Monoterpenoids, Potent Inducers of Apoptosis in the Cells of *Marchantia polymorpha*

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The cultured cells of *Marchantia polymorpha* suffered apoptosis when treated with monoterpenoids, e.g. bornyl acetate. When the apoptosis occurred, the cultured cells of *M. polymorpha* produced H_2O_2, which takes part in a defense reaction. The inhibitors of oxidizing agents, such as cysteine and *N*-acetylcysteine, had an apparent effect on monoterpenoid-induced apoptosis. These results showed that monoterpenoid-induced apoptosis occurs via the production of active oxygen species, e.g. H_2O_2.

In general, when in contact with pathogenic fungi or bacteria, higher plants (vascular plants) have the ability to respond with defense reaction, e.g. the formation of antimicrobial phytoalexins, the reinforcement of plant cell walls and the induction of certain hydrolytic enzymes.[1] It is well known that the responses of higher plants are triggered by elicitors.[2,3] However, in contrast to the study of the defense mechanisms in higher plants, the defense mechanisms of liverworts and algae are poorly understood.[4] In a previous paper, we showed that the cultured cells of *Marchantia polymorpha* act a defense reaction against monoterpenoids, e.g. bornyl acetate (**1**), which are formed as secondary metabolites in higher plants.[5] In the present study, we will show that some monoterpenoids exhibit potent apoptosis-inducing activities toward the cultured cells of *M. polymorpha*.

According to the reported procedure,[5] the cultured suspension cells of *M. polymorpha* were incubated for 2 weeks in a 300-ml conical flask containing 100 ml of fresh MSK-II medium[6] at 25 °C. Various terpenoids (final concentration; 300 μM) were administered to the flask. The color of the cultured cells turned brown when stimulation with geraniol and bornyl acetate (**1**). After 5 h incubation, cellular DNA was extracted by a reported method.[7,8] Figure 1 shows electrophoresis of DNA extracted from the cultured cells of *M. polymorpha* which were administered terpenoids. Treatment of the cultured cells with geraniol and bornyl acetate (**1**) led to a DNA break with a pattern characteristic of internucleosomal fragmentation in agarose gel electrophoresis. In the case of geraniol treatment, genomic DNA was also fragmented in a ladder form. These DNA fragmentations indicated that bornyl acetate (**1**) and geraniol had apoptosis-inducing activities. On the other hand, DNA fragmentation was not observed either in the control or in the dimethylallyl alcohol, farnesol, and geranylgeraniol-treated cells. Since DNA was fragmented with bornyl acetate (**1**) and geraniol, various monoterpenoids were examined for the DNA fragmentation activity. As shown in Table 1, DNA fragmentation was formed indifferently of the stereochemistry of bornyl acetates (**1a** and **1b**). Therefore, (-)-bornyl acetate

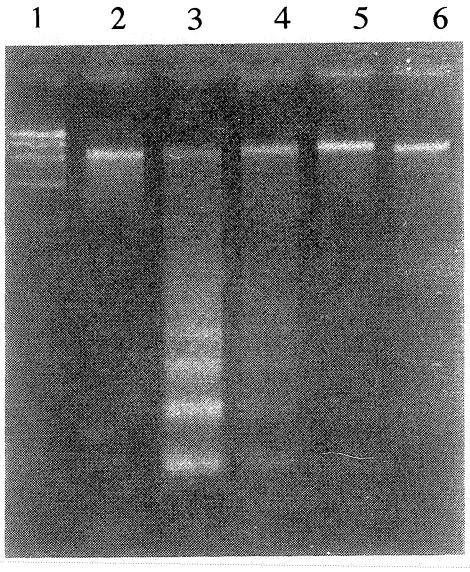
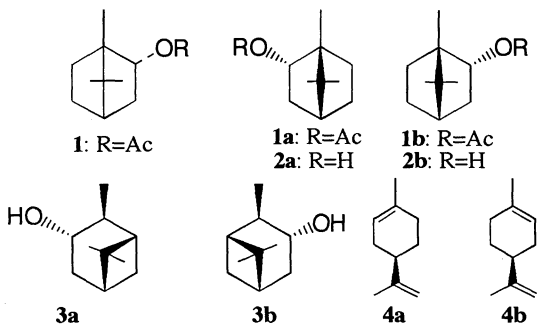


Figure 1. Agarose gel electrophoresis of DNA extracts from the cultured cells of *M. polymorpha*. The cultured cells were treated with the following terpenoids (300 μM). Lane 1, marker (λ/Hind Ⅲ); lane 2, dimethylallyl alcohol; lane 3, bornyl acetate; lane 4, geraniol; lane 5, farnesol; lane 6, geranylgeraniol .



1: R=Ac
1a: R=Ac **1b**: R=Ac
2a: R=H **2b**: R=H
3a **3b** **4a** **4b**

Scheme 1.

Table 1. Effect of various monoterpenoids on DNA fragmentation and release of H_2O_2 from the cultured cells of *M. polymorpha*

Monoterpenoids[a]	Relative DNA fragmentation (%)	Relative H_2O_2 concentration (%)
(±)-Bornyl acetate (**1**)	98	95
(+)-Bornyl acetate (**1a**)	99	91
(-)-Bornyl acetate (**1b**)	100	100
(+)-Borneol (**2a**)	15	4
(-)-Borneol (**2b**)	7	3
(+)-Isopinocampheol (**3a**)	73	41
(-)-Isopinocampheol (**3b**)	72	40
(+)-Limonene (**4a**)	86	64
(-)-Limonene (**4b**)	89	59

[a] The concentration of each monoterpenoid was 300 μM.

(**1b**) were used unless otherwise noted. In the case of borneols (**2a** and **2b**), DNA was slightly fragmented. The genomic DNA of *M. polymorpha* was fragmented not only when treated with bornyl acetate but also with isopinocampheols (**3a** and **3b**) and limonenes (**4a** and **4b**). We have now little information on this "substrate specificity" of the DNA fragmentation. Further works are necessary to clarify this substrate specificity, but these substrate effects may be due to the mobility of the substrate in the suspension cells.

This DNA fragmentation occurred in a dose- and time-dependent manner. The DNA fragmentation was observed at (-)-bornyl acetate (**1b**) concentration of 0.2 mM to 5 mM. Figure 2 shows the time courses of the DNA fragmentation of the cultured

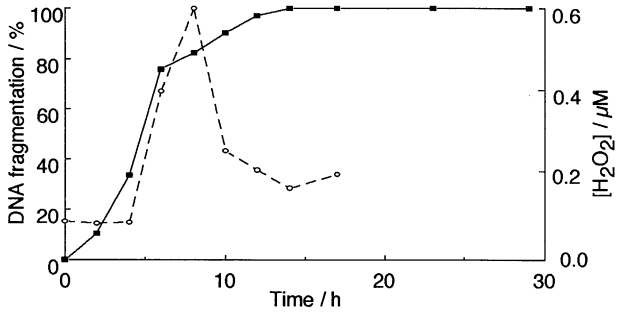


Figure 2. Time dependency of the induction of DNA fragmentation (-■-) and H_2O_2 production (--○--) in the cultured cells of *M. polymorpha* by 300 μM of (-)-bornyl acetate (**1b**) treatment.

cells of *M. polymorpha*. The fragmentation of DNA started in less than 1 h. DNA was completely fragmented in less than 12 h. These results showed that monoterpenoids have the potent apoptosis-inducing activities.

In general, when a plant is subjected to stress, active oxygen species, mainly H_2O_2, are produced as a defense reaction.[2,3,10] The question arises as to whether the production of H_2O_2 when the monoterpenoids are introduced induces the apoptosis. Time courses of the H_2O_2 formation from the cultured cells of *M. polymorpha* are shown in Figure 2.[11] The H_2O_2 concentration in the medium began to increase dramatically in 4 h after treatment with 300 μM of (-)-bornyl acetate (**1b**), reaching a maximum at about 8 h, and declined thereafter. The cultured cells of *M. polymorpha* without (-)-bornyl acetate (**1b**) treatment exhibited less than 0.2 μM H_2O_2 in the medium. The timing of rapid increase of H_2O_2 in the medium synchronized with the timing of fragmentation of DNA. Furthermore, the cultured cells of *M. polymorpha* responded to the treatment with (-)-bornyl acetate (**1b**) in a dose-dependent manner. About 300 μM of (-)-bornyl acetate (**1b**) were sufficient to cause a significant increase in H_2O_2 formation.

The H_2O_2 formation in the cultured suspension cells of *M. polymorpha* was induced not only with (-)-bornyl acetate (**1b**) but also with its stereoisomer (**1a**) (Table 1). H_2O_2 were also formed in the cases of isopinocampheols (**3a** and **3b**) and limonenes (**4a** and **4b**). However, in the case of borneols (**2a** and **2b**), H_2O_2 was slightly formed. Similar substrate effects were seen in the DNA fragmentation of the cultured cells of *M. polymorpha*. These observations suggest that the production of H_2O_2 induces the DNA fragmentation.

Table 2. Quantitative evaluation of DNA fragmentation in the cultured cells treated with 300 μM of (-)-bornyl acetate (**1b**) and various antioxidizing agents

Antioxidizing agents	DNA fragmentation (%)
None	82.3
1 mM *N*-Acetylcysteine	0.5
1 mM Cysteine	23.8
0.5 mM Cystine	85.3

Is the production of H_2O_2 necessary for the DNA fragmentation? In the case of animal cells, antioxidizing agents have been used as a method to remove the intrinsic H_2O_2.[13] To examine whether the DNA fragmentation was inhibited by antioxidizing agents, *N*-acetylcysteine and cysteine were administered to the cultured cells of *M. polymorpha*.[14] As shown in Table 2, *N*-acetylcysteine and cysteine inhibited the fragmentation of DNA. On the other hand, cystine, which does not have the antioxidizing effect, did not inhibit the fragmentation of DNA. These results are powerful evidence of the necessity of the active oxygen species, e.g. H_2O_2, for the fragmentation of DNA.

From these results, we conclude that apoptosis, which is programmed cell death, occurred in the cultured cells of *M. polymorpha* by the addition of monoterpenoids. It was also clear that production of H_2O_2 is necessary for the occurrence of apoptosis. If plant cells can evade outbreak of H_2O_2, the cells may continue to live. These results suggest that the mechanism of defense reaction of plants against chemical stress is similar to that of animals. Studies on the apoptosis of the plant cells have just began. Further study must be done about the mechanism of the apoptosis after the stimulation with monoterpenoids.

References and Notes

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14 The cultured cells of *M. polymorpha* were incubated for 5 h in the presence of antioxidizing agents (1 mM), and then, after addition of (-)-bornyl acetate (**1b**) (300 μM), the cultures were further incubated for 5 h.