Bull. of Yamagata Univ., Nat. Sci., Vol.15, No.3, Feb. 2003

Inhibitions of Growth and Lateral Branch Development by Calmodulin Antagonists in Hairy Roots of *Lithospermum erythrorhizon*, *Atropa belladonna* and *Daucus carota*

Ryoichi Kato[†], Miyuki Arashida[†], Masahiro Kodama[†], Hiroshi Kamada[‡] and Takashi Suzuki[††]

(Received March 29, 2002)

Abstract

Hairy roots of *Lithospermum erythrorhizon*, belladonna and carrot, which were induced by inoculation with *Agrobacterium rhizogenes* harboring the Ri plasmid, were cultured on a medium containing 0.1, 1, 10, 30 or 100 μM W-7 or W-5, calmodulin antagonists. Growth rates of *L. erythrorhizon* and belladonna hairy roots cultured on all media containing W-7 were lower than that of the roots cultured without W-7. Growth of carrot hairy roots was inhibited by W-7 above 30 μM. Inhibition rates of the root growth by high concentrations of W-5 were lower than those of the growth by the same concentrations of W-7. In the case of the development of lateral roots on hairy roots, 30 and 100 μM W-7 or W-5 inhibited formation of lateral roots. The number of lateral roots formed by culturing on a medium containing W-7 was lower than that of the roots formed on the medium containing W-5. These results strictly suggest that calmodulin acts upon the growth of hairy roots and the development of lateral roots on hairy roots.

[†]Biology Laboratory, Faculty of Education, Yamagata University, Yamagata, 990–8560 Japan

[‡]Institute of Biological Sciences, University of Tsukuba, Tsukuba, Ibaragi, 305–8572 Japan

[††]Science Laboratory, Faculty of Education, Yamagata University, Yamagata, 990–8560 Japan

Abbreviations

W-7, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; W-5, N-(6-aminohexyl)-naphthalenesulfonamide.

Key words

Atropa belladonna—Calmodulin antagonist—*Daucus carota*—Hairy roots—Root growth and lateral branch development—*Lithospermum erythrorhizon*.

1 Introduction

Calmodulin plays important roles in many physiological processes in plant cells as well as animal cells (Dieter 1984, Sun 2000, Chung et al. 2000, Lenartowska et al. 2001). Muto and Hirosawa (1987) reported that growth of adventitious roots formed in stem cuttings of *Tradescantia fluminensis* could be inhibited by incubation in a solution containing a low concentration of a calmodulin antagonist: trifluoperazine or chlorpromazine, and they discussed involvement of calmodulin in the growth of the roots. These antagonists bind very tightly to isolated calmodulin (Levin and Weiss 1976, 1977). However, an affinity of the antagonists for cytoplasmic membrane is so high that the antagonists seriously damage cultured mammalian cells (Osborn and Weber 1980). These reports suggest that using of trifluoperazine or chlorpromazine in *in vivo* experiments is unsuitable for detecting the action of calmodulin.

It has been clearly demonstrated that the Ri plasmid present in *Agrobacterium rhizogenes* causes the transformation of plant cells via the introduction of T-DNA from the Ri plasmid into the genomic DNA of plant cells. It has also been demonstrated that the transformed plant cells give rise to massive roots, the so-called hairy roots (White and Nester 1980, Tepfer 1984, Shanks and Morgan 1999, Giri and Narasu 2000). The transformed plant cells can continue to produce hairy roots even after *Agrobacterium* was eliminated. Hairy roots grow vigorously in a phytohormone-free medium and provide a useful material for studies on secondary metabolite production (Kamada et al. 1986, Bourgaud et al. 2001, Facchini 2001, Kim et al. 2002). But, there are very few physiological investigations into hairy root growth (Kato et al. 1989, Bonhomme et al. 2000, Tanaka et al. 2001) and lateral branch development on hairy roots (Morgan and Shanks 2000, Balvanyos et al. 2001).

In this report, we investigated the involvement of calmodulin in the growth of hairy roots and the development of lateral branches on the roots,

using W-7 and W-5 as calmodulin antagonists. W-7 and W-5 can penetrate cytoplasmic membrane and distribute mainly in cytoplasm (Hidaka et al. 1981).

2 Materials and Methods

Plant material—The procedures for the induction of hairy roots were described by Kato et al. (1989) and by Shimomura et al. (1991). Carrot seeds (*Daucus carota* L. cv. US-Harumakigosun) were sown on vermiculite at 25°C under the continuous light (ca. 3 k lux). One-week-old seedlings were surface-sterilized with 1% sodium hypochlorite solution for 15 min and rinsed three times with sterilized distilled water. Sterile belladonna (*Atropa belladonna* L.) and *Lithospermum erythrorhizon* Sieb. et Zucc. plants were obtained by culturing the shoot tips of field-grown plants. They were maintained by repeated shoot culture on a Murashige and Skoog's (MS) medium containing 3% sucrose and 1% agar, at 25°C under a 18-h light (ca. 4 k lux) / 6-h dark condition. Carrot hypocotyls, belladonna and *L. erythrorhizon* internodes were cut into 20-mm-long segments and placed with the basal part uppermost on the MS medium. The cut ends of these segments were inoculated by a needle with *Agrobacterium rhizogenes*, which had been grown on YEB medium (Vervliet et al. 1974) containing 1.5% agar. The segments of belladonna and *L. erythrorhizon* were infected with *A. rhizogenes* strain 15834 and those of carrot were infected with strain 2659. Hairy roots appeared at the inoculation sites three weeks later. The growing root tips of the hairy roots were cut off and cultured on an MS medium containing 3% sucrose, 1% agar and an antibiotic (1 mg/ml carbenicillin) at 25°C under a 16-h light (ca. 3 k lux) / 8-h dark condition, to eliminate the bacteria from the roots. The root tips were subcultured three more times at two-week intervals on a fresh MS medium supplemented with the antibiotic. The root tips of belladonna and carrot hairy roots were re-subcultured on an MS medium containing 3% sucrose and 0.2% Gellan Gum (Kelco, Division of Merck & Co., Inc., San Diego, U.S.A) without antibiotic at monthly intervals at 25°C. The tips of *L. erythrorhizon* hairy roots were re-subcultured at two-month intervals at 25°C on the same medium and later on a Root Culture (RC) medium [EMBO course manual 1982 as follows (mg/l): $Ca(NO_3)_2 \cdot 4H_2O$ (288), KNO_3 (80), KCl (65), $MgSO_4 \cdot 7H_2O$ (370), $Na_2SO_4 \cdot 10H_2O$ (226.7), $NaH_2PO_4 \cdot 4H_2O$ (21.5), H_3BO_3 (1.5), $ZnSO_4 \cdot 7H_2O$ (2.65), KI (0.75), $Na_2MoO_4 \cdot 2H_2O$ (0.25), $CuSO_4 \cdot 5H_2O$ (0.02), $MnCl_2 \cdot 4H_2O$ (6.0), $FeCl_3$ (3.2), EDTA-2Na (7.4), thiamine-HCl (0.1), nicotinic acid (0.5), pyridoxin-HCl (0.1) and glycine (3.0)] containing 1.5% sucrose and 0.3% Gellan Gum (pH 4.9).

Incubation with calmodulin antagonists—Segments (length: 15 mm) of root tips were cut from these hairy roots. Ten segments of belladonna and carrot hairy roots were put side by side in a Petri dish (diameter: 94 mm) on an MS medium containing 3% sucrose, 0.2% Gellan Gum and various concentrations (zero, 0.1, 1, 10, 30 and 100 μM) of W-7 or W-5. Ten segments of *L. erythrorhizon* hairy roots were put side by side in the same dish on a RC medium containing 1.5% sucrose, 0.3% Gellan Gum and various concentrations of W-7 or W-5. The dishes were covered with a double thickness of aluminum foil. They were incubated at 24°C for 6, 8, 13 or 16 days. Growth of each hairy root was measured with a slide caliper. At the carrot hairy roots, the number of lateral branches formed on the roots was visually counted.

W-7 and W-5 were obtained from Seikagaku Corporation, Tokyo, Japan.

3 Results

Established cultures of hairy roots exhibited rapid growth with lateral roots on the hormone-free media. In order to confirm that true transformation had occurred, the presence of opines (mannopine and agropine, or cucumopine) was monitored by the method described earlier (Kamada et al. 1986, Isogai et al. 1990), and both opines were detected (data not shown).

Growth rates—Thirty segments of *Lithospermum erythrorhizon* hairy roots were cultured on a RC medium containing zero, 0.1, 1, 10, 30 or 100 μM W-7 for 13 days. Growth rates of the hairy roots cultured on the all media containing W-7 were lower than that of the roots cultured without W-7 (zero), and then the rates of the roots became lower as W-7 concentrations increased (Fig. 1). Segments of *L. erythrorhizon* hairy roots were cultured for 13 days on the medium containing W-5 in the same concentrations of W-7. Growth rates of hairy roots cultured on the media containing W-5 below 10 μM were equal to that of the roots cultured without W-5 (zero), but then the rates of the roots cultured on the media containing W-5 above 30 μM were lower than that of the roots cultured without W-5 (Fig. 1).

We tried to determine whether the calmodulin antagonists inhibit the growth of hairy roots in other plant species. Segments of belladonna hairy roots were cultured on an MS medium containing zero, 0.1, 1, 10, 30 or 100 μM of W-7 or W-5 for 6 days. Growth of the roots was inhibited by W-7 above 0.1 μM or by W-5 above 1 μM (Fig. 2). Growth rates of the roots cultured on the medium containing W-7 were lower than those of the roots cultured on the medium containing W-5 at 10, 30 and 100 μM antagonists, respectively. And then a difference between inhibition rates by W-7 and W-5

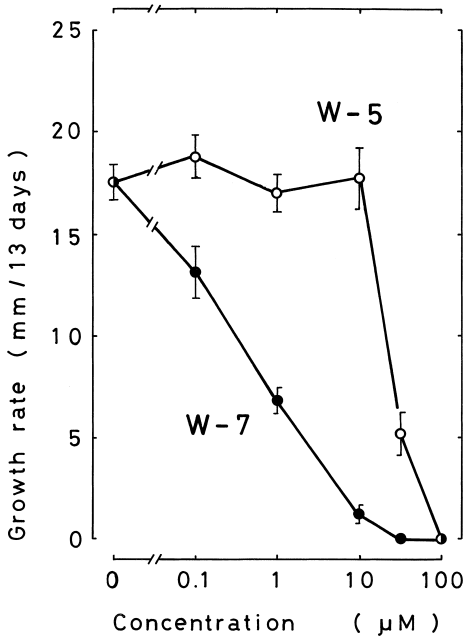

Fig. 1. Effects of W-7 and W-5 on the growth of *Lithospermum erythrorhizon* hairy roots. Segments (length: 15 mm) of the tips of *L. erythrorhizon* hairy roots were cultured on an MS medium containing zero, 0.1, 1, 10, 30 or 100 μM W-7 (●) or W-5 (○), in the dark at 24°C for 13 days. Each point represents the average of measurements from at least 30 segments of hairy roots, with the standard error.

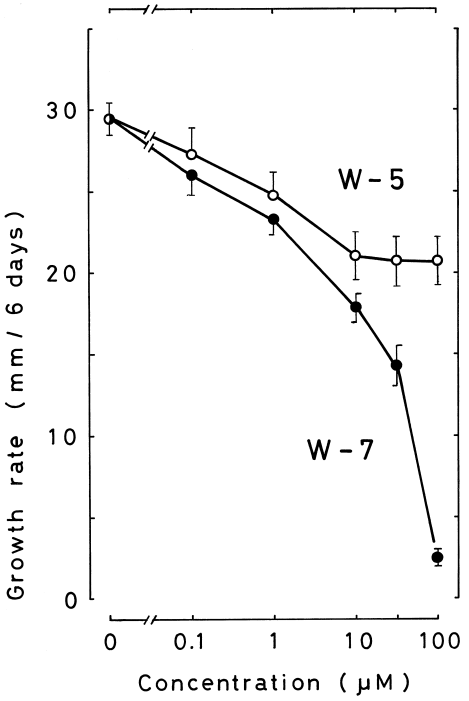

Fig. 2. Effects of W-7 and W-5 on the growth of belladonna hairy roots. Segments of the tips of belladonna hairy roots were cultured on an MS medium containing zero, 0.1, 1, 10, 30 or 100 μM W-7 (●) or W-5 (○), in the dark at 24°C for 6 days. Each point represents the average of measurements from at least 30 segments of hairy roots, with the standard error.

became higher as antagonists concentrations increased (Fig. 2).

Segments of carrot hairy roots were cultured on an MS medium containing zero, 0.1, 1, 10, 30 or 100 μM of W-7 or W-5 for 8 days. Growth rates of the roots cultured on three media containing 0.1, 1 and 10 μM antagonists were equivalent to those of the roots cultured without antagonist (zero) (Fig. 3). Thirty micro-mole W-5 did not inhibit the root growth, but 30 μM W-7 did (Fig. 3). Inhibition rate of the root growth by 100 μM W-7 was higher than that of the growth by 100 μM W-5 (Fig. 3).

Number of lateral branches—The effects of the calmodulin antagonists on the development of lateral roots on hairy roots were investigated in the next experiments. Thirty segments of carrot hairy roots were cultured on an MS medium containing zero, 0.1, 1, 10, 30 or 100 μM of W-7 or W-5 for 16 days. The number of the lateral roots formed on the media containing antagonists

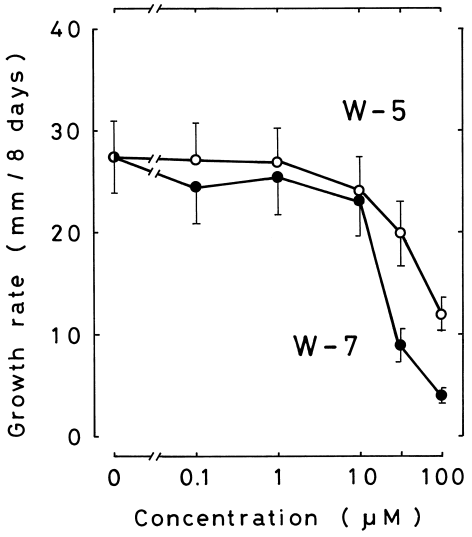

Fig. 3. Effects of W-7 and W-5 on the growth of carrot hairy roots. Segments of the tips of carrot hairy roots were cultured on an MS medium containing zero, 0.1, 1, 10, 30 or 100 μM W-7 (●) or W-5 (○), in the dark at 24°C for 8 days. Each point represents the average of measurements from at least 30 segments of hairy roots, with the standard error.

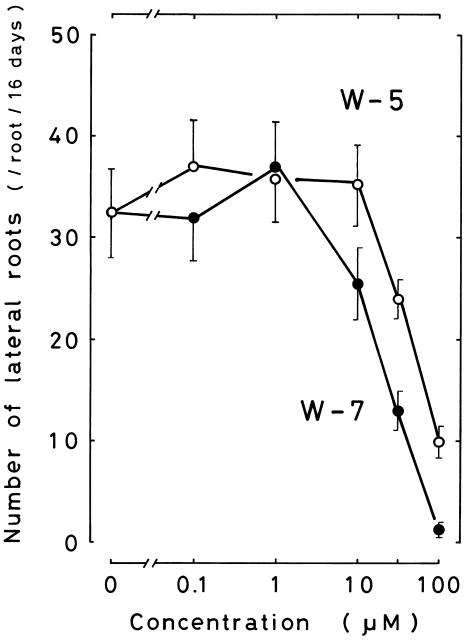

Fig. 4. Effects of W-7 and W-5 on the development of lateral roots on carrot hairy roots. Segments of the tips of carrot hairy roots were cultured on an MS medium containing zero, 0.1, 1, 10, 30 or 100 μM W-7 (●) or W-5 (○), in the dark at 24°C for 16 days. Each point represents the average of measurements from at least 30 segments of hairy roots, with the standard error.

below 10 μM was equal to that of the roots formed on an antagonist-free medium (zero), and then the number of lateral roots formed on antagonists above 30 μM was lower than that of the roots formed on an antagonist-free medium (Fig. 4). At 30 and 100 μM the antagonists, the number of lateral roots induced on the medium containing W-7 was lower than that of the roots induced on the medium containing W-5 (Fig. 4).

4 Discussion

Calmodulin regulates various physiological responses in animal and plant cells (Dieter 1984, Sun 2000, Chung et al. 2000, Lenartowska et al. 2001). It was shown that calmodulin involves in growth of adventitious roots induced in stem cuttings of *Tradescantia fluminensis*, by an incubation of the roots in a solution containing trifluoperazine or chlorpromazine (Muto and Hirosawa 1987). These calmodulin antagonists, however, tightly combine

with cytoplasmic membrane and damage the cells without direct inhibition of the calmodulin action (Osborn and Weber 1980). Applications of these antagonists to intact cells or tissues are unfit for a detection of the calmodulin function. Hairy roots, which are originated from transformation of plant cells by the Ri plasmid of *Agrobacterium rhizogenes*, have been induced from some plant materials to produce secondary metabolites (Kamada et al. 1986, Bourgaud et al. 2001, Facchini 2001, Kim et al. 2002). But, only a few studies have been performed on the analyses of hairy roots growth (Kato et al. 1989, Bonhomme et al. 2000, Tanaka et al. 2001) and lateral branch development on hairy roots (Morgan and Shanks 2000, Balvanyos et al. 2001).

We determined involvement of calmodulin in the growth of hairy roots using W-7 and W-5, which are able to penetrate cytoplasmic membrane (Hidaka et al. 1981). Segments of *L. erythrorhizon*, belladonna and carrot hairy roots were cultured on media containing various concentrations of W-7. Growth rates of *L. erythrorhizon* and belladonna hairy roots cultured on the media containing W-7 were lower than those of the roots cultured without W-7 (Figs. 1 and 2). Growth of carrot hairy roots was inhibited by W-7 above 30 μM (Fig. 3). Segments of those roots were cultured on media containing W-5, a chlorine-deficient analogue of W-7 that weakly interacts with calmodulin (Hidaka et al. 1981), at the same concentrations of W-7 to make a close examination of the role of calmodulin in the growth of hairy roots. Inhibition rates of the root growth by high concentrations of W-5 were lower than those of the growth by the same concentrations of W-7 (Figs. 1, 2 and 3). These results strictly suggest that calmodulin is concerned in hairy root growth. To the best of our knowledge, this is the first report proved exactly on involvement of calmodulin in the growth of plant roots.

In order to determine whether the calmodulin antagonists inhibit development of lateral roots on hairy roots, segments of carrot hairy roots were cultured on an MS medium containing various concentrations of W-7 or W-5. At 30 and 100 μM the antagonists, both W-7 and W-5 inhibited formation of lateral roots and the number of lateral roots formed on the medium containing W-7 was lower than that of the roots formed on the medium containing W-5 (Fig. 4). These results show that calmodulin acts upon the development of lateral roots on hairy roots.

The involvement of calmodulin in cell division in plant cells is suggested by the following facts. (1) Young dividing and growing cells contained more calmodulin than matured non-growing cells in pea seedlings (Muto and Miyachi 1984). (2) Distribution of calmodulin related to that of tubulin in the cell cycle of onion and pea root meristematic cells (Wick et al. 1985). (3) Calmodulin was localizing at kinetochore microtubles in the mitotic apparatus in *Haemanthus* endosperm cells (Vantard et al. 1885). Growth of hairy

roots consists of cell division and cell elongation. An important event in the development of lateral roots on hairy roots is cell division, which causes primordium formation of lateral roots. Inhibition rate of hairy root growth by each concentration of W-7 or W-5 shown in Figure 3 is similar to that of formation of lateral roots of the roots by W-7 or W-5 shown in Figure 4. These results suggest that calmodulin plays an important role in cell division in hairy roots. These may have some reference to the reports that calmodulin-binbing protein is involved in cell division in pea seedlings (Day et al. 2000) and that expression of calmodulin mRNA is closely correlated with cell division in tobacco anthers (Chen et al. 1999).

References

[Balvanyos et al. 2001] Balvanyos, I., L. Kursinszki and E. Szoke, Plant Growth Regul. 34: 339-345. 2001.

[Bonhomme et al. 2000] Bonhomme, V., D. Laurain-Mattar and M. A. Fliniaux, J. Nat. Prod. 63: 1249-1252. 2000.

[Bourgaud et al. 2001] Bourgaud, F., A. Gravot, S. Milesi and E. Gontier, Plant Sci. 161: 839-851. 2001.

[Chen et al. 1999] Chen, S. R., Y. T. Lu and H. Y. Yang, Chinese Sci. Bull. 44: 142-146. 1999.

[Chung et al. 2000] Chung, W. S., S. H. Lee, J. C. Kim, W. D. Heo, M. C. Kim, C. Y. Park, H. C. Park, C. O. Lim, W. B. Kim and J. F. Harper, Plant Cell 12: 1393-1407. 2000.

[Day et al. 2000] Day, I. S., C. Miller, M. Golovkin and A. S. N. Reddy, J. Biol. Chem. 275: 13737-13745. 2000.

[Dieter 1984] Dieter, P., Plant Cell Environ. 7: 371-380. 1984.

[EMBO course manual 1982] EMBO course manual, The use of Ti plasmid as cloning vector for genetic engineering in plants, August 4-23, p. 109. 1982.

[Facchini 2001] Facchini, P. J., Annu. Rev. Plant Physiol. 52: 29-66. 2001.

[Giri and Narasu 2000] Giri, A. and M. L. Narasu, Biotechnol. Adv. 18: 1-22. 2000.

[Hidaka et al. 1981] Hidaka, H., Y. Sasaki, T. Tanaka, T. Endo and S. Ohno,

Proc. Natl. Acad. Sci. U.S.A 78: 4354-4357. 1981.

[Isogai et al. 1990] Isogai, A., N. Fukuchi, M. Hayashi, H. Kamada, H. Harada and A. Suzuki, Phytocem. 29: 3131-3134. 1990.

[Kamada et al. 1986] Kamada, H., H. Okamura, M. Satake, H. Harada and K. Shimomura, Plant Cell Reports 5: 239-242. 1986.

[Kato et al. 1989] Kato, R., H. Kamada and M. Asashima, Plant Cell Physiol. 30: 605-608. 1989.

[Kim et al. 2002] Kim, Y., B. E. Wyslouzil and P. J. Weathers, In Vitro Cell Dev-Pl. 38: 1-10. 2002.

[Lenartowska et al. 2001] Lenartowska, M., M. I. Rodriguez-Garcia and E. Bednarska, Acta Biol. Cracov. Bot. 43: 117-123. 2001.

[Levin and Weiss 1976] Levin, R. M. and B. Weiss, Mol. Pharmacol. 12: 581-589. 1976.

[Levin and Weiss 1977] Levin, R. M. and B. Weiss, Mol. Pharmacol. 13: 690-697. 1977.

[Morgan and Shanks 2000] Morgan, J. A. and J. V. Shanks, J. Biotechnol. 79: 137-145. 2000.

[Muto and Hirosawa 1987] Muto, S. and T. Hirosawa, Plant Cell Physiol. 28: 1569-1574. 1987.

[Muto and Miyachi 1984] Muto, S. and S. Miyachi, Z. Pflanzenphysiol. 114: 421-431. 1984.

[Osborn and Weber 1980] Osborn, M. and K. Weber, Exp. Cell Res. 130: 484-488. 1980.

[Shanks and Morgan 1999] Shanks, J. V. and J. Morgan, Curr. Opin. Biotech. 10: 151-155. 1999.

[Shimomura et al. 1991] Shimomura, K., H. Sudo, H. Saga and H. Kamada, Plant Cell Reports 10: 282-285. 1991.

[Sun 2000] Sun, D. Y., Acta, Bot. Sin. 42: 441-445. 2000.

[Tanaka et al. 2001] Tanaka, N., Y. Fujikawa, M. A. M. Aly, H. Saneoka, K. Fujita and I. Yamashita, Plant Cell Tiss. Org. 66: 175-182. 2001.

[Tepfer 1984] Tepfer, D. A., Cell 37: 959-967. 1984.

[Vantard et al. 1985] Vantard, M., A.-M. Lambert, J. De Mey, P. Picquot and L. J. Van Eldik, J. Cell Biol. 101: 488-499. 1985.

[Vervliet et al. 1974] Vervliet, G., M. Holsters, H. Teuchy, M. Van Montagu and J. Schell, J. Gen. Virol. 26: 33-48. 1974.

[White and Nester 1980] White, F. F. and E. W. Nester, J. Bacteriol. 141: 1134-1141. 1980.

[Wick et al. 1985] Wick, S. M., S. Muto and J. Duniec, Protoplasma 126: 198-206. 1985.