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Wounding stress induces phenylalanine ammonia lyases, leading to the accumulation of phenylpropanoids in the model liverwort *Marchantia polymorpha*

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ABSTRACT

Wounding stress induces the biosynthesis of various specialized metabolites in plants. In this study, wounding induced the biosynthesis of luteolin, apigenin, and isoriccardin C, which are biosynthesized through the phenylpropanoid pathway, in the model liverwort *Marchantia polymorpha* L (Marchantiaceae). Recombinant *M. polymorpha* phenylalanine ammonia lyases (MpPALs) exhibited PAL activity *in vitro* and converted phenylalanine into *trans*-cinnamic acid. Based on semi-quantitative RT-PCR analysis, the expression levels of the Mp*PAL* genes were up-regulated after wounding. α-Aminooxy-β-phenylpropionic acid, a PAL inhibitor, suppressed the production of wounding-induced phenolic compounds, luteolin, apigenin, and isoriccardin C, in *M. polymorpha*. Thus, PAL is a committed step in the biosynthesis of phenylpropanoids in response to wounding in *M. polymorpha*. This study suggests that wound-induced specialized metabolites such as phenylpropanoids comprise a conserved defense system in land plants.

1. Introduction

Plants are constantly subjected to environmental stresses; therefore, sophisticated mechanisms for adaptation to adverse environmental conditions have been developed. Because wounding by pathogens and insects can be fatal to plants, a system in plants spontaneously produces specialized metabolites to restrict pathogenic infection and insect attack in response to wounding, including antibacterial compounds and insect antifeedants (Ramakrishna and Ravishankar, 2011; War et al., 2012). Wounding stimulates the biosynthesis of various specialized metabolites in plants. Many of these bioactive plant specialized metabolites are also useful for humans as medicines and treatments for diseases. The activation of specialized metabolites following wounding is a practical and effective method to increase the concentrations of bioactive compounds.

Among the specialized metabolites produced by plants, phenolic compounds are widely distributed between algae and angiosperms, with important roles in plant physiology. Thus, plants produce a diverse array of phenolic compounds that are suitable for different stages of development and environments. These phenolic compounds are primarily classified into flavonoids, stilbenes, coumarins, monolignols, and lignans, among others, according to their chemical structures. Phenolic compounds have many biological functions and a great diversity of functions for plant defense, including UV absorbance, protection from pathogen and herbivore attack, allelopathic effects, cell wall reinforcement, and antioxidation (Kutchan et al., 2015).

Most phenolic compounds produced in plants are biosynthesized through the phenylpropanoid pathway, which begins with phenylalanine. Phenylalanine ammonia lyase (PAL) is the enzyme responsible for the conversion of phenylalanine into *trans*-cinnamic acid. PAL is the first and committed step in the phenylpropanoid pathway (Emiliani et al., 2009; Thomas, 2010). Plants respond to stress with alterations in PAL activity and phenylpropanoid accumulation (Dixon and Paiva, 1995; McConn et al., 1997). Wounding is a type of abiotic stress that induces *PAL* gene expression (Diallinas and Kanellis, 1994; Fukasawa-Akada et al., 1996) and elevates PAL activity (Kamo et al., 2000; Ke and Saltveit, 1989).

Bryophytes, such as liverworts, mosses and hornworts, are plants that are taxonomically intermediate between algae and vascular plants (Asakawa et al., 2013a, 2013b; Asakawa, 1982; Bowman et al., 2007; Qiu et al., 2006) and constitute an early, diverging lineage of land plants. Therefore, bryophytes are at a key position in plant evolution,

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and comprehension of the physiology of bryophytes should provide significant information on how plants colonized land (Bowman et al., 2007). Notably, the complete genome of the model liverwort *Marchantia polymorpha* has been sequenced and will provide insights into the evolutionary history of land plants (Bowman et al., 2017).

Bryophytes produce a wide variety of specialized metabolites; however, information regarding the mechanisms that regulate the induction of specialized metabolites in bryophytes is scarce. In the model moss *Physcomitrella patens,* UV-B irradiation triggers flavonoid biosynthesis and expression of the genes encoding chalcone synthases, important enzymes for flavonoid biosynthesis (Wolf et al., 2010). The moss *Hypnum plumaeforme* accumulates momilactones A and B, which were originally found as allelochemicals in rice in response to UV-C irradiation, elicitors, and $CuCl_2$ (Kato-Noguchi, 2011; Okada et al., 2016). In the model liverwort *Marchantia polymorpha* L. (Marchantiaceae), UV-C irradiation induces bisbibenzyls, specialized metabolites typically produced in liverworts by the abscisic acid (ABA) signaling pathway (Kageyama et al., 2015). These studies suggest that activation of production of specialized metabolites in response to UV irradiation is a conserved defense mechanism against UV irradiation in land plants. Wounding, another serious environmental stress, induces volatile production in *M. polymorpha* (Kihara et al., 2014), suggesting that wounding may also act as a cue for specialized metabolite synthesis in *M. polymorpha*. Previous work suggests that specialized metabolites (volatiles) are defensive compounds in bryophytes and that wounding can stimulate specialized metabolite accumulation in *M. polymorpha*; thus, we might anticipate volatile production to be a defensive response to wounding. However, volatile production as a defensive response to wounding remains to be investigated in *M. polymorpha.*

In this study, wounding in *M. polymorpha* induced the expression of genes encoding PALs, which was accompanied by increases in phenylpropanoids such as a bisbibenzyl and flavonoids. Given that wounding stimulates the biosynthesis of specialized metabolites in flowering plants, wounding-induced specialized metabolite production is suggested to be a conserved physiological response in land plants.

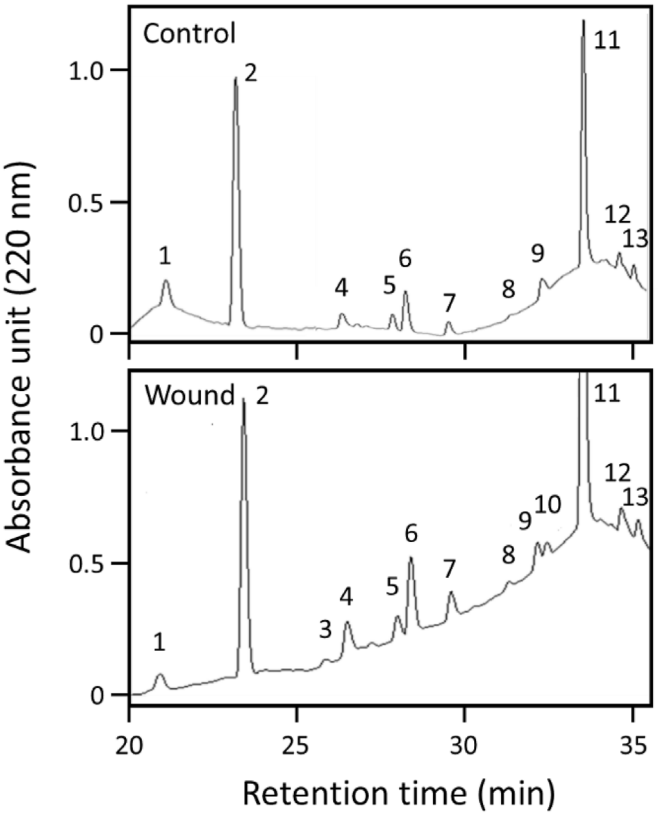

Fig. 1. HPLC chromatograms of the *M. polymorpha* extract with or without wounding treatment. Upper chromatogram, control plant; lower chromatogram, wounded plant. Plants were grown on 1/2 Gamborg's B5 medium containing 1.4% agar at 22 °C under continuous white fluorescent light for 30 days. Plants were harvested at 1 h after wounding. HPLC conditions are described in the Experimental Methods.

2. Results and discussion

2.1. Identification of compounds induced by wounding

The effects of wounding on the production of nonvolatile compounds were examined in *M. polymorpha* grown on 1/2 Gamborg's B5 agar medium. Plants were harvested at 1 h after wounding, and metabolites were extracted with methanol. The resultant extract was analyzed by reversed-phase high-performance liquid chromatography (HPLC) (Fig. 1 and Fig. S1). Comparison of HPLC profiles between extracts of wounded and control plants revealed that wounding significantly increased the intensities of peaks 8, 10, 11, 12, and 13. Peaks 8 and 10 appeared only in the extract of the wounded plant. Thus, wounding induced the production of nonvolatile specialized metabolites in *M. polymorpha*. To identify the compounds induced by wounding in *M. polymorpha,* wounded plants (510 g) were soaked in methanol, and several steps of chromatography yielded compounds **1** (0.9 mg, peak 8), **2** (2.0 mg, peak 10), and **3** (33.3 mg, peak 11). All isolated compounds had potent UV absorbing capacity.

Based on high resolution field desorption mass spectrometry (HR-FDMS) analysis, the molecular formula of compound **1** was $C_{15}H_{10}O_6$ (found at *m/z* 286.04823, calcd. 286.04774). For compound **1**, the ^{1}H NMR spectrum revealed that all the proton signals were derived from sp^2 protons (δ_H 6.5–7.5 ppm). Analysis of the MS and NMR spectroscopic data (Supplemental data) determined that compound **1** was luteolin (Fig. 2) (Lee et al., 2013).

From HR-FDMS spectral data, the molecular formula of compound **2** was determined to be $C_{15}H_{10}O_4$ (found at *m/z* 270.05392, calcd. 270.05870). The ^{1}H NMR spectrum of compound **2** revealed that most proton signals were sp^2 proton signals (δ_H 6.2–8.0 ppm) and that the structure of compound **2** was similar to that of compound **1**, which suggested that compound **2** was also a flavonoid. Analysis of the MS and NMR spectroscopic data (Supplemental data) revealed that compound **2** was apigenin (Fig. 2) (Lee et al., 2013). Production of luteolin (**1**) and apigenin (**2**) has been previously reported in *M. polymorpha* (Markham et al., 1998).

The molecular formula of compound **3** was $C_{28}H_{24}O_6$ (found at *m/z* 424.16679, calculated 424.16746), based on HR-FDMS spectral data. The ^{1}H NMR spectrum of compound **3** revealed that most of its proton signals were derived from aromatic protons. Moreover, the characteristic olefin proton signal of bisbibenzyls, which was shifted at approximately δ_H 5.5 ppm, was observed in the ^{1}H NMR spectrum of compound **3**. Detailed investigations of the MS and NMR spectral data (Supplemental data) revealed that compound **3** was isoriccardin C (Fig. 2) (Asakawa et al., 1987). This compound is categorized as a bisbibenzyl, a characteristic secondary metabolite in liverworts.

These results showed that wounding induced the production of phenylpropanoids in *M. polymorpha.* Accordingly, a wounding-induced system for the biosynthesis of specialized metabolites is probably conserved in land plants. However, the structures of the compounds at peaks 12 and 13, which were not separated by any HPLC conditions in this study, could not be determined.

2.2. Accumulation profiles of compounds 1–3 after wounding

Based on analysis of specialized metabolites in *M. polymorpha*, compounds **1–3** accumulated 1 h after wounding. The time course of the accumulation of compounds **1–3** was examined by HPLC analysis. The concentrations of compounds **1–3** were transiently elevated at 1