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Identification and functional analysis of brassicicene C biosynthetic gene cluster in *Alternaria brassicicola*

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ABSTRACT

The biosynthetic gene cluster of brassicicene C was identified in *Alternaria brassicicola* strain ATCC 96836 from genome database search. In vivo and in vitro study clearly revealed the function of Orf8 and Orf6 as a fusicoccadiene synthase and methyltransferase, respectively. The understanding toward the biosynthetic pathway promises construction of this type of diterpene compounds with genetic engineering.
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Fusicoccin (FC) A[1,2] (**1**) and J[3,4] are diterpene glycosides produced by the plant-pathogenic fungus *Phomopsis amygdali* (Fig. 1). FC and structurally related cotylenins (CN),[5–7] e.g., cotylenin A (**2**) (Fig. 1), isolated from *Cladosporium* sp. 501-7W, display a phytohormone-like activity via the activation of the plasma membrane H^+-ATPase.[8] Crystallographic analysis of the ternary complex of a plant 14-3-3 protein, FC, and a phosphopeptide derived from the C-terminus of H^+-ATPase reveals that FC stabilizes the interaction between 14-3-3 protein and H^+-ATPase by forming ternary complex resulting in the continuous activation of H^+-ATPase.[9] Since 14-3-3 proteins are highly conserved in eukaryotes, FC/CNs are anticipated to act to animal cells. Actually, recent studies also show that CN induces the differentiation of human myeloid leukemia cells[10–12] and acts synergistically with IFN-α to induce apoptosis in a wide array of cancer cells.[13,14] These biological activities of FC/CNs show that FC/CN analogs are attractive tool to investigate the physiological roles of 14-3-3 proteins in intracellular signal transductions. Since complete understanding of the FC/CN biosynthesis facilitates biological and biochemical production of novel FC/CN analogs, the characterization and functional analysis of biosynthetic gene cluster of FC/CN and structurally related compounds are of great importance.

Biosynthetic study of FC has been investigated in detail from the analysis of biosynthetic intermediates and feeding experiments with labeled compounds. Concerning the characteristic 5-8-5 fused ring system of FC, fusicocca 2,10(14)-diene was determined to be the first tricyclic hydrocarbon metabolite in FC biosynthesis.[15] Feeding experiments with [19-$^2H_2$]-fusicocca 2,10(14)-diene clearly showed that oxidation of fusicocca 2,10(14)-diene results in the fusicocca 2,10(14)-dien-8β-ol.[16] Furthermore, based on the chemical structures and feeding experiments of FC H, FC J, isolated from *P. amygdali*, [1–4,17] the biosynthetic pathway of FC A is suggested to be that as shown in Figure 2A.

More recently, we identified fusicoccadiene synthase (PaFS) from *P. amygdali*, which is a unique chimeric enzyme possessing both a geranylgeranyl diphosphate (GGDP) synthase domain and a diterpene cyclase domain (Fig. 2B).[18] In addition, it was also revealed that a part of genes (*dioxygenase, cytochrome P450, short-chain dehydrogenase/reductase, mannosidase*), probably involved in the FC biosynthesis, were clustered near the *PaFS* (Fig. 2B).[18] However, the other genes, responsible for the biosynthesis of FC such as glycosyltransferase, acetyltransferase, prenyltransferase etc. were not identified around *PaFS* (data not shown). This indicates that FC biosynthetic genes are located in at least two loci, and it makes difficult to study the whole biosynthetic pathway of FC. Concerning CN biosynthetic gene cluster, on the other hand, lost of proliferation activity of *Cladosporium* sp. 501-7W, a CN producer, misses an opportunity to obtain its biosynthetic gene cluster. To clarify the whole biosynthetic machinery of FC/CN type compounds at the enzymatic level, therefore, screening of additional gene sources related to FC/CN biosynthesis are essential.

In order to obtain additional gene sources, genome databases were searched with *PaFS*, a characteristic gene possessing both a

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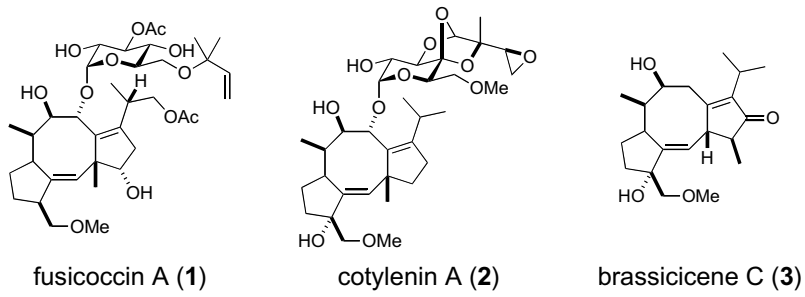

Figure 1. Structure of fusicoccin A (**1**), cotylenin A (**2**), and brassicicene C (**3**).

GGDP synthase domain and a terpene cyclase domain, as a query. As a result, one gene, *orf8*, was identified in *A. brassicicola* ATCC 96836, partial genome sequences of which were released.[19] Full-length cDNA of *orf8* encodes 697 aa residue (47% identity to the *PaFS*) with DDXXE and DDXXD motifs, both of which are important for the coordination of divalent metal cation,[20] in the N-terminus and C-terminus region, respectively. Taking these facts together, the *orf8* gene product would perhaps catalyze the formation of fusicoccadiene from GGDP. In addition, based on the BLAST search,[21] it was thought that five cytochrome P450 genes (*orf1*, *orf2*, *orf5*, *orf7*, *and orf11*), one short-chain dehydrogenase/reductase (SDR) gene (*orf3*), one acetyltransferase gene (*orf4*), one methyltransferase gene (*orf6*), one dioxygenase gene (*orf9*), and one hypothetical gene (*orf10*) are located near *orf8* (Fig. 3A). Especially, *orf5* and *orf9* show significant homology to the P450 gene (47% identity over 94 amino acids) and to the dioxygenase gene (61% identity over 160 amino acids) of *P. amygdali*. These results strongly suggest that *A. brassicicola* ATCC 96836 has the potential to produce an FC/CN-like metabolite.

To investigate metabolites produced by *A. brassicicola* ATCC 96836, the strain was cultured at 25 °C for 7 days in 2 L Sakaguchi flasks each containing 400 mL medium of 0.5% of yeast extract, 5.0% of saccharose, 0.5% of KH_2PO_4, 0.1% of $MgSO_4{\cdot}7H_2O$, and then the supernatant (13.2 L) was extracted with ethyl acetate. The obtained crude extract was purified by silica gel column chromatography to afford a metabolite (2.9 mg). The molecular formula was established as $C_{21}H_{32}O_4$ on the basis of HRTOF-MS data. The ^1H NMR spectrum was similar to that of the brassicicene C (**3**), isolated from *A. brassicicola* strain UAMH 7474.[22,23] From H–H COSY, HSQC, and HMBC experiments, the planar structure of the isolated compound, which completely corresponds to that of brassicicene C, was determined (Fig. 3B).

These data allowed us to determine the metabolite isolated from *A. brassicicola* ATCC 96836 as brassicicene C. By searching genome database of *A. brassicicola* ATCC 96836, the strain perhaps has no paralogs *orf8*, and the *orf1* to *orf11* would participate in brassicicene C biosynthesis. Then, we propose a model for the biosynthetic pathway of brassicicene C (Fig. 3B). In the first step, the Orf8, which shows significant identity toward PaFS, catalyzes the formation of fusicoccadiene from isopentenyl diphosphate and dimethylallyl diphosphate, and then P450 or dioxygenase catalyzes oxidation at the C-8 position. Although the next step is unclear, one possible biosynthetic pathway is as follows; oxidation at C-16 position to aldehyde catalyzed by P450 or dioxygenase, followed by isomerization of the double bond and reduction of aldehyde to alcohol catalyzed by SDR to produce a diol compound. The next

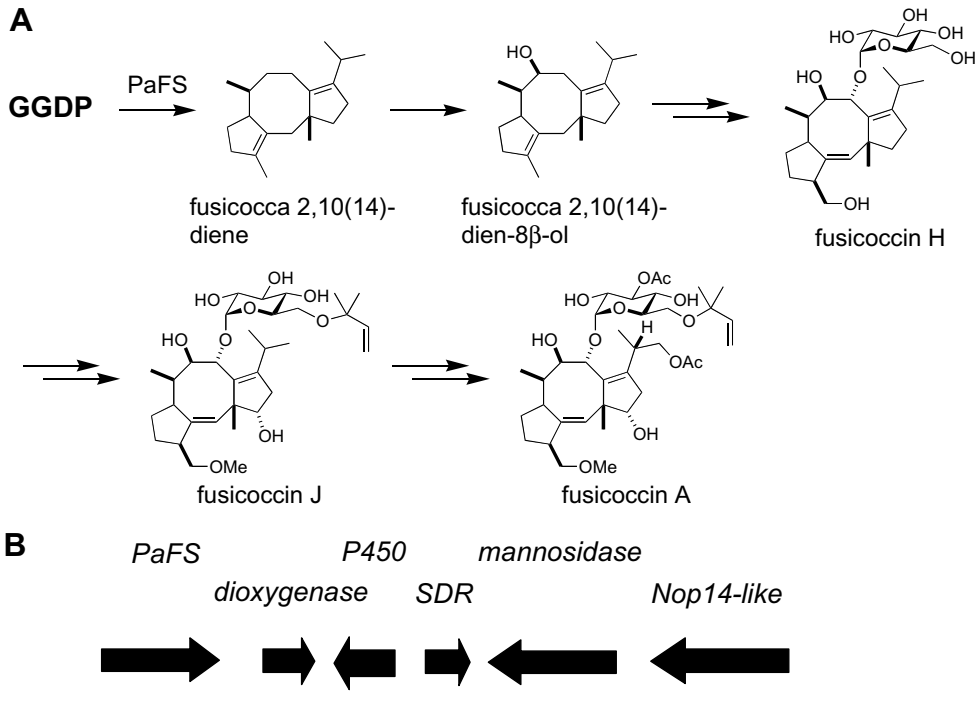

Figure 2. (A) Proposed biosynthetic pathway of FC A and FC J. (B) Putative biosynthetic gene cluster of FC A.

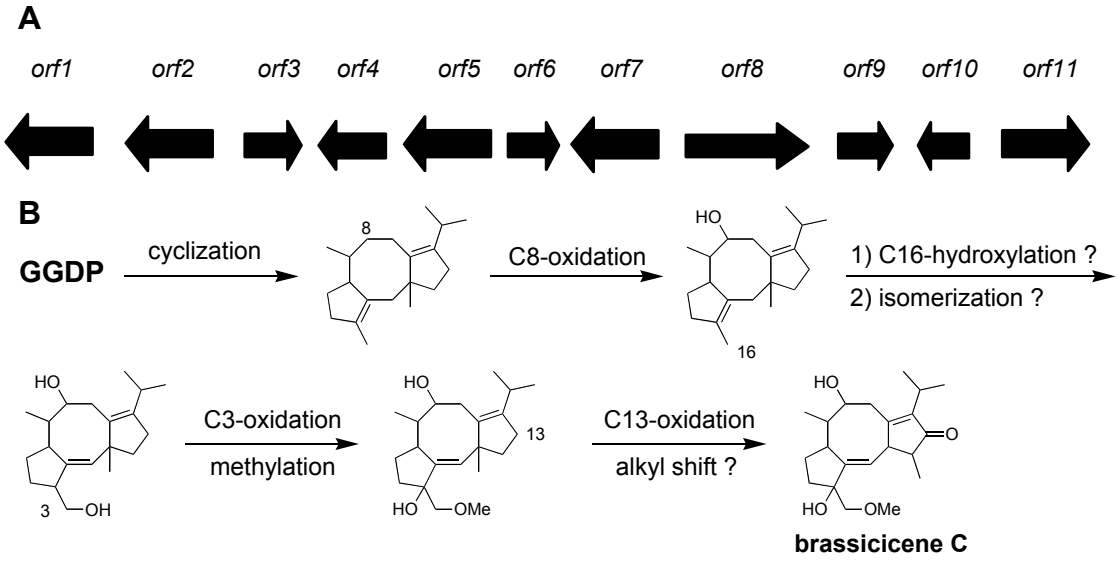

Figure 3. (A) Biosynthetic gene cluster, (B) Proposed biosynthetic pathway of brassicicene C.

step would be a turning point between FC biosynthesis and brassicicene C biosynthesis. In the case of FC biosynthesis, oxidation at the C-9 position followed by glycosylation produces FC H. In contrast, in brassicicene C biosynthesis, oxidation at the C-3 position catalyzed by P450 or dioxygenase may produce a triol compound. Finally, methylation of the hydroxy group at position 16 (methyltransferase; Orf6) followed by oxidation at the C-13 position (P450 or dioxygenase) to ketone and an alkyl shift of the methyl group (P450) to produce brassicicene C. Although the probable acetyltransferase gene (*orf4*) was included in the gene cluster,

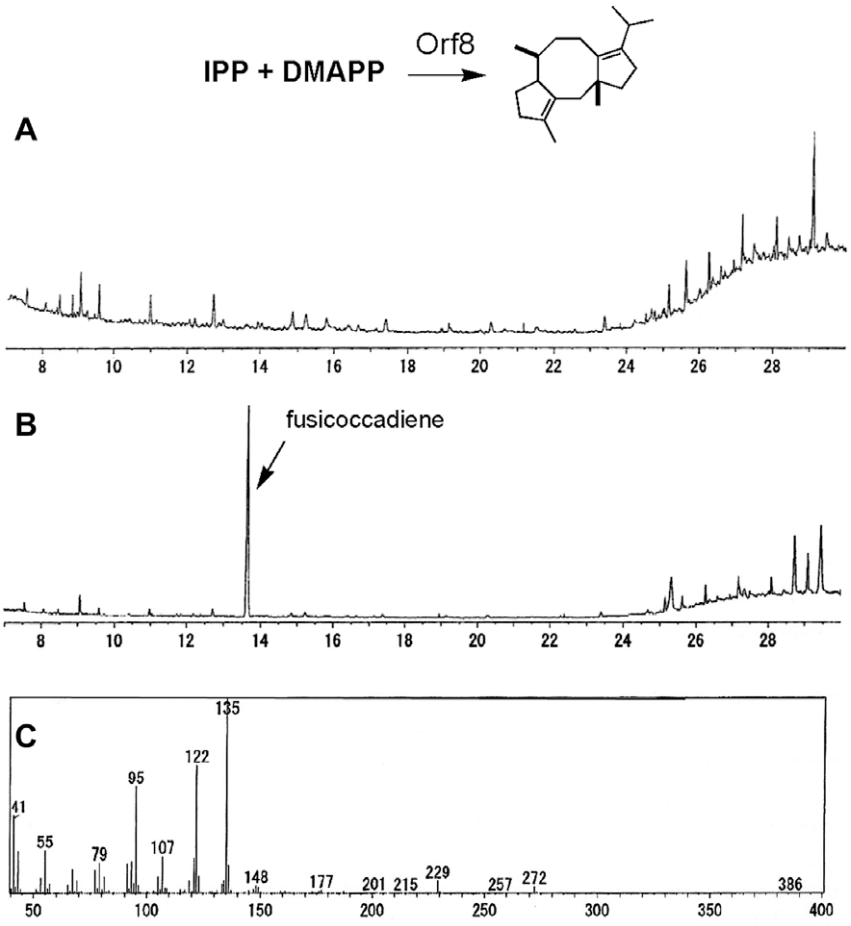

Figure 4. GC profiles of the analytical samples prepared from (A) *E. coli* M15/pQE30, (B) *E. coli* M15/pQE30-orf8. (C) MS spectrum of the reaction product.

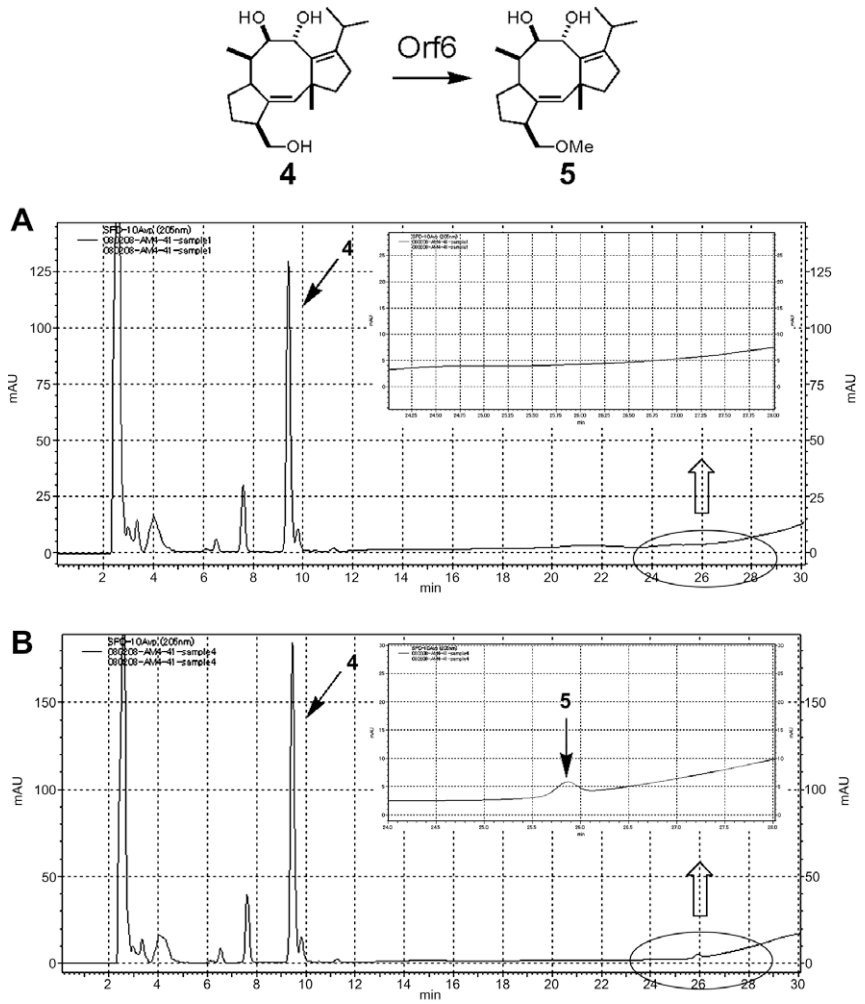

Figure 5. HPLC profiles of the enzyme reaction products with FC H aglycon **4** and SAM using cell-free extracts from (A) *E. coli* M15/pQE30, and (B) *E. coli* M15/pQE30-orf6.

no acetylation reactions are necessary for brassicicene C biosynthesis. However, the fact that brassicicene E,[22] which is a structurally related compound having an acetoxy group at position 12, was previously isolated from another strain of *A. brassicicola* suggests that the ATCC 96836 strain might also produce a small amount of brassicicene E. As described, all of the gene products are provisionally assigned to each of the reactions in brassicicene C biosynthesis.

To confirm that the gene cluster identified in *A. brassicicola* ATCC 96836 would indeed be responsible for brassicicene C biosynthesis, functional analyses of *orf8* and *orf6* gene products were carried out. At first, *orf8*,[24] a putative fusicoccadiene synthase gene, was amplified from first-strand cDNA, prepared from total-RNA of *A. brassicicola* ATCC 96836. The subcloned PCR fragment was ligated into an expression vector, pQE30, by which *E. coli* M15 was transformed.[25] In our previous study, we showed that *E. coli* harboring the plasmid with *PaFS* produce fusicoccadiene,[18] therefore, after extraction of the metabolites from *E. coli* cells harboring *orf8*, the extract was partially purified by silica gel column chromatography to analyze the fusicoccadiene formation by GC–MS. As a result, one new peak, the retention time and MS fragment patterns of which completely correspond to those of the authentic fusicoccadiene, was detected (Fig. 4). The result clearly showed that Orf8 catalyzes GGDP formation using isopentenyl diphosphate and dimethylallyl diphosphate, followed by fusicoccadiene formation from generated GGDP in a similar manner to that of *PaFS*.

Then, *orf6*,[24] a putative methyltransferase, was also cloned into an expression vector, pQE30, by which *E. coli* M15 was transformed.[26] SDS–PAGE analysis of the transformant lysates clearly showed that Orf6 was obtained as a soluble protein. As described above, Orf6 perhaps participates in methylation of the hydroxy group at C-16 position of the C-3, C-8 and C-16 hydroxylated triol intermediate. However, because we do not have this substrate in hand, its substrate analog, an aglycon part of FC H (FC H aglycon) (**4**), which is a C-8, C-9 and C-16 hydroxylated compound, was used as a substrate for the Orf6 reaction. An enzyme assay was performed with cell lysate and FC H aglycon, together with *S*-adenosyl L-methionine as a cofactor. HPLC analysis showed a new peak from the enzyme reaction products, the retention time of which completely corresponded to the authentic 16-methylated compound (**5**) (Fig. 5). On the other hand, no peak was detected at all from the enzyme reaction mixtures of the control experiment (Fig. 5). This result clearly showed that Orf6 catalyzes a methyltransfer reaction towards a hydroxy group at position 16 utilizing *S*-adenosyl L-methionine. Considering that **4** is a substrate analog for Orf6, the low yield of the methylated product **5** is readily understood. Although we also tried to purify recombinant Orf6 for further analysis, purification of Orf6 was unsuccessful due to the instability of Orf6. The results described above strongly suggest that the gene cluster is involved in brassicicene C biosynthesis.

In summary, we successfully identified brassicicene C biosynthetic gene cluster in *A. brassicicola* ATCC 96836, and determined the function of two enzymes, Orf8 and Orf6 as a fusicoccadiene

synthase and methyltransferase, respectively. Because complete understanding of this biosynthetic pathway promises (chemo-)enzymatic synthesis of various analogs of these type of compounds, functional analysis of the other enzymes are in progress.

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References and notes

1. Ballio, A.; Brufani, M.; Casinovi, C. G.; Cerrini, S.; Fedeli, W.; Pellicciari, R.; Santurbono, B.; Vaciago, A. *Experientia* **1968**, *24*, 631.
2. Barrow, K. D.; Barton, D. H. R.; Chain, E. B.; Ohnsorge, U. F. W.; Thomas, R. *Chem. Commun.* **1968**, 1198.
3. Ballio, A.; Casinovi, C. G.; D'Alessio, V.; Grandolini, G.; Randazzo, G.; Rossi, C. *Experientia* **1974**, *30*, 1108.
4. Barrow, K. D.; Barton, D. H. R.; Chain, E.; Bageenda-Kasujja, D.; Mellows, G. *J. Chem. Soc. Perkin Trans. 1.* **1975**, 877.
5. Sassa, T. *Agric. Biol. Chem.* **1971**, *35*, 1415.
6. Sassa, T.; Ooi, T.; Nukina, M.; Kato, N. *Biosci. Biotechnol. Biochem.* **1998**, *62*, 1815.
7. Sassa, T.; Togashi, M.; Shindo, T. *Agric. Biol. Chem.* **1975**, *39*, 1735.
8. Marre, E. *Ann. Rev. Plant. Physiol.* **1979**, *30*, 273.
9. Würtele, M.; Jelich-Ottmann, C.; Wittinghofer, A.; Oecking, C. *EMBO J.* **2003**, *22*, 987.
10. Asahi, K.; Honma, Y.; Hazeki, K.; Sassa, T.; Kubohara, Y.; Sakurai, A.; Takahashi, N. *Biochem. Biophys. Res. Commun.* **1997**, *238*, 758.
11. Honma, Y. *Leuk. Lymphoma* **2002**, *43*, 1169.
12. Matsunawa, W.; Ishii, Y.; Kasukabe, T.; Tomoyasu, S.; Ota, H.; Honma, Y. *Leuk. Lymphoma* **2006**, *47*, 733.
13. Honma, Y.; Ishii, Y.; Yamamoto-Yamaguchi, Y.; Sassa, T.; Asahi, K. *Cancer Res.* **2003**, *63*, 3659.
14. Honma, Y.; Kasukabe, T.; Yamori, T.; Kato, N.; Sassa, T. *Gynecol. Oncol.* **2005**, *99*, 680.
15. Kato, N.; Zhang, C. -S.; Matsui, T.; Iwabuchi, H.; Mori, A.; Ballio, A.; Sassa, T. *J. Chem. Soc. Perkin Trans. 1* **1998**, 2473.
16. Sassa, T.; Zhang, C.-S.; Sato, M.; Tajima, N.; Kato, N.; Mori, A. *Tetrahedron Lett.* **2000**, *41*, 2401.
17. Barrow, K. D.; Barton, D. H. R.; Chain, E.; Ohnsorge, U. F. W.; Sharma, R. P. *J. Chem. Soc. C* **1973**, 1590.
18. Toyomasu, T.; Tsukahara, M.; Kaneko, A.; Niida, R.; Mitsuhashi, W.; Dairi, T.; Kato, N.; Sassa, T. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 3084.
19. Available from: <http://genome.wustl.edu/index.cgi>.
20. Hohn, T. M.. In *Comprehensive Natural Products Chemistry*; Cane, D., Ed.; Elsevier Science: Oxford, 1999; vol. 2, p 201.
21. Available from: <http://blast.ddbj.nig.ac.jp/top-j.html>.
22. Mackinnon, S. L.; Keifer, P.; Ayer, W. A. *Phytochemistry* **1999**, *51*, 215.
23. Spectrum data of isolated brassicicene C is as follows: ^{1}H NMR (500 MHz, CD_3OD); δ 6.08 (dd, J = 1.6, 10.1 Hz, 1H), 4.17 (ddd, J = 3.7, 5.5, 11.5 Hz, 1H), 3.47 (d, J = 9.9 Hz, 1H), 3.38 (d, J = 9.9 Hz, 1H), 3.36 (s, 3H), 3.35–3.38 (m, 1H), 3.12 (m, 1H), 2.91 (m, 2H), 2.79 (br s d, J = 7.7 Hz, 1H), 2.44 (dd, J = 11.5, 13.7 Hz, 1H), 2.03 (m, 1H), 1.96 (m, 1H), 1.87 (m, 1H), 1.53 (m, 1H), 1.44 (m, 1H), 1.32 (d, J = 6.9 Hz, 3H), 1.25 (d, J = 7.4 Hz, 3H), 1.14 (d, J = 6.9 Hz, 3H), 1.00 (d, J = 7.5 Hz, 3H). ^{13}C NMR (125 MHz, CD_3OD); δ 207.5, 176.9, 151.2, 147.7, 127.8, 84.1, 79.5, 75.5, 59.6, 58.7, 50.9, 47.8, 44.9, 38.0, 36.6, 30.6, 28.9, 22.0, 19.0, 13.0, 12.4. HRTOF-MS: calcd for $C_{21}H_{32}O_4Na$: 371.2193. Found: 371.2204. $[\alpha]_D^{27} = -75.2$ (c 0.016, MeOH).
24. The coding sequences of *orf6* and *orf8* from *A. brassicicola* ATCC 96836 was deposited at GeneBank under Accession No. AB465603 and AB465604, respectively.
25. The *orf8* gene was amplified by PCR from previously prepared first-strand cDNA with primers, 5′-GGGGGATCCAAATACCAATTTTCCATCATTGTGGA-3′ and 5′-ACGCGTCGACTCAAAGCTTGAGCATCATTAGCATC-3′. The subcloned PCR product was ligated into the pQE30 (QIAGEN) to give pQE30-orf8. The pQE30-orf8 was transformed into *E. coli* M15 for overexpression. In vivo analysis of fusicoccadiene was performed according to Ref. 18.
26. The *orf6* gene was amplified by PCR from previously prepared first-strand cDNA with primers, 5′-GGGGGATCCGCAGTCCAAGAGACAGACCTGGACAA-3′ and 5′-ACGCGTCGACTTATGCATTCTGTGCCGCAGGCTTA-3′. The subcloned PCR product was ligated into the pQE30 (QIAGEN) to give pQE30-orf6. The pQE30-orf6 was transformed into *E. coli* M15 for overexpression. The reaction mixture (20 mM of MOPS (pH 6.9), 10% of glycerol, 0.5 mM of FC H aglycon, 1 mM of *S*-adenosyl L-methionin) with cell-free extract, prepared from *E. coli* cells having pQE30-orf6, was incubated in 30 °C. After 20 h, the reaction was terminated by addition of ethyl acetate, and the mixture was extracted with ethyl acetate. The combined organic layer was concentrated, and the residue was dissolved in 30 μL of methanol. The sample was analyzed by HPLC.