Ethylene Production during Clubroot Development in Turnip

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(Received January 31, 2011)

Summary

Auxin and cytokinin play a crucial role for initiation stage of clubroot development in *Plasmodiophora brassicae*-infected cruciferous plants. On the other hand, the roles of phytohormones in a later maturation stage of clubroot remain unclear, regardless of significant accumulation of endogenous auxin indole-3-acetic acid (IAA) in fully developed clubroot getting deteriorated. Here we analyzed the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) contents and ACC oxidase (ACO) activity during clubroot development in turnip (*Brassica rapa*) to evaluate involvement of ethylene in clubroot development. Ethylene biosynthesis capacity estimated from endogenous ACC contents and ACO activity was not significantly affected in early growing clubroot but was elevated in later maturating tissues. This result suggests that ethylene biosynthesis is affected by *P. brassicae* infection and its level was coordinated with IAA during the maturation phase. Real-time PCR analysis of nitrilase, an IAA biosynthetic enzyme, demonstrated that treatment of turnip roots with ACC inhibits nitrilase expression, whereas ACC-dependent ethylene biosynthesis is well-known to be upregulated by IAA. These insights imply that IAA biosynthesis via nitrilase precedes and induces ethylene production in the maturating clubroot. This is the first report that ethylene could be involved in the maturation and deterioration of clubroot.

Bull.Facul.Agric.Niigata Univ., 63(2):83-87, 2011

Key words : 1-aminocyclopropane-1-carboxylic acid, auxin, clubroot, ethylene

Clubroot disease is one of the most serious diseases of cruciferous plants worldwide. The pathogen *Plasmodiophora brassicae* parasitizes roots of various agriculturally important crops, causing abnormal gall formation. Recent works in our laboratory and others have revealed that plant hormones auxin and cytokinin play a crucial role in the early growth of clubroot (Devos *et al*., 2006; Ishikawa *et al*., 2007, 2010). In turnip, BrNIT-T1, which is an isoform of auxin-biosynthetic and cytokinin-responsible nitrilase, is upregulated and contributes to de novo synthesis of an endogenous auxin, indole-3-acetic acid (IAA) (Ishikawa *et al*., 2007, 2010). On the other hand, a significant accumulation of IAA was also observed in mature clubroot tissues, which almost finished growing and started to deteriorate (Grsic *et al*., 1999; Ugajin *et al*., 2003; Ishikawa *et al*., 2007, 2010). Compared to the recent progression for the initiation stage, very little is known about involvement of plant hormones in the maturation stage of clubroot.

Of the three turnip nitrilase isoforms that we identified previously, BrNIT-T4 has homology to another type of plant nitrilases, which catalyzes hydration and hydrolysis of β-cyano-L-alanine (AlaCN). Since AlaCN is known as a byproduct of ethylene, this type of nitrilases is believed to be associated with ethylene biosynthesis by detoxification of the byproduct. Interestingly, *BrNIT-T4* expression was drastically affected during clubroot development, e.g., significant downregulation during an early growing stage and a following increase in maturating clubroots (Ishikawa *et al*., 2007). These insights prompted us to evaluate effects of clubroot infection on ethylene biosynthesis during disease development. Due to the difficulties in direct measurement of ethylene synthesized in the underground root tissues, we determined endogenous levels of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), which is well-investigated as an indicator of the ethylene production level.

The level of ACC contents in non-infected roots was constant during 20 to 35 days after germination (dag) but sharply decreased at 40 dag **(Fig. 1A)**. The ACC level in infected roots was comparable to that in non-infected roots between 20 and 30 dag, but increased at 35 dag and the level remained higher over 45 dag **(Fig. 1A)**. The relative contents of ACC and IAA determined elsewhere (Ishikawa *et al*., 2007) are summarized in **Table 1.**

We then determined ACC oxidase (ACO) activity, which catalyzes the ethylene production from ACC. In the early clubroot-growing phase, ACO specific activity normalized with protein amounts was strongly reduced **(Fig. 1B; Table 1)**. However, ACO activity when normalized with fresh weight of root tissues did not decrease in the early phase **(Fig. 1C; Table 1)**, because of higher protein contents in infected tissues compared to those in non-infected roots. As mentioned above, ACC contents per fresh weight did not change significantly during the early phase **(Fig. 1A; Table 1)**. Therefore, it was estimated that ethylene production levels in the growing clubroot tissues were comparable to that in the non-infected roots, suggesting that ethylene might

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not involve in the early clubroot growth.

On the other hand, in the later phase, ACO specific activity per protein content was recovered to that in control roots, like the *BrNIT-T4* expression as reported previously **(Fig. 1B; Table 1**; Ishikawa *et al*., 2007). Both ACC content and ACO activity per tissue weight were significantly higher in the infected roots than those in the non-infected roots **(Fig. 1A, C; Table 1)**, indicating that ethylene production was very active in the later phase.

Based on these results, we hypothesize that in a late phase of clubroot development, upregulation of *BrNIT-T2* and the resulting free IAA accumulation (Ishikawa *et al*., 2007, 2010) could induce ethylene production, despite the fact that the increases of ACC contents and ACO activity were already initiated by 35 dag prior to the significant increase of free IAA observed at 40 dag **(Table 1)**. The first reason is that auxin is known to induce ethylene production by upregulation of ACC synthase activity (Yoshii and Imaseki, 1982; Abel *et al*., 1995). On the other hand, we observed that *BrNIT-T1* and *BrNIT-T2* expressions were strongly downregulated by treatment with ACC **(Table 2)**, indicating that the turnip nitrilases are negatively regulated by ethylene. Thus, upregulation of *BrNIT-T2* and following increase of free IAA could promote ACC accumulation, but not *vice versa*. The second reason is that the local increase of free IAA may be underestimated when analyzed using whole tissues. The level of free IAA in infected tissues was lower than that in control roots during the intermediate phase (30-35 dag) **(Table 1)**, but our previous study of IAA immunolocalization revealed the presence of abundant IAA only within the cells containing pathogen in infected tissues at 30 dag (Ishikawa *et al*., 2010). In fact, free IAA contents increased from 30 to 35 dag in the infected roots (Ishikawa *et al*., 2007), although the level was lower compared to that in the non-infected roots **(Table 1)**. We speculate that ACC production was induced by IAA that accumulated in the pathogen-infected cells at 35 dag, whose amount might be diluted in the extract from whole tissues.

However, other possibilities can not be excluded so far. Instead, there are several reports insisting that ethylene promotes IAA biosynthesis in Arabidopsis seedlings (Růžiĉka *et al*., 2007; Stepanova *et al*., 2007; Swarup *et al*., 2007), although nothing is known which IAA synthetic pathways are influenced by ethylene. In addition to nitrilase, aldehyde oxidase (Ando *et al*., 2006) and IAA-amide conjugate hydrolase (Ludwig-Müller *et al*., 1996) are possible to contribute to free IAA production in Chinese cabbage clubroot. Since *BrNIT-T1* and *BrNIT-T2* were clearly downregulated by ACC **(Table 2)**, it should be addressed whether other IAA biosynthetic enzymes are involved in crosstalk between IAA and ethylene in maturation phase of clubroot development in future studies.

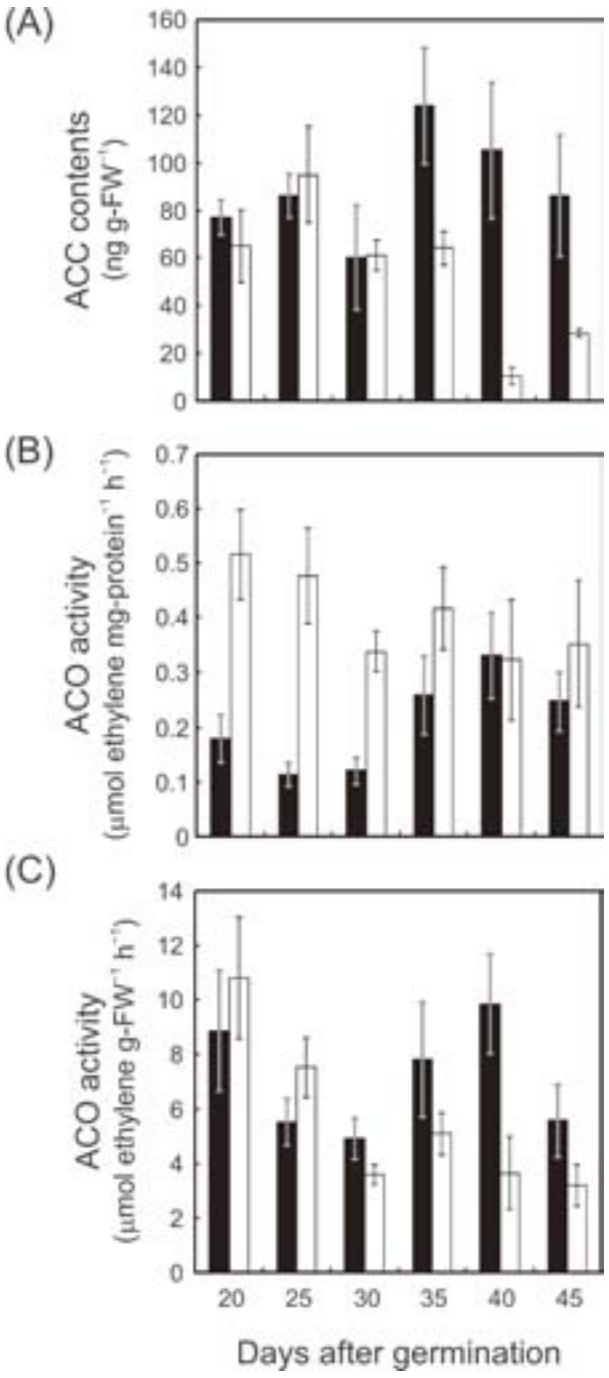

Fig. 1　ACC content and ACO activity during clubroot development.

(A) Free ACC contents in infected (closed bar) and healthy (open bar) roots. (B) ACC oxidase activity was standardized by protein amounts (B) or by tissue weights (C). Values show mean ± SD (n = 5).

Table 1. Comparisons of auxin contents and ethylene producing activity between infected and non-infected roots.

		(Dag)					
		20	25	30	35	40	45
Free IAA	Ratio[a]	1.64	0.87	0.39	0.41	1.61	0.35
	Significance[b]	*		***	***	*	***
Total IAA	Ratio[a]	7.15	8.89	11.14	13.58	9.91	7.30
	Significance[b]	***	***	***	***	***	***
ACC	Ratio[a]	1.19	0.91	0.98	1.93	10.16	3.03
	Significance[b]				**	**	**
ACO	Ratio[a]	0.35	0.24	0.36	0.62	1.02	0.70
(mg protein^{-1})	Significance[b]	***	***	***	**		
ACO	Ratio[a]	0.82	0.73	1.37	1.54	2.70	1.75
(g FW^{-1})	Significance[b]		**	*	*	***	*

[a]Ratio of values in infected roots to those in non-infected roots.
[b]Statistical analysis (Student's *t*-test between infected and non-infected) was performed using seven independent data: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Table 2. Effects of various reagents on expression of *BrNIT-Ts* in turnip roots.

		Expression ratio to *β-actin* (% control)[†]		
Reagents	μM	*BrNIT-T1*	*BrNIT-T2*	*BrNIT-T4*
IAN ‡	100	113 ± 27	73 ± 11	132 ± 75
IAA ‡	10	60 ± 18	71 ± 15	127 ± 48
ACC	100	26 ± 9	36 ± 11	141 ± 19
KCN	100	56 ± 21	102 ± 29	114 ± 49
AlaCN	100	92 ± 20	97 ± 20	118 ± 64

[†]mRNA amounts of *BrNIT-Ts* were referenced to those of *β-actin* and expressed as percentage of non-treated control. Values show means ± SD from independent three experiments.
[‡]Referred from Ishikawa *et al.* (2010).

MATERIALS AND METHODS

1. Plant growth and pathogen inoculation

Cultivation of turnip (*Brassica rapa* L. cv Natsumaki 13-gou kokabu, The Musashino Seed Co., Ltd., Tokyo, Japan) and inoculation with *Plasmodiophora brassicae* were performed as described previously (Ishikawa *et al.*, 2007).

2. Determination of endogenous ACC

1-Aminocyclopropane-1-carboxylic acid (ACC) was measured by gas-chromatography followed by chemical conversion to ethylene according to Lizada and Yang (1979) with some modifications by Coleman and Hodges (1991). Briefly, plant tissue (0.5-1 g) was homogenized and extracted twice in 80% (v/v) methanol. After vacuum concentration of the supernatants, the residue was resuspended in 450 μl of distilled water and treated with perchloric acid to remove interfering protein contamination. Four hundred μl of the de-proteinized extract was transferred to a vial (17×60 mm) with a silicon septum and mixed with 500 μl of ice-cold 20 mM $HgCl_2$. Reaction was started by addition of 200 μl of an ice-cold mixture of 5% NaOCl and saturated NaOH (2:1, v/v) with a syringe through the septum. The vial was allowed to stand on ice for 5 min followed by vortexing for 15 sec, and 1 ml of the head space gas was injected to a gas-chromatograph (GC-4000, GL Sciences Inc., Tokyo, Japan) equipped with an activated alumina column (60-80 mesh, 2 m ×3 mm). The injector and column were maintained at 80℃ and helium was used as carrier gas at 30 ml min^{-1}. A flame ionization detector was used at 200℃ with a flow of 35 ml min^{-1} of hydrogen and 400 ml min-1 of air. ACC concentrations were determined by a calibration curve using authentic standard.

3. Determination of ACO activity

Enzyme activity of ACC oxidase (ACO) was measured according to the method described by Rodriguez-Gacio and Matilla (2001) with slight modifications. Frozen tissue (200

mg) was powdered in liquid N_2 with a mortar and pestle and extracted in twice volumes of extraction buffer [200 mM HEPES, pH 7.0, 10 mM ascorbic acid, 10% (v/v) glycerol, 5 mM DTT]. The insoluble residue was removed by centrifugation at 15,000×*g* for 5 min at 4℃ and the supernatant was used as protein sample for ACC oxidase assay. The protein sample (100 μl) was reacted with 1 mM ACC in a 2 ml total volume of reaction buffer [200 mM HEPES, pH 7.0, 10 μM $FeSO_4$, 30 mM $NaHCO_3$, 24 mM ascorbic acid, 10% (v/v) glycerol, 1 mM DTT] in a 13-ml vial with a rubber septum. After incubation for 60 min at 30℃, 1 ml of head space gas was collected and analyzed by gas chromatography as described above. Protein concentration was determined by Bradford's method (Bradford, 1976).

4. Real-time PCR analysis

Sterilized turnip seeds were sown in 40 ml of 1/2 strength MS medium (Sigma, St. Louis, MO) supplemented with 1% (w/v) sucrose in a 200-ml flask and incubated at 25℃ with a constant rotation at 60 rpm under continuous light at 80 μmol m^{-2} s^{-1}. One week after sowing, the culture was added with reagents and incubated under the same conditions for additional 24 h. Roots from the 8 seedlings were pooled and used for total RNA extraction. Real-time RT-PCR analysis was performed according to the published method (Ishikawa *et al*., 2007, 2010). Measurement was repeated three times using independent pools.

REFERENCES

Abel, S., Nguren, M.D., Chow, W. and Theologis, A. 1995. ACS4, a primary indoleacetic acid-responsive gene encoding 1-aminocyclopropane-1-carboxylate synthase in *Arabidopsis thaliana*. Structural characterization, expression in *Escherichia coli*, and expression characteristics in response to auxin. *J. Biol. Chem*. **270**: 19093-19099.

Ando, S., Tsushima, S., Tagiri, A., Kamachi, S., Konagaya, K., Hagio, T. and Tabei, Y. 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.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*. **72**: 248-254.

Coleman, L.W. and Hodges, C.F. 1991. Interference with the determination of 1-aminocyclopropane-1-carboxylic acid by various plant proteins. *J. Plant Physiol*. **138**: 7-11.

Devos, S., Laukens, K., Deckers, P., van der Straeten, D., Beeckman, T., Inze, D., Van Onckelen, H., Writters, E. and Prinsen, E. 2006. A hormone and proteome approach to picturing the initial metabolic events during *Plasmodiophora brassicae* infection on Arabidopsis. *Mol. Plant-Microbe Interact*. **19:** 1431-1443.

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

Ishikawa, T., Okazaki, K., Kuroda, H., Itoh, K., Mitsui, T. and Hori, H. (2007) Molecular cloning of *Brassica rapa* nitrilases and their expression during clubroot development. *Mol. Plant Pathol*. **8**: 623-637.

Ishikawa, T., Okazaki, K., Nagaoka, T., Itoh, K., Mitsui, T. and Hori, H. 2010. Isoform-specific localization of *Brassica rapa* nitrilases in root infected with *Plasmodiophora brassicae* revealed using in situ hybridization probes improved with locked nucleic acids. *J. Plant Growth Regul*. **29**: 210-222.

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

Rodriguez-Gacio, M.C. and Matilla, A.J. 2001. The last step of the ethylene biosynthesis pathway in turnip tops (*Brassica rapa*) seeds: Alterations related to development and germination and its inhibition during desiccation. *Physiol. Plant*. **112**: 273-279.

Růžička, K., Ljung, K., Vanneste, S., Podhorska, R., Beeckman, T., Friml, J. and Benkova, E. 2007. Ethylene regulates root growth through effects on auxin biosynthesis and transport-dependent auxin distribution. *Plant Cell* **19**: 2197-2212.

Stepanova, A.N., Yun, J., Likhacheva, A.V. and Alonso, J.M. 2007. Multilevel interactions between ethylene and auxin in Arabidopsis roots. *Plant Cell* 19: 2169-2185.

Swarup, R., Perry, P., Hagenbeek, D., van der Straeten, D., Beemster, G. T., Sandberg, G., Bhalerao, R., Ljung, K. and Bennett, M. J. 2007. Ethylene upregulates auxin biosynthesis in Arabidopsis seedlings to enhance inhibition of root cell elongation. *Plant Cell* **19**: 2186-2196.

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

Yoshii, H. and Imaseki, H. 1982. Regulation of auxin-induced ethylene biosynthesis. Repression of inductive formation of 1-aminocyclopropane-l-carboxylate synthase by ethylene. *Plant Cell Physiol*. **23**: 632-649.

カブ根こぶ病感染組織におけるエチレン合成

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（平成23年１月31日受付）

要　約

Plasmodiophora brassicae 感染による根こぶ組織形成の初期段階におけるオーキシンやサイトカイニンといった植物ホルモンの関与がよく知られる一方で、根こぶ組織の成熟、腐熟段階における植物ホルモンの関与はほとんどわかっていない。本研究では、根こぶ組織成熟過程へのエチレンの関与に着目し、根こぶ病の進行に伴うエチレン前駆体1- アミノシクロプロパン -1- カルボン酸（ACC）含量と ACC 酸化酵素（ACO）活性の変動を解析した。ACC 含量と ACO 活性から推定されるエチレン生合成活性は、肥大初期の根こぶ組織で大きな変化は無かったが、肥大後期から腐熟期にある根こぶ組織では大きく増加していることが示された。リアルタイム定量 PCR 解析の結果、ACC はオーキシン合成酵素ニトリラーゼの mRNA 発現を強く抑制することが明らかとなり、根こぶ成熟期におけるオーキシンの増加はエチレンの蓄積に先立って起こることが示唆された。

新大農研報, 63(2):83-87, 2011

キーワード：1- アミノシクロプロパン -1- カルボン酸、オーキシン、根こぶ病、エチレン

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