ACTA BIOLOGICA CRACOVIENSIA Series Botanica 56/1: 54–58, 2014
DOI: 10.2478/abcsb-2014-0007

THE ROLE OF JASMONATES IN THE FORMATION OF A COMPOUND OF CHALCONES AND FLAVANS WITH PHYTOALEXIN-LIKE PROPERTIES IN MECHANICALLY WOUNDED SCALES OF *HIPPEASTRUM* × *HYBR.* BULBS

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Received February 24, 2013; revision accepted February 5, 2014

Mechanical damage to scales of *Hippeastrum* × *hybr.* bulbs leads to the formation of phytoalexin-like compounds which redden the wounded tissue. The reaction is accompanied by an increase in methyl jasmonate (JA-Me). Applying 2-(4-isobutylphenyl) propionic acid, a jasmonate biosynthesis inhibitor, decreases the level of endogenous jasmonates and decreases the plant's ability to produce the red pigment. Experimental results indicate that jasmonates are involved in the defense response to wounding in *Hippeastrum*, which is manifested in the formation of red pigment, a compound of chalcones and flavans with phytoalexin-like properties.

Key words: *Hippeastrum*, jasmonates, phytoalexins, wounding.

INTRODUCTION

Higher plants have to cope with abiotic stresses such as salinity, drought, chilling, anaerobic conditions, UV radiation and wounding. Wounding breaks tissue continuity and opens the way for invasion of pathogenic fungi, viruses and bacteria. It is well established that plants can react to wounding and infection by inducing de novo synthesis and accumulation of defense compounds such as phytoalexins (Richard et al., 2000; Grayer and Kokubun, 2001; Naoumkina at al., 2007; Hasegawa et al., 2010). Plant phytoalexins are a class of low-molecular-weight compounds that accumulate in response to biotic and abiotic elicitors. Hundreds of phytoalexins have been identified. Other substances that protect particular species against pathogenic microorganisms are under study.

Various mechanically wounded organs of *Hippeastrum* produce a mixture of an orange-colored chalcone and flavans which can be oxidized to red-colored dimers or polymers (Wink and Lehmann, 1996) which prevent penetration of injured tissues by *Phoma narcissi*, *Botrytis cinerea*, *Fusarium oxysporum* and *Phoma poolensis* (Saniewska and Budzianowski, 1997; Saniewska et al., 2005; Saniewski et al., 2006). The biosynthesis pathways of these phytoalexin-like compounds in wounded tissues of *Hippeastrum* are unknown, but efficient induction of immune processes is known to depend on different internal signaling pathways, among which the jasmonate pathway is the most important one (Wasternack et al., 2006, 2007; Koo et al., 2009). Exogenous jasmonates induce typical responses to wounding in intact plants (Farmer et al., 2003) and restore the activity of many genes involved in plant protective mechanisms repressed due to application of jasmonate biosynthesis inhibitors (Peña-Cortés et al., 1995).

Many data indicate that jasmonates play a crucial role in phytoalexin accumulation (Nojiri et al., 1996; Jaber-Vazdekis et al., 2008). Here we investigated the correlation between jasmonate biosynthesis and the formation of a red compound with phy-

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PL ISSN 0001-5296

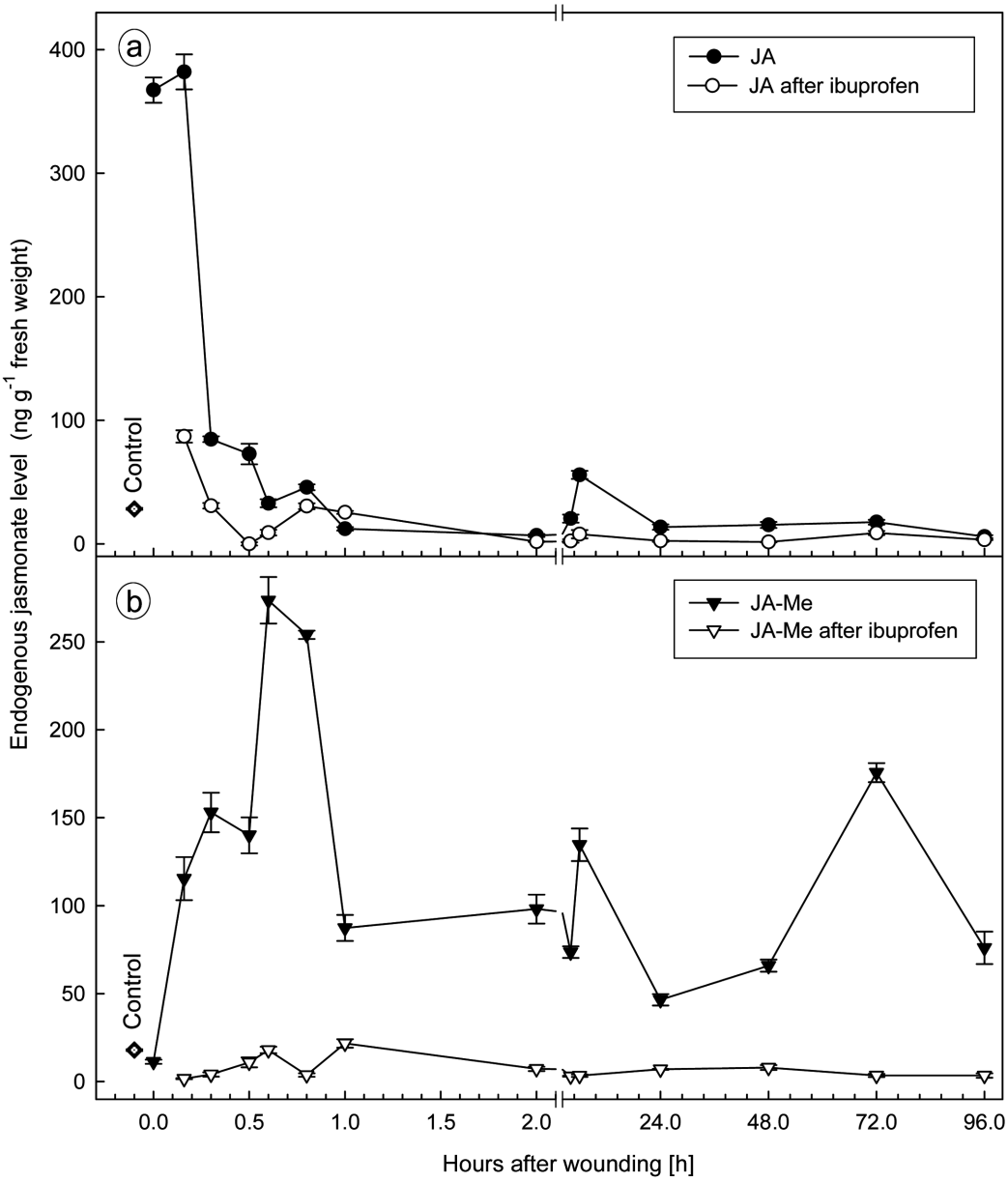

Fig. 1. Changes in JA (**a**) and JA-Me (**b**) content (ng g^{-1} fresh weight) in mechanically wounded scales of *Hippeastrum* bulbs and after treatment with a jasmonate biosynthesis inhibitor (1 mM ibuprofen). Values are means of two replicates of each of three separate samples. SE is marked at the bars. Unwounded storage leaves are the control.

toalexin-like properties in wounded scales of *Hippeastrum* bulbs.

MATERIALS AND METHODS

Hippeastrum × *hybr.* Jan was used in the study. The storage leaves were cut into small pieces (4 × 4 mm) and kept on Petri dishes at 20–25°C under continuous cool white fluorescent light (Polam, Warsaw; 130 μmol m^{-2} s^{-1}) and high humidity. Table 1 and Figure 1 give the time of collection of plant material.

In experiments examining the effect of 2-(4-isobutylphenyl)propionic acid (ibuprofen) on the level of endogenous jasmonates and the red compound with phytoalexin-like properties in mechanically wounded scales of *Hippeastrum* bulbs, 1 mM ibuprofen was administered directly after the wounding event (0 h). High inhibitor concentration was maintained by 6 applications at intervals of 10 min. Material for

TABLE 1. Effect of ibuprofen (1 mM) on red pigment level in wounded scales of *Hippeastrum* bulbs

Treatments	Absorbance at 480 nm after incubation time [hours]				
	24	48	72	96	120
Control	0.0 ± 0.09	0.05 ± 0.03	0.16 ± 0.02	0.51 ± 0.01	0.52 ± 0.01
	Ibuprofen applied in				
0 min	0.01 ± 0.08	0.06 ± 0.02	0.21 ± 0.05	0.58 ± 0.06	0.76 ± 0.12*
0,10 min	0.0 ± 0.06	0.05 ± 0.02	0.26 ± 0.06*	0.60 ± 0.08	0.72 ± 0.09*
0,10,20 min	0.0 ± 0.02	0.02 ± 0.01	0.06 ± 0.09	0.23 ± 0.01**	0.41 ± 0.02*
0,10,20,30 min	0.0 ± 0.09	0.0 ± 0.01	0.01 ± 0.02**	0.14 ± 0.03**	0.23 ± 0.03**
0,10,20,30,40 min	0.0 ± 0.08	0.0 ± 0.03	0.02 ± 0.01**	0.11 ± 0.08**	0.13 ± 0.04**
0,10,20,30,40,50 min	0.0 ± 0.06	0.0 ± 0.02	0.02 ± 0.01**	0.09 ± 0.03**	0.08 ± 0.04**
0,10,20,30,40,50,60 min	0.0 ± 0.02	0.0 ± 0.01	0.0 ± 0.05**	0.02 ± 0.06**	0.08 ± 0.01**

Values are means of three independent replicates (biological). **P<0.01, *P<0.05 (by Student's t-test); ibuprofen-treated plants versus untreated control.

endogenous jasmonate examination was collected 10 min after inhibitor application as well as 24, 48, 72, and 96 h after wounding. The controls were unwounded storage leaves.

For analyses of endogenous jasmonates (jasmonic acid – JA, methyl jasmonate – JA-Me) we used the methods of Gundlach et al. (1992) and Fan et al. (1998) with modifications. *Hippeastrum* leaves (5 g) were frozen in liquid N_2 and homogenized in a chilled mortar with a pestle. Jasmonates were extracted twice with 90% (v/v) 40 ml methanol. Internal standards were 500 ng d_2-JA-Me and 500 ng d_5-JA added to the crude extract. The extract was reduced to the aqueous phase, acidified to pH 2 with 12 M HCl and centrifuged at 12,000 g for 30 min to remove precipitated chlorophyll. The supernatant was partitioned three times against chloroform and dried under vacuum. The pellet was dissolved in 3 ml n-hexane and applied to a silica gel solid-phase extraction column (Backer-bound SPE silica gel, 500 mg, 3 ml; J.T. Backer, Philipsburg, NJ, USA). The column was washed with 5 ml n-hexane and then eluted with 5 ml n-hexane and diethyl ether (2:1, v/v) with 0.5% (v/v) acetic acid. The eluate was evaporated to dryness, methylated with diazomethane, dissolved in 50 µl methanol and analyzed by gas chromatography/mass spectroscopy-selective ion monitoring (GC/MS-SIM; Auto-System XL coupled to TurboMass, Perkin Elmer) using a DB-5 column (30 m × 0.25 mm, 0.5 µm phase thickness). The GC temperature program was as follows: 80°C for 1 min, 80–160°C at 10°C min^{-1}, 160-230°C at 5°C min^{-1}, flow rate 1 ml min^{-1}, injection port temperature 250°C. GC/MS-selected ion monitoring was performed by monitoring m/z 193, 195, 198, 224, 226 and 229.

In experiments examining the effect of ibuprofen on formation of the red compound in mechanically wounded scales of *Hippeastrum* bulbs, ibuprofen was administered as described above. The material for study was collected 24, 48, 72, and 96 h after wounding. Control samples were treated with water.

The mixture of chalcones and flavans that can be oxidized to red dimers or polymers with phytoalexin-like properties was analyzed spectrophotometrically on the basis of redness intensity in extracts taken from wounded tissue 24, 48, 72, 96 and 120 h after mechanical stress. Control storage leaves were collected from unwounded plants. The tissue (0.3 g) was extracted with 1 ml 90% methanol and absorbance was measured at 480 nm.

RESULTS AND DISCUSSION

Numerous investigations have shown that wounding causes intensified biosynthesis of jasmonates JA and JA-Me (e.g., Glauser et al., 2008). The changes in the level of these hormones appear in particular plant species from several seconds up to several hours after the stress stimulus action (Creelman and Mullet, 1995; Glauser et al., 2009).

We showed that JA content increases immediately after wounding of *Hippeastrum* bulb scales (Fig. 1). At 20 min after wounding the JA level begins to decrease gradually and slightly oscillates but remains lower than the level of JA-Me (Fig. 1). The JA-Me level increases from the moment of stress application and at 40 min reaches its maximum value of ~270 ng g^{-1} fresh weight, which is 25 times the level in intact scales. The increased endogenous JA-Me accompanied by decreased JA may be due to wounding-caused stimulation of the activity of jasmonic acid carboxyl methyltransferase (*JMT*), which is responsible for conversion of JA to

JA-Me (Pauw et al., 2005; Wasternack et al., 2006). The excess of JA-Me over JA during the defense reaction may be a consequence of higher metabolic activity and stability, as well as better permeability of the ester form (Weiler et al., 1993). In *Hippeastrum*, stimulation of defense reactions leading to the formation of a red pigment with properties similar to those of phytoalexins may be associated with activation of *JMT* gene expression. Seo et al. (2001) demonstrated that expression of *JMT* was induced both locally and systemically by wounding or methyl jasmonate treatment, suggesting a vital role of JA-Me as a diffusible intercellular signal transducer.

Ibuprofen administered to wounded *Hippeastrum* bulb scales decreased the level of endogenous JA-Me and also inhibited red pigment formation (Tab. 1).

Our results indicate that jasmonates are involved in the defense response to wounding in *Hippeastrum*. They are required for initiating synthesis of a phytoalexin-like red pigment in reaction to mechanical wounding of *Hippeastrum* bulb scales. Further experiments, including study of JMT expression in mechanically wounded tissues of *Hippeastrum*, should shed more light on the role of jasmonates during processes leading to achievement of immunity. Recent studies revealed that jasmonoyl-L-isoleucine (JA-Ile) acts as a key jasmonate signal (Suza and Staswick, 2008; Suza et al., 2010), so the role of JA-Ile in the response to wounding in *Hippeastrum* bulb scales also presents an area for investigation.

AUTHORS' CONTRIBUTIONS

EW, KF carried out the experiments, analyzed the data and wrote the manuscript; WG, AK carried out the experiments; JK, MB were responsible for the preparation of figures and table; AS-J, MS helped in preparing the manuscript. The authors declare that there are no conflicts of interests.

ACKNOWLEDGEMENTS

This research was supported by the Polish Ministry of Science and Higher Education (grant no. N N310 141435) and the National Science Centre (grant no. N N310 301839).

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