Tomatidine and lycotetraose, hydrolysis products of α-tomatine by *Fusarium oxysporum* tomatinase, suppress induced defense responses in tomato cells

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Abstract **Many fungal pathogens of tomato produce extracellular enzymes, collectively known as tomatinases, that detoxify the preformed antifungal steroidal glycoalkaloid α-tomatine. Tomatinase from the vascular wilt pathogen of tomato *Fusarium oxysporum* f. sp. *lycopersici* cleaves α-tomatine into the aglycon tomatidine (Td) and the tetrasaccharide lycotetraose (Lt). Although modes of action of α-tomatine have been extensively studied, those of Td and Lt are poorly understood. Here, we show that both Td and Lt inhibit the oxidative burst and hypersensitive cell death in suspension-cultured tomato cells. A tomatinase-negative *F. oxysporum* strain inherently non-pathogenic on tomato was able to infect tomato cuttings when either Td or Lt was present. These results suggest that tomatinase from *F. oxysporum* is required not only for detoxification of α-tomatine but also for suppression of induced defense responses of host.**

Keywords: Reactive oxygen species; Tomato; Plant defense; α-Tomatine

1. Introduction

Antimicrobial secondary metabolites of plants are grouped into two types: the preformed compounds known as phytoanticipins, which are produced constitutively, and the phytoalexins, which are produced only in response to infection by a potential pathogen [1,2]. In tomato plants, the main phytoanticipin is the steroidal glycoalkaloid α-tomatine, which consists of an aglycon moiety (tomatidine) and a tetrasaccharide moiety (lycotetraose) [3,4]. α-Tomatine shows uniform distribution in all organs of tomato plants [4] and high concentrations enough to inhibit most microbes in vitro [5], suggesting that this compound would provide a chemical barrier against the attack of pathogenic fungi.

Many fungal tomato pathogens detoxify α-tomatine by the production of specific enzymes known as tomatinases (for review, see [2,6]). Although tomatinases from these fungi have different mechanisms of action in hydrolysis of α-tomatine, the ability to hydrolyze sugar from α-tomatine is common among tomato pathogens. The metabolites originated by the hydrolysis of α-tomatine have little or no antifungal activity to most tomato pathogens [5]. Thus, for many phytopathogenic fungi the production of tomatinase may be a determinant to successfully infect tomato plants [6].

In addition to the detoxification of α-tomatine, another role of tomatinase in establishing the disease has been reported recently [7]. β_2-Tomatine, the hydrolysis product of tomatinase activity on α-tomatine by the tomato leaf spot fungus *Septoria lycopersici*, suppressed induced defense responses of host plants by interfering with fundamental signal transduction processes leading to disease resistance, although the mechanism is not clear [7]. This report prompted us to examine whether hydrolysis products of α-tomatine by tomatinase from other tomato pathogens might suppress host's defense responses. The vascular wilt pathogen of tomato *Fusarium oxysporum* f. sp. *lycopersici* produces tomatinase that cleaves α-tomatine into the tomatidine (Td) and lycotetraose (Lt) [8,9]. Physiological roles of these degradation products of α-tomatine in plant-pathogen interactions are unknown. We examined whether Td and Lt suppress host's defense responses as reported in β_2-tomatine [7]. In the present study, we show that both Td and Lt suppress the oxidative burst, which is thought to be required for subsequent defense responses in many plant species [10]. We also demonstrate that defense systems of tomato cuttings against a non-pathogen of *F. oxysporum* were actually compromised by the action of both Td and Lt.

2. Materials and methods

2.1. Plant culture and fungal strains

The *Lycopersicon esculentum* L. cultivar 'Ponderosa' was used to generate cell suspension cultures according to the methods described previously [11]. Every 7 days, cells growing in the log phase were transferred into fresh medium containing Murashige–Skoog salts and B_5 vitamins supplemented with 0.5 mg/l 2,4-dichlorophenoxyacetic acid, 0.1 mg/l kinetin, and 2% (w/v) sucrose, pH 5.7, and incubated under continuous shaking at 100 rpm in the dark at 25 °C. Suspension cultured cells used for all experiments were 3 days old. For obtaining tomato plantlets and cuttings, seeds of tomato cultivar 'Ponderosa' were planted in minipots containing vermiculite and

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maintained in a growth chamber at 25 °C with 16 h light and 8 h dark. *F. oxysporum* f. sp. *raphani* MAFF103058 (FOR#21) and *F. oxysporum* f. sp. *lycopersici* MAFF103036 (FOL#24) were grown on potato dextrose agar at 25 °C.

2.2. Chemicals

α-Tomatine and 3,3-diaminobenzidine (DAB) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Tomatidine and all other reagents were from Wako Pure Chemical (Osaka, Japan). Lycotetraose was prepared from α-tomatine by enzymatic hydrolysis with crude tomatinase [12] followed by gel-filtration with Sephadex LH20 (Pharmacia) and silica gel chromatography. Stock solutions of α-tomatine, tomatidine, and lycotetraose were made at 10 mM in dimethylsulfoxide (DMSO) (α-tomatine) and methanol (tomatidine and lycotetraose). DAB (10 mg/ml) was made up in DMSO as a stock solution.

2.3. Detection of resistance responses in tomato cells

Fungal elicitor suspended in distilled water (50 mg/ml) was prepared from FOR#21cells according to the method described previously [13]. Three day old tomato cell-suspension cultures were treated with FOR#21elicitor (200 μg/ml) and DAB (100 μg/ml) for 24 h in the presence of either α-tomatine, tomatidine, or lycotetraose. DAB polymerizes instantly and locally as soon as it comes into contact with H_2O_2 forming a reddish brown compound in the presence of peroxidase [14]. Production of H_2O_2 was measured with a luminometer BLR-201 (Aloka, Tokyo, Japan) by peroxide-dependent chemiluminescence of luminol [15]. Cell suspension cultures of tomato were resuspended in 50 mM potassium phosphate buffer (pH 7.8) to give a final concentration of 300 mg cell mass per ml and incubated for 1 h. Fungal elicitor or mixtures of fungal elicitor with tomatidine or with lycotetraose were added to the cell suspensions and immediately filtrated with a membrane filter (pore size 0.45 μm). Resultant filtrates (200 μl) were mixed with 700 μl of 50 mM potassium phosphate buffer (pH 7.8) and 100 μl of 1.2 mM luminol in 50 mM potassium phosphate buffer. The reaction was started by adding 100 μl of 10 mM potassium ferricyanide. Hypersensitive cell death was determined by Evans blue staining [16]. Extracellular pH of tomato cell-suspension cultures was continuously monitored by a glass pH electrode.

2.4. Measurement of the superoxide (O_2^-)-scavenging activity

The O_2^--scavenging activity was measured by the nitrite method modified by Ooyanagui [17]. The reaction mixture consisted of 15 mM potassium phosphate–borax–EDTA buffer (pH 8.2), 0.1 mM hypoxanthine, 1 mM hydroxylamine, 0.1 mg/ml hydroxylamine *o*-sulfonic acid, 2.9 mU/ml of xanthine oxidase, and a 200 μl sample solution in a final volume of 1 ml. After incubation at 37 °C for 30 min, diazo dye formation was induced at 37 °C for 30 min by adding 2 ml of a mixture of 30 μM *N*-(1-naphthyle) ethylenediamine dihydrochloride, 3 mM sulfanilic acid, and 4.2 M acetic acid. The absorbance at 550 nm was determined with a spectrophotometer. Calculations of the concentration of the antioxidant sample (IC_{50}) were made from zero to full inhibition at the point where 50% inhibition of diazo dye formation took place.

2.5. Plant colonization assays

Seeds of tomato (cultivar 'Ponderosa') were planted in minipots containing vermiculite and maintained in a growth chamber at 25 °C with 16 h light and 8 h dark cycle. Seedlings in the third true leaf stage were cut at right above the crown and placed in a vial containing 10 ml of a conidial suspension (1×10^6 conidia per ml) of FOR#21, and maintained in a growth chamber at 25 °C with 16 h light and 8 h dark cycle. Five days after incubation, a 7-mm section of hypocotyls was cut from right below the cotyledons. The plant sections were surface sterilized by submerging in 70% ethanol for 20 s followed by 1% sodium hypochloride for 10 min and washing in sterilized deionized water for 1 min. Each plant section was then placed on a *Fusarium*-selective medium containing K_2HPO_4 (1 g/l), KCl (500 mg/l), $MgSO_4 \cdot 7H_2O$ (500 mg/l), Fe-EDTA(10 mg/l), L-asparagine (2 g/l), D-galactose (20 g/l), pentachloronitrobenzene 75% wettable powder (1 g/l), sodium cholate (500 mg/l), streptomycin sulfate (300 mg/l), and agar (15 g/l) (pH 4.0) [18]. A tomato pathogen FOL#24 was used as positive control.

3. Results and discussion

To examine the effects of Td and Lt on the inducible H_2O_2 production of tomato cells, cell suspension cultures of tomato were treated with fungal elicitor in the presence or absence of Td and Lt. Accumulation of H_2O_2 was visualized by DAB staining as a reddish brown coloration (Fig. 1). Time-course experiments showed that within 2 h, the elicitor induced the accumulation of H_2O_2 in tomato cell suspensions treated with elicitor. At 7 h, approximately 80% of tomato cells exhibited whole-cell DAB coloration (data not shown). In contrast, very limited number of tomato cells showed H_2O_2 accumulation when the cell cultures were treated with a mixture of elicitor with Td, that with Lt, or that with Td and Lt, although among those more browning was evident in tomato cells treated with a mixture of elicitor with Lt. α-Tomatine did not show such inhibitory activity on H_2O_2 accumulation in tomato cells (Fig. 1). Control treatments showed that the solvents used (DMSO and methanol), α-tomatine, Td, and Lt themselves had no effect on the accumulation of H_2O_2 in tomato cell suspensions (data not shown).

We next examined the effects of Td and Lt on elicitor-induced generation of H_2O_2, the so-called oxidative burst. It has been known that the oxidative burst commonly occurs in two distinct phases [19]. As expected, two distinct phases of the oxidative burst were observed: the first burst peaking at 20 min and the more prolonged second burst peaking at 7 h after addition of elicitor (data not shown). The second phase lasted for at least 6 h, suggesting that the prolonged production of H_2O_2 correlates with hypersensitive disease resistance response as described previously [20]. The oxida-

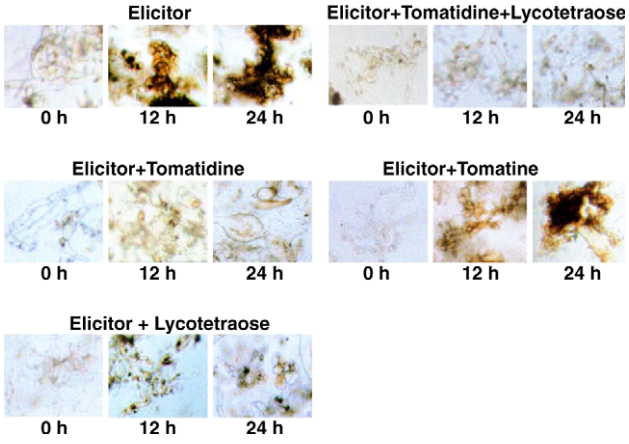

Fig. 1. Histochemical staining of tomato cell suspension cultures with DAB to visualize H_2O_2 accumulation. Tomato cell suspension cultures were treated with fungal elicitor, a mixture of fungal elicitor with 40 μM tomatidine, a mixture of fungal elicitor with 40 μM lycotetraose, a mixture of fungal elicitor with 40 μM tomatidine and 40 μM lycotetraose, and a mixture of fungal elicitor with 40 μM α-tomatine, in the presence of DAB for 24 h. The reddish-brown coloration in tomato cells treated with fungal elicitor (Elicitor) and with a mixture of fungal elicitor with α-tomatine (Elicitor + Tomatine) at 12 h- and 24 h-incubation indicates the accumulation of H_2O_2. In contrast, the reddish-brown coloration was not observed in tomato cells treated with a mixture of fungal elicitor with tomatidine (Elicitor + Tomatidine), a mixture of fungal elicitor with lycotetraose (Elicitor + Lycotetraose), and a mixture of fungal elicitor with tomatidine and lycotetraose (Elicitor + Tomatidine + Lycotetraose).

tive burst in tomato cultures was completely abolished by both tomatidine and lycotetraose in a dose-dependent manner (Fig. 2). The second phase, which is thought to be the principal determinant of disease resistance, was not measurable when the first burst was abolished by either Td or Lt. Td and Lt inhibited also the elicitor-induced hypersensitive cell death (HCD) of tomato cultures (Fig. 3). Control treatments showed that the solvents used (DMSO and methanol), α-tomatine, Td, and Lt themselves had no effect both on the oxidative burst and on the elicitor-induced HCD in tomato cultures (data not shown). These results suggest that Td and Lt suppress induced defense responses in suspension-cultured tomato cells. In addition, alkalization of extracellular medium, an indicator of elicitor perception in plant cells [21], was also inhibited by both tomatidine and lycotetraose (Fig. 4), suggesting that these two molecules may inhibit a sequence of signal steps linking elicitor perception to initiation of oxidative burst because alkalization precedes the oxidative burst [22].

It is possible that Td and Lt may directly scavenge reactive oxygen species (ROS). Thus, Td and Lt were subjected to the antioxidant assay [17]. Td showed the superoxide (O_2^-) scavenging activity (IC_{50}: 80 μM) roughly comparable to that of ascorbic acid (IC_{50}: 60 μM), although Lt showed no antioxidant activity. These results suggest that Td and Lt act differentially in inhibiting the oxidative burst. Td may inhibit the oxidative burst by scavenging ROS directly although the mechanism is unknown. As to the mode of action of Lt, further biochemical investigation is required to establish where the block may be.

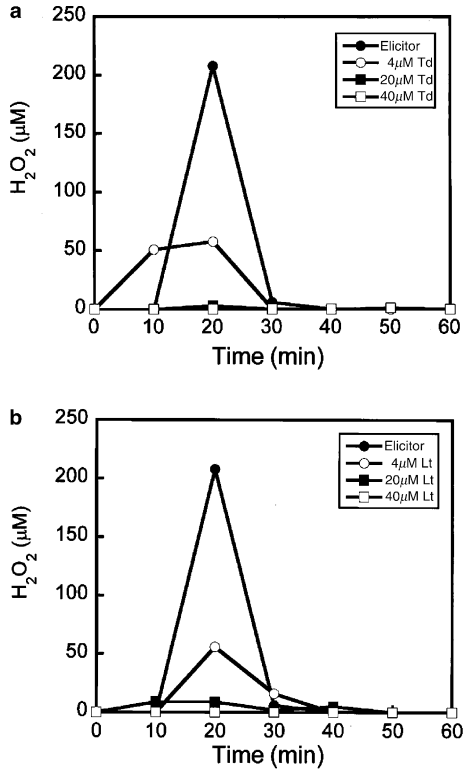

Fig. 2. Suppression of the elicitor-induced oxidative burst reaction in tomato cell suspension cultures by tomatidine (a) and lycotetraose (b). Suspension-cultured cells of tomato were treated either with fungal elicitor alone (●), or a mixture of fungal elicitor with each 4 μM (○), 20 μM (■), and 40 μM (□) of tomatidine (Td) and of lycotetraose (Lt). Generation of H_2O_2 was monitored in the supernatant by luminol-induced luminescence. Results are typical of those obtained in at least three independent experiments.

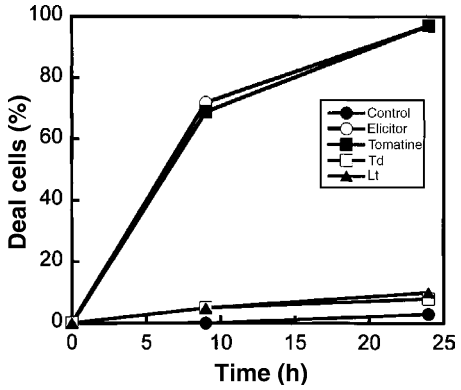

Fig. 3. Hypersensitive cell death of tomato cultured cells. Suspension-cultured cells of tomato were treated with fungal elicitor alone (○), a mixture of fungal elicitor with 40 μM α-tomatine (■), that with 40 μM tomatidine (□), or that with 40 μM lycotetraose (▲). Tomato cells were stained with the viability stain Evans blue at a final concentration of 500 μg/ml for 10 min. The cells were examined with a light microscope and the percentage of Evans Blue-stainable (dead cells) was calculated from the observations of at least 300 cells. Suspension-cultured cells of tomato with no treatment were stained with Evans blue as negative control (●).

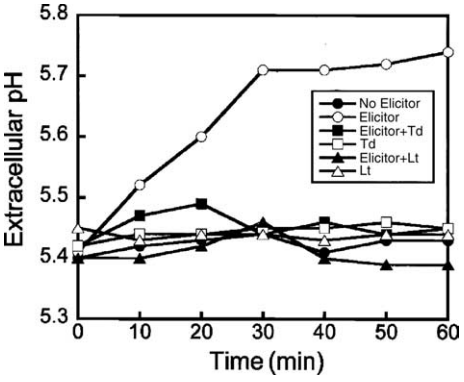

Fig. 4. Time course of elicitor-stimulated alkalization responses in tomato cultured cells. Suspension-cultured cells of tomato were treated with fungal elicitor alone (○), a mixture of fungal elicitor with 40 μM tomatidine (■), that with 40 μM lycotetraose (▲), 40 μM tomatidine alone (□), or 40 μM lycotetraose alone (△). No elicitor control (●) consisted of cells treated with the same volume of water.

We next examined the effect of Td and Lt on the infection of tomato with the Japanese radish yellow pathogen, *F. oxysporum* f. sp. *raphani* (FOR#21), which does not infect tomato plants and does not have tomatinase [12]. Tomato cuttings (plants with excised root systems) were exposed to conidial suspensions of the fungus in the presence or absence of Td and Lt, and then transferred onto the selective medium for the growth of *Fusarium* species to detect the fungus colonized in the xylem vessels of tomato cuttings. FOR#21was observed around the ends of cuttings that had been exposed to the fungus in the presence of either Td or Lt, indicating that the fungus had colonized in the

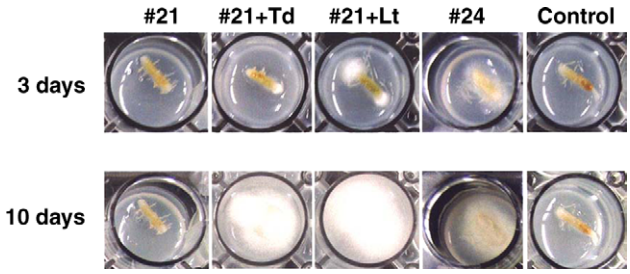

Fig. 5. Effects of tomatidine and lycotetraose on infection of tomato cuttings with a non-pathogenic strain of *F. oxysporum* (FOR#21). Tomato cuttings were placed in a vial containing conidial suspension of FOR#21in the presence of 40 μM tomatidine (#21+Td) or 40 μM lycotetraose (#21+Lt) and incubated for 5 days. After the incubation, a 7-mm section of hypocotyls was cut from right below the cotyledons, surface sterilized, placed on a *Fusarium*-selective medium, and then incubated for 4 weeks. Positive control with a tomato pathogen *F. oxysporu* m f. sp. *lycopersici* (#24) and negative controls with no fungal inoculation (Control) or with no treatment of tomatidine and lycotetraose (#21) are also performed.

xylem vessels of tomato cuttings (Fig. 5). No fungal colonies were observed from the cuttings inoculated with FOR#21 in the absence of Td and Lt during the 4-weeks incubation. Neither Td nor Lt promoted the growth of FOR#21 on the medium in the absence of the plant materials (data not shown). These results suggest that Td and Lt would suppress defense responses of tomato cuttings and thereby FOR#21, a non-pathogen of tomato, was able to infect them.

Successful phytopathogens must be able to overcome or suppress ROS-mediated defense system in plants. In fact, microbial suppression of ROS-mediated defenses by secretion of ROS-scavenging enzymes such as superoxide dismutase and catalase [23–26] and by non-enzymatic metabolites such as mannitol [27] and oxalic acid [28] has been reported. In the present study, we have demonstrated that *F. oxysporum* would utilize α-tomatine, an arsenal of tomato plants against the attack of pathogens, as the material for production of Td and Lt that act as suppressors of host's defense responses. This implicates an important role of tomatinase in tomato-*F. oxysporum* interactions, because both Td and Lt are derived from α-tomatine solely by the action of the tomatinase produced by *F. oxysporum*. Indeed, a mutant strain of *F. oxysporum* f. sp. *lycopersici* with low tomatinase activity showed reduced pathogenicity on tomato plants [29]. Thus, *F. oxysporum* having tomatinase may be a fungus which has evolved an efficient counter-defensive strategy to utilize host's antibiotic α-tomatine.

References

[1] VanEtten, H.D., Mansfield, J.W., Bailey, J.A. and Farmer, E.E. (1994) Plant Cell 9, 1191–1192.
[2] Morrissey, J.P. and Osbourn, A.E. (1999) Microbiol. Mol. Biol. Rev. 63, 708–724.
[3] Roddick, J.G. (1974) Phytochemistry 13, 9–25.
[4] Arneson, P.A. and Durbin, R.D. (1968) Plant Physiol. 43, 683–686.
[5] Sandrock, R.W. and VanEtten, H.D. (1998) Phytopathology 88, 137–143.
[6] Ruiz-Rubio, M., Pérez-Espinosa, A., Lairini, K., Roldán-Arjona, Dipietro, A. and Anaya, N. (2001) in: Studies in Natural Products Chemistry (Rahman, A., Ed.), vol. 25, pp. 293–326, Elsevier Science, Oxford.
[7] Bouarab, K., Melton, R., Peart, J., Baulcombe, D. and Osbourn, A. (2002) Nature 418, 889–892.
[8] Ford, J.E., McCance, D.J. and Drysdale, R.B. (1977) Phytochemistry 16, 545–546.
[9] Lairini, K. and Ruiz-Rubio, M. (1997) Phytochemistry 45, 1371–1376.
[10] Wojtaszek, P. (1997) Biochem. J. 322, 681–692.
[11] Honee, G., Buitink, J., Jabs, T., De Kloe, J., Sijbolts, F., Apotheker, M., Weide, R., Sijen, T., Stuiver, M. and De Wit, J.G.M. (1998) Plant Physiol. 117, 809–820.
[12] Ito, S., Kawaguchi, T., Nagata, A., Tamura, H., Matsushita, H., Takahara, H., Tanaka, S. and Ikeda, T. (2004) J. Gen. Plant Pathol. 70, 195–201.
[13] Ndimba, B.K., Chivasa, S., Hamilton, J.M., Simon, W.J. and Slabas, A.R. (2003) Proteomics 3, 1047–1059.
[14] Thordal-Christensen, H., Zhang, Z., Wei, Y. and Collinge, D.B. (1997) Plant J. 11, 1187–1194.
[15] Schröder, S., Hildebrandt, U., Bothe, H. and Niehaus, K. (2001) Mycorrhiza 11, 101–106.
[16] Baker, C.J. and Mock, N. (1994) Plant Cell Tissue Org Cult. 39, 7–12.
[17] Ooyanagui, Y. (1984) Anal. Biochem. 142, 290–296.
[18] Komada, H. (1975) Rev. Plant Prot. Res. 8, 115–125.
[19] Levine, A., Tenhaken, R., Dixon, R. and Lamb, C. (1994) Cell 79, 583–593.
[20] Chandra, S., Martin, G.B. and Low, P.S. (1996) Proc. Natl. Acad. Sci. USA 93, 13393–13397.
[21] Felix, G., Duran, J.D., Volko, S. and Boller, T. (1999) Plant J. 18, 265–276.
[22] Wojtaszek, P. (1997) Acta Physiol. Plant 19, 581–589.
[23] Katsuwan, J. and Anderson, A.J. (1990) Appl. Environ. Microbiol. 56, 3576–3582.
[24] Klotz, M.G. and Hutcheson, S.W. (1992) Appl. Environ. Microbiol. 58, 2468–2473.
[25] DeGroote, M.A., Ochsner, U.A., Shiloh, M.U., Nathan, C., McCord, J.M., Libby, S.J., Vazquez-Torres, A., Xu, Y. and Fang, F.C. (1997) Proc. Natl. Acad. Sci. USA 94, 13997–14001.
[26] San Mateo, L.R., Hobbs, M.M. and Kawula, T.H. (1998) Mol. Microbiol. 27, 391–404.
[27] Jennings, D.B., Ehrenshaft, M., Mason Pharr, D. and Williamson, J.D. (1998) Proc. Natl. Acad. Sci. USA 95, 15129–15133.
[28] Cessna, S.G., Sears, V.E., Dickma, M.B. and Low, P.S. (2000) Plant Cell 12, 2191–2199.
[29] Ito, S., Takahara, H., Kawaguchi, T., Tanaka, S. and Kameya-Iwaki, M. (2002) J. Phytopathol. 150, 474–480.