagu and J. Schell. 1984. Genetic analysis of the individual T-DNA genes of *Agrobacterium tumefaciens*; further evidence that two genes are involved in indole-3-acetic acid synthesis. *Mol. Gen. Genet.*, **194**: 265-274.

Ishikawa, T., K. Okazaki, H. Kuroda, K. Itoh, T. Mitsui and H. Hori. 2007. Molecular cloning of *Brassica rapa* nitrilases and their expression during clubroot development. *Mol. Plant Pathol.*, **8**: *in press*.

Jones, D. R. and D. S. Ingram. 1982a. Characterization of isolates derived from single resting spores of *Plasmodiophora brassicae* and studies of their interaction. *Plant Physiol.*, **31**: 239-246.

Jones, D. R. and D. S. Ingram. 1982b. Factors affecting tests for differential pathogenicity in populations of *Plasmodiophora brassicae*. *Plant Physiol.*, **31**: 229-238.

Kawaguchi, M., S. Fujioka, A. Sakurai, Y.T. Yamaki and K. Syono. 1993. Presence of a pathway for the biosynthesis of auxin via indole-3-acetamide in trifoliata orange. *Plant Cell Physiol.*, **34**: 121-128.

Kawaguchi, M., M. Kobayashi, A. Sakurai and K. Syono. 1991. The presence of an enzyme that converts indole-3-acetamide into IAA in wild and cultivated rice. *Plant Cell Physiol.*, **32**: 143-149.

Kuginuki, Y., H. Yoshikawa, and M. Hirai. 1999. Variation in virulence of *Plasmodiophora brassicae* in Japan tested with clubroot-resistant cultivars of Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*). *Eur. J. Plant Pathol.*, **105**: 327-332.

Ludwig-Müller, J., E. Epstein and W. Higenberg. 1996. Auxin-conjugate hydrolysis in Chinese cabbage: characterization of an amidohydrolase and its role during infection with clubroot disease. *Physiol. Plant.*, **97**: 627-634.

Magie, A. R., E. E. Wilson and T. Kosuge. 1963. Indoleacetamide as an intermediate in the synthesis of indoleacetic acid in *Pseudomonas savastanoi*. *Science*, **141**: 1281-1282.

Neuhaus, K., S. Grsic-Rausch, S. Sauerteig and J. Ludwig-Müller. 2000. Arabidopsis plants transformed with nitrilase 1 or 2 in antisense direction are delayed in clubroot development. *J. Plant Physiol.*, **156**: 756-761.

Piao, Z. Y., Y. Q. Deng, S. R. Choi, Y. J. Park and Y. P. Lim. 2004. SCAR and CAPS mapping of CRb, a gene conferring resistance to *Plasmodiophora brassicae* in Chinese cabbage (*Brassica rapa* ssp. *pekinensis*). *Theor. Appl. Genet.*, **108**: 1458-1465.

Pollmann, S., D. Neu, T. Lehmann, O. Berkowitz, T. Schafer and E. W. Weiler. 2006. Subcellular localization and tissue specific expression of amidase 1 from *Arabidopsis thaliana*. *Planta,* **224**:1241-1253

Pollmann, S., D. Neu and E. W. Weiler. 2003. Molecular cloning and characterization of an amidase from *Arabidopsis thaliana* capable of converting indole-3-acetamide into the plant growth hormone, indole-3-acetic acid. *Phytochemistry*, **62**: 293-300.

Rajagopal, R., K. Tsurusaki, G. Kannangara, S. Kuraishi and N. Sakurai. 1994. Natural occurrence of indoleacetamide and amidohydrolase activity in etiolated aseptically-grown squash seedlings. *Plant Cell Physiol.*, **35**: 329-339.

Seo, M., S. Akaba, T. Oritani, M. Delarue, C. Bellini, M. Caboche and T. Koshiba. 1998. Higher activity of an aldehyde oxidase in the auxin-overproducing *superroot1* mutant of *Arabidopsis thaliana*. *Plant Physiol.*, **116**: 687-693.

Siemens, J., M. Nagel, J. Ludwig-Müller and M. D. Sacristán. 2002. The interaction of *Plasmodiophora brassicae* and *Arabidopsis thaliana*: parameters for disease quantification and screening of mutant lines. *J. Phytopathol.*, **150**: 592-605.

Schröder G., S. Waffenschmidt, E. W. Weiler and J. Schröder. 1984. The T-region of Ti plasmids codes for an enzyme synthesizing indole-3-acetic acid. *Eur. J. Biochem.*, **138**: 387-391.

Suwabe, K., H. Tsukazaki, H. Iketani, K. Hatakeyama, M. Fujimura, T. Nunome, H. Fukuoka, S. Matsumoto and M. Hirai. 2003. Identification of two loci for resistance to clubroot (*Plasmodiophora brassicae* Woronin) in *Brassica rapa* L. *Theor. Appl. Genet.*, **107**: 997-1002.

Thomashow, L. S., S. Reeves and M. F. Thomashow. 1984. Crown gall oncogenesis: evidence that a T-DNA gene from the *Agrobacterium* Ti plasmid pTiA6 encodes an enzyme that catalyzes synthesis of indoleacetic acid. *Proc. Natl. Acad. Sci. USA*, **81**: 5071-5075.

Ugajin, T., K. Takita, H. Takahashi, S. Muraoka, T. Tada, T. Mitsui, T. Hayakawa, T. Ohyama and H. Hori. 2003. Increase in indole-3-acetic acid (IAA) level and nitrilase activity in turnips induced by *Plasmodiophora brassicae* infection. *Plant Biotechnol.*, **20**: 215-220.

インドール-3-アセトアミドをインドール-3-酢酸に変換する酵素アミダーゼのカブ根こぶ病における役割の評価

石川寿樹・黒田春香・岡崎桂一・伊藤紀美子・三ツ井敏明・堀　秀隆 *

（平成19年 6 月29日受付）

要　約

根こぶ病菌 *Plasmodiophora brassicae* による根こぶ形成の分子機構解明を目的にオーキシン合成酵素の1つアミダーゼ活性を、カブ組織から可溶性粗酵素画分を調製し測定した。活性最適温度は45及び55℃であり、最適 pH は、反応温度45℃で pH 8.5、55℃で pH 7.5付近であった。熱安定性を調べる為、粗酵素画分を45または55℃で加熱し、その後45及び55℃の反応温度で残存活性を測定した。45℃熱処理では、両測定温度で失活パターンは同一であった。55℃熱処理では、45℃での活性は70% 失活し、55℃では60％の活性が残存した。

発芽後20～40日の間 5 日毎に健全カブと根こぶ病感染カブの葉、胚軸、根のアミダーゼ活性と IAA 含量を測定し根こぶ病とアミダーゼの関係を調べた。健全カブでは、上記全組織で安定なアミダーゼ活性があり恒常的な機能が推測された。一方感染カブの根で初期に55℃での活性が、後期には45℃の活性がそれぞれ健全カブより増加した。本研究で、複数のアミダーゼが存在し、それらが独立で根こぶ病発現に関与する可能性が初めて示唆された。

新大農研報, 60:53-60, 2007

キーワード：*Brassica rapa*、*Plasmodiophora brassicae*、アミダーゼ、インドール-3-酢酸、インドール-3-アセトアミド

新潟大学自然科学研究科

* 代表著者 : hide-hri@gs.niigata-u.ac.jp