ISSN 1021-4437, Russian Journal of Plant Physiology, 2011, Vol. 58, No. 2, pp. 238–247. © Pleiades Publishing, Ltd., 2011.

RESEARCH PAPERS

Role of Lipoxygenase and Allene Oxide Synthase in Wound-Inducible Defense Response of Pea[1]

H. R. Yang[a, b, 2], H. T. Liu[c, 2], K. Tang[a, b], and W. D. Huang[a]

[a]*College of Food Science and Nutritional Engineering, China Agricultural University, Beijing 100083, China; fax: +86-010-6273-7553; e-mail: huanggwd@263.net*

[b]*School of Biotechnology, Jiangnan University, Wuxi 214122, China;*

[c]*Institute of Geographic Sciences and Natural Resources Research, the Chinese Academy of Sciences, Beijing 100101, China*

Received April 8, 2010

Abstract—Lipoxygenase (LOX) and allene oxide synthase (AOS) are key enzymes in the jasmonic acid (JA) biosynthesis pathway. In this work, the role of LOX and AOS in defense response induced by wounding was investigated using pea (*Pisum sativum* L., cv. Ning Xia) seedlings as the material. The results showed that wound-induced "JA burst" was accompanied by the activation of LOX and AOS and the accumulation of their mRNAs; applied JA also stimulated the accumulation of LOX and AOS. Further experiments conducted with inhibitors demonstrated that the wound-induced JA was regulated by LOX and AOS at both transcriptional and enzymic levels. Their activation was necessary in wound-mediated defense response and could enhance the tolerance of pea seedlings to wounding.

Keywords: Pisum sativum, allene oxide synthase, lipoxygenase, jasmonic acid, defense response.

DOI: 10.1134/S1021443711020233

INTRODUCTION

Plants have evolved complex defense mechanisms to cope with the stress caused by mechanical wounding, which is produced by either abiotic factors or herbivory and results in the induction of defense genes. The activation of wound-responsive genes is triggered by a complex signaling network, in which the plant hormone jasmonic acid (JA) plays a pivotal role.

In generally, JA and its derivatives, such as methyl jasmonic acid (MeJA), collectively called jasmonates (JAs), are synthesized from linolenic acid (LeA) or linoleic acid (LA) by consecutive actions of 13-lipoxygenase (13-LOX), allene oxide synthase (AOS), allene oxide cyclase (AOC), 12-oxophytodienoic acid reductase (OPR3), and β-oxidative enzymes [1]. JAs exist widely in plants and regulate plant development on molecular, cell, and organ levels. They are involved in plants growth, fruit maturation, tuber formation, trichome formation, senescence, and flower development [2], as well as in various stress responses [3, 4]. JA is an important signal molecule in plants functioning in response to biotic and abiotic stresses. In most plant stress responses, the role of JA is indicated by its endogenous rise following the onset of stresses or pathogenic attack. Also distinct developmental stages, organs, and tissues are characterized by remarkably different and specific contents of JA and related compounds. Besides, it is possible that JA acts as a systemic signal, leading also to systemic expression of genes encoding proteinase inhibitors and other foliar compounds with negative effects on herbivore performance. Consequently, the plants become immunized against a subsequent herbivore attack. Grafting experiments with JA-deficient and JA-signaling mutants performed in G. Howe laboratory have shown that JA signaling was necessary in the systemic leaf defense [1].

Most LOXs (EC 1.13.11.12) are non-heme iron-containing dioxygenases that catalyze hydroperoxidation of polyunsaturated fatty acids with a *cis,cis*-1,4-pentadiene structure. The products of LOX activity are 9-C or 13-C fatty acid hydroperoxides synthesized by 9-LOX and 13-LOX, respectively [1]. Many 9- and 13-LOXs are located in the cytosol, but chloroplasts contain only 13-LOXs. The products of the LOX reaction are then converted to different oxylipins in at least six biosynthetic branches [5]. The AOS branch leads to JAs, like JA or 12-oxo-oxophytodienoic acid (OPDA). The participation of chloroplastic LOXs in the AOS branches has been clearly established in several plants. In *Arabidopsis thaliana*, cosupression of

[1] This text was submitted by the authors in English.
[2] These authors contributed equally to this work.

Abbreviations: AOC—allene oxide cyclase; AOS—allene oxide synthase; ASA—acetylsalicylic acid; JA—jasmonic acid; HPOT—hydroperoxyoctadecatrienoic acid; JAs—jasmonates; LOX—lipoxygenase; LA—linoleic acid; LeA—linolenic acid; MeJA—methyl jasmonic acid; NDGA—nordihydroguaiaretic acid; OPDA—12-oxo-oxophytodienoic acid; OPR3—12-oxophytodienoic acid reductase; PAL—phenylalanine ammonia-lyase.

the *AtLOX2* gene reduced wound-induced JA accumulation and wound- and JA-inducible gene expression [6]. In *Nicotiana attenuata*, antisense expression of *NaLOX3* specifically reduced JA accumulation, expression of JA-induced genes, and resistance to *Manduca sexta* attack [7]. Activity, protein, or mRNA accumulation of chloroplastic LOXs in response to wounding or pathogen interaction has been also described in a number of nonlegume plants, like tomato [8], wheat [9], *N. attenuate* [7], maize [10], and others. Besides, LOXs played important roles in some other stresses, such as water and drought stresses [11, 12].

Conversion of hydroperoxyoctadecatrienoic acid (HPOT), the first step in JA biosynthesis, is carried out by AOS, a cytochrome P450 enzyme of the CYP74A family, leading to an unstable allene oxide [13]. Then the short-lived allene oxide is cyclized to *cis*-(+)-OPDA by AOC. The synthesis of OPDA from LA occurs in plastids [6], and it is now becoming increasingly clear that, in addition to JA or OPDA, the first cyclic metabolite in the pathway, is a genuine endogenous signaling compound [1]. Thus, it is to be expected that the level of OPDA in plants is tightly controlled. Therefore, the entrance reaction of a biosynthetic pathway may be an appropriate control point for the entire pathway. The observation that the levels of AOS mRNA rose drastically and transiently in *A. thaliana* leaves after wounding [13] and that the AOS promoter was strongly wound-responsive [14] is in line with this notion.

All genes encoding enzymes of JA biosynthesis are JA-inducible, and promoters analyzed so far increase their activity upon JA treatment. This has led to the suggestion that JA biosynthesis is regulated by a positive feedback [4]. LOX and AOS are the two key enzymes of this pathway and could be induced by wounding. However, the relation between LOX/AOS and JA accumulation as well as their roles in wound-inducible defense response were investigated little. In this study, pea seedlings were used as the material for studying the relation between LOX/AOS and JA in the terms of enzymic and transcriptional levels, as well as investigating the roles of LOX/AOS in wound-mediated defense response using the inhibitors of LOX/AOS activity.

MATERIALS AND METHODS

Materials. Ten-day-old pea (*Pisum sativum* L., cv. Ning Xia) seedlings with six leaves grown in the greenhouse in China Agricultural University were used as the material. After being excised at the base of stems with a razor blade, the seedling shoots were placed in white porcelains with a little water and then recovered for 12 h at 25°C in a growth chamber with light intensity of 200 μmol/(m^2 s). The recovered seedlings were used for the following treatments.

All of the seedlings treated were maintained in a growth chamber for 15 h of light (200 μmol/(m^2 s) at 25°C and 9 h of dark at 19°C at 60% relative humidity.

Wounding treatment was performed as follows: one of the upper fully expanded leaves in each recovered seedling was quickly wounded twice at the central zone perpendicular to the main vein with a pair of scissors. After the treatment was finished, samples were harvested at the times indicated in figures, immediately frozen in liquid nitrogen, and stored at −80°C until further use.

Exogenous JA (10 μM, Sigma, United States) treatment was performed as follows: the recovered seedlings were immersed in 10 μM (±)JA (Sigma) under light at 25°C for 1 h. After treatment was completed, the pea leaves were harvested at the times indicated in figures, immediately frozen in liquid nitrogen, and stored at −80°C until further use.

Nordihydroguaiaretic acid (NDGA) (0.2 mM, Sigma) treatment was performed as follows: NDGA was sprayed on the pea seedling leaves until drips formed, for a 40 min infiltration period, then wounding treatment was conducted as described above. After treatment was completed, the pea leaves were harvested at the times indicated in figures, immediately frozen in liquid nitrogen, and stored at −80°C until further use.

Acetylsalicylic acid (ASA, 2.5 mM) and linoleic acid (LA, 75 μM) treatments were conducted similarly as NDGA treatment.

The leaves from nonpretreated seedlings were used as the control; they were harvested at the same period as the treated ones. At least three independent experiments were conducted for each treatment. All chemicals were purchased from Sigma unless otherwise noted.

Determination of MDA content. MDA content was measured as described by Heath and Packer [15]. The pea leaves (0.3 g) were homogenized in 5 ml of 0.1% trichloroacetic acid and centrifuged at 10000 *g* for 5 min. After centrifugation, 1 ml of the supernatant was mixed with 4 ml of 0.5% thiobarbituric acid, and the mixture was incubated in boiling water for 30 min; thereafter, it was transferred to an ice bath to stop the reaction. The absorbance was read at 532 nm and adjusted for nonspecific absorbance to 600 nm. MDA content was estimated by using an extinction coefficient of 155/(mM cm).

Determination of JA content. JA content was determined according to Deng et al. [16].

Analysis of LOX activity. Leaf tissues were ground to a fine powder in liquid nitrogen, and 1 g of powdered tissue was homogenized in 3 ml of ice-cold extraction buffer containing 50 mM sodium phosphate, pH 6.5, 1% (w/v) insoluble polyvinylpolypyrolidone, 5 mM DL-dithiothreitol, and 0.1 mM phenylmethanesulfonyl fluoride. The homogenate was centrifuged at 10000 *g* for 20 min at 4°C, and the

supernatant was used as the enzyme source. LOX activity was determined spectrophotometrically by measuring the formation of conjugated dienes at 234 nm and 30°C using LA (Acros Organics, United States) as a substrate according to a method described previously with some modification [17]. The absorption at 234 nm was recorded as a function of time for 4–5 min, and the activity was determined from the slope of the linear portion of the curve. One unit of enzyme activity was defined as an increase in absorbance at 234 nm per minute per milligram of protein under assay conditions. In order to determine the optimum pH of the reaction, the assays of the LOX activity were carried out at various pH values: 0.1 M sodium acetate buffer (pH 4.0, 4.5, 5.0, and 5.5), 0.1 M sodium phosphate buffer (pH 6.0, 6.5, 7.0, and 7.5), 50 mM Tris–HCl (pH 8.0, 8.5, and 9.0). Protein concentration was determined using the Bradford assay kit (Bio-Rad, United States) and BSA as a standard protein according to the manufacturer's instructions.

Analysis of AOS activity. AOS activity was measured as previously described by Zimmeraman [18] with some modifications. The reaction mixture contained 3 ml of PBS (pH 7.0), 200 μl of hydroperoxide substrate solution (0.4 ml of soybean LOX solution (1.0 mg/ml borate buffer, pH 9.0), 0.2 ml of linoleic acid (25 mM), and 10 ml of water for 2 h at 30°C), and 100 μl of the enzyme extract. The absorbance was measured at 234 nm before and after incubation.

Analysis of phenylalanine ammonia-lyase (PAL) activity. PAL activity was measured as previously described by Solecka and Kacperska [19] with some modifications. Briefly, the pea leaves (4 g) were homogenized with mortar and pestle in 12 ml of extraction buffer (50 mM Tris–HCl, pH 8.9, 15 mM β-mercaptoethanol, 5 mM EDTA, 5 mM ascorbic acid, 10 mM leupeptin, 1 mM PMSF, and 0.15% (w/v) PVP). The homogenate was filtrated through four layers of cheesecloth and centrifuged at 12000 *g* for 20 min at 4°C. The supernatant was used as a source of crude enzyme for assaying PAL activity. The reaction mixture (3 ml) contained 16 mM L-phenylalanine, 50 mM Tris–HCl (pH 8.9), 3.6 mM NaCl, and 0.5 ml of the crude enzyme. Incubation was performed at 37°C for 1 h, and the reaction was stopped by the addition of 500 ml of 6 M HCl. The reaction mixture was then centrifuged for 10 min at 12000 *g* to pellet the denatured protein. The absorbance was measured at 290 nm before and after incubation. One unit of enzyme activity was defined as an increase in absorbance at 290 nm per hour per milligram of protein under assay conditions. Protein concentration was determined using the Bradford assay kit (Bio-Rad) and BSA as a standard protein according to the manufacturer's instructions. To determine whether the reaction was enzymatic, a sample extract was boiled and assayed.

Determination of H_2O_2 content. Hydrogen peroxide was determined by the method of Patterson [20], and the result was presented as nmol H_2O_2/g fr wt.

Total RNA extraction and RT-PCR analysis. Total RNA was isolated from pea leaves with the method described by Logemann et al. [21] with some modifications. All steps were performed at 4°C. The pea leaves (0.5 g) were ground in liquid nitrogen and transferred into 2 ml of washing buffer comprising 0.1 M Tris–boracic acid (pH 7.4), 0.35 M sorbitol, 10% PEG 6000 (w/v), and 2% β-mercaptoethanol (v/v). After centrifugation at 12000 *g* for 8 min, 2 ml of the extraction buffer containing 0.1 M Tris–boracic acid (pH 7.4), 1.4 M NaCl, 0.02 M EDTA, and 2% cetyltrimethyl ammonium bromide was added, and the mixture was kept at 55°C for 20 min. Then 200 ml of 5 M acetate, 200 ml of ethanol, and 2 ml of chloroform were added. After centrifugation at 12000 *g* for 10 min, 1/3 volume of 10 M LiCl and 0.8 volume of isopropylalcohol were added before centrifugation at 15000 *g*. The pellet was dried and then resuspended in 0.5 ml of diethylpyrocarbamate (DEPC)-treated water, and 0.5 ml of water-saturated phenol was added. After centrifugation at 15000 *g* for 15 min, 0.5 ml of chloroform/isoamylalcohol was added before centrifugation at 15000 *g*. Total RNA was then precipitated overnight after addition of 1/3 volume of 10 M LiCl. After centrifugation (15000 *g*, 30 min), the pellet was washed in 75% ethanol and resuspended in DEPC-treated water. RNA yield was determined spectrophotometrically, and its quality was evaluated by electrophoresis in an agarose gel followed by ethidium bromide staining and UV light visualization.

A 2-μg aliquot of RNA was used as a template for reverse transcription–polymerase chain reaction, using an AMV reverse transcriptase (Promega A3500, United States) according to procedures specified by the manufacturer. According to published sequences of pea, gene-specific primers were designed (table). The amplification of *Actin* cDNA was used as an internal control. The PCR reactions were conducted in 25 μl total volume containing cDNA (0.5 μl), 0.5 mM dNTP, 2 units of *Taq* DNA polymerase (TaKaRa), 10 pmol of each gene-specific amplification primer. PCR conditions were as follows: 8 min initial heating at 94°C, followed by 26(*ACTIN*)/30(*LOX*)/35(*AOS*) three-step cycles of 1 min denaturation at 94°C, 30 s annealing at 53°C (*ACTIN*)/49°C(*LOX*)/47°C(*AOS*), and 30 s elongation at 72°C, followed by a final extension at 72°C for 8 min. The identity of all PCR products was confirmed by sequencing analysis at Invitrogen (Beijing, China).

Statistical analysis. Data were analyzed by analysis of variance and Student-Newman-Keuls test. Significance tests were performed on three independent experiments ($n = 3$; each replicate an average of 10 seedlings).

Primers for RT-PCR

Gene	Sequences 5'–3'	Size of PCR production	Genbank accession no.
LOX	Sense: ACCCTTATGCTGTTGATGGACT	327 bp	AJ49704
	Antisense: CAGGCATGAATCTTCTGCTTTT		
AOS	Sense: CTTCACCGTAGGTTAGCG	323 bp	AB095985
	Antisense: TGTTTCAACAACTTCTCCC		
ACTIN	Sense: GATTCTGGTGATGGTGGTGTGAGT	578 bp	PSU81048
	Antisense: GACAATTTCCCGTTCAGCAGT		

RESULTS

Activation of LOX and AOS and Accumulation of Their mRNAs Could Be Induced by Mechanical Wounding and Applied JA

LOX and AOS are two key enzymes involved in JA biosynthesis; they catalyze LA to form hydroperoxide and unstable allene oxide. Thus, the response of LOX and AOS to JA was investigated in pea seedling leaves.

The optimum pH of LOX activity in *Pisum sativum* L., cv. NingXia was determined firstly because it varies substantially in different materials. As shown in Fig. 1, the LOX activity determined in pea leaves was the highest at pH 6.0. In acid or alkaline environment, LOX activity was low or even undetectable. Therefore, pH 6.0 was selected for LOX activity determination in this study.

Although 9- and 13-LOXs always exist simultaneously with variation during plant development, they are responsible for different physiological functions. 13-LOXs had been identified to play a predominant role in wound-induced LOX activation [22]. After mechanical wounding, two peaks of LOX activity were observed in pea seedlings: a smaller peak (in 5 h, twice higher than control) and a higher peak (36 h, threefold higher than control). The first smaller peak was induced and disappeared rapidly, but the latter one accumulated gradually and maintained at a higher level for a long time (Fig. 2a). LOX activity increased gradually and reached its maximum in 24 h after JA application (threefold higher than control), then declined almost to its control level (Fig. 2b). The pattern of AOS response to wounding and exogenous JA was similar to that of LOX (Figs. 2c, 2d).

Additionally, changes in the accumulation of *LOX* and *AOS* mRNA induced by wounding and exogenous JA showed the same trends except for time parameters (Fig. 3). The two phases of *LOX* mRNA accumulation stimulated by wounding occurred in 3 and 24 h, and *AOS* mRNA appeared in 1 and 8 h, respectively (Fig. 3a). The accumulation of wound- and JA-inducible *LOX/AOS* mRNA occurred a little earlier than their activities (Fig. 3b).

Effect of Applied Inhibitors and Stimulator of JA Biosynthesis on LOX/AOS Activity and JA Accumulation Induced by Wounding

The results above indicated that LOX and AOS in pea seedlings responded to wounding and applied JA in a manner, which was similar to the response of endogenous JA to wounding. This may imply that LOX and AOS played instrumental roles in JA accumulation induced by wounding.

NDGA is a nonselective LOX inhibitor in terms of conversion of active ferric ions into the inactive ferrous state and chelating ferric ions [12]. The wound-induced LOX activity could be impaired to some degree after plant spraying with 100, 200, and 500 μM NDGA before wounding, and it was totally inhibited after pretreatment with 200 and 500 μM NDGA (Fig. 4a). Moreover, wound-induced JA was also inhibited greatly by pretreatment with 200 and 500 μM NDGA (Fig. 4b). Given the lower the better, the concentration of NDGA applied in the following experiments was 200 μM.

ASA is an inhibitor of AOS activity. As shown in Fig. 4c, AOS activity was partly inhibited after pretreatment with 0.5 and 1 mM ASA, and it was totally inhibited by pretreatment with 2.5 mM ASA. Similarly, wound-induced endogenous JA content was

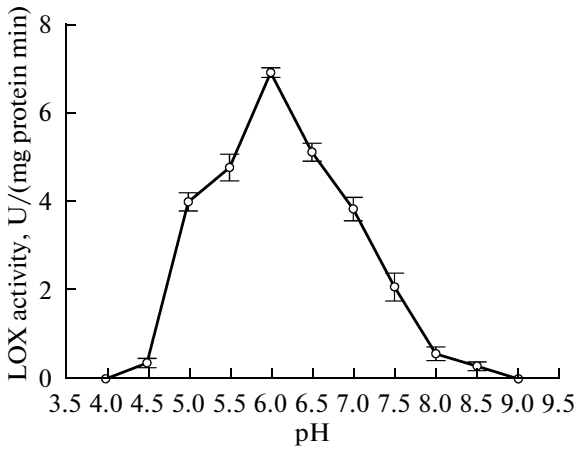

Fig. 1. Effect of pH values on LOX activity in pea seedlings.

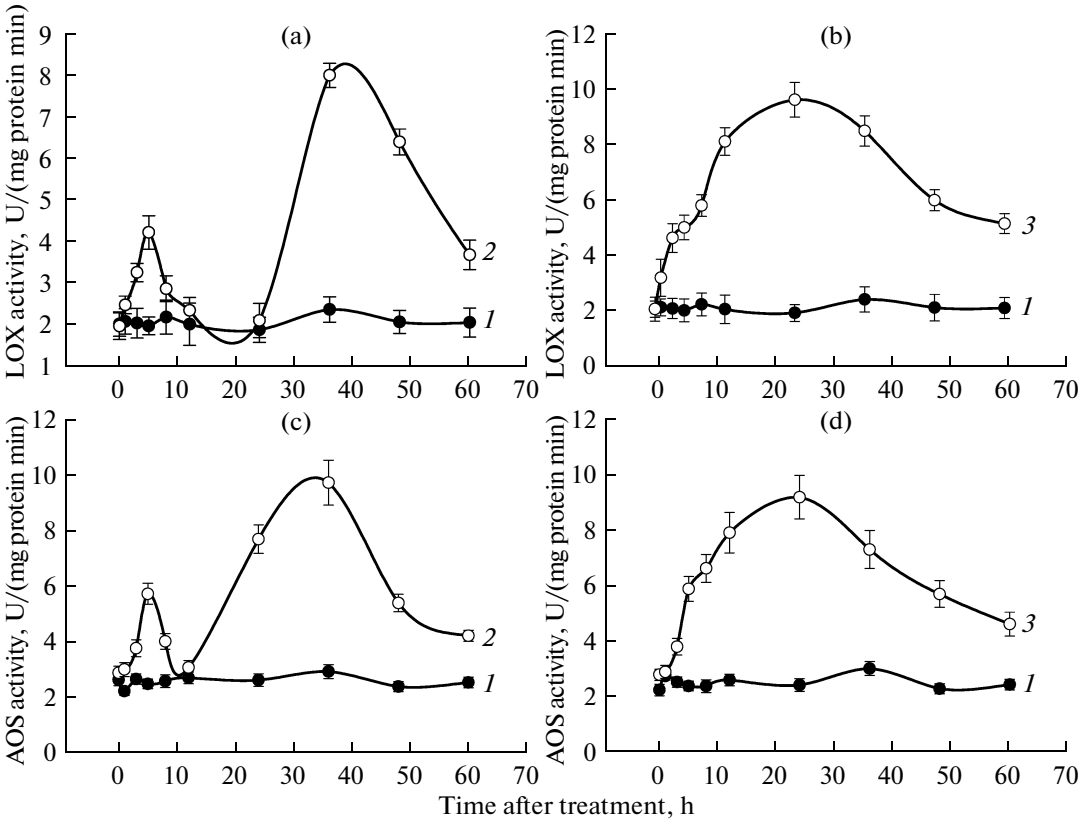

Fig. 2. Effect of wounding (a, b) and exogenous JA (b, d) on LOX (a, b) and AOS (c, d) activity. Intact pea seedling leaves were used as a control. (*1*) Control; (*2*) wounding; (*3*) JA.

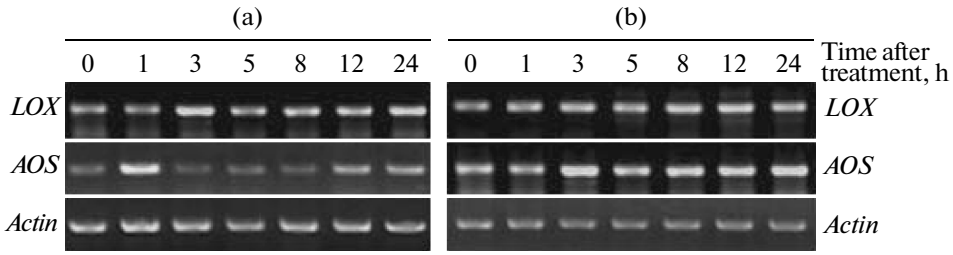

Fig. 3. Accumulation of *LOX* and *AOS* mRNA induced by wounding and exogenous JA.
(a) Wounding treatment; (b) treatment with 10 μM JA. *LOX* and *AOS* mRNA in response to wounding and applied JA were analyzed by RT-PCR. Amplification of β-*ACTIN* cDNA was used as a control to standardize the expression of the genes.

impaired little by 0.5 and 1 mM ASA pretreatment, but it was inhibited intensely by 2.5 mM ASA pretreatment (Fig. 4d). Therefore, 2.5 mM ASA was used as the inhibitor of JA biosynthesis in the following experiments.

As shown in Fig. 4e, LA could activate LOX activity effectively. When 25 μM LA was applied, LOX activity was not increased evidently; but when 75 and 100 μM LA were applied, LOX activity reached 83 and 93% of that induced by applied JA, respectively. Only 75 and 100 μM LA could enhance endogenous JA accumulation effectively, which was about 75 and 78% of that induced by wounding, respectively (Fig. 4f). Thus, 75 μM LA was used in the following experiments.

Effect of Applied Inhibitors and Stimulator of JA Biosynthesis on H_2O_2 Accumulation, PAL Activity, and MDA Content Induced by Wounding

Plants injury caused by various stresses is mainly the result of action of reactive oxygen species (ROS), which main form is H_2O_2. Hydrogen peroxide burst was observed immediately after wounding treatment of pea seedlings and reached its maximum in 3 h after

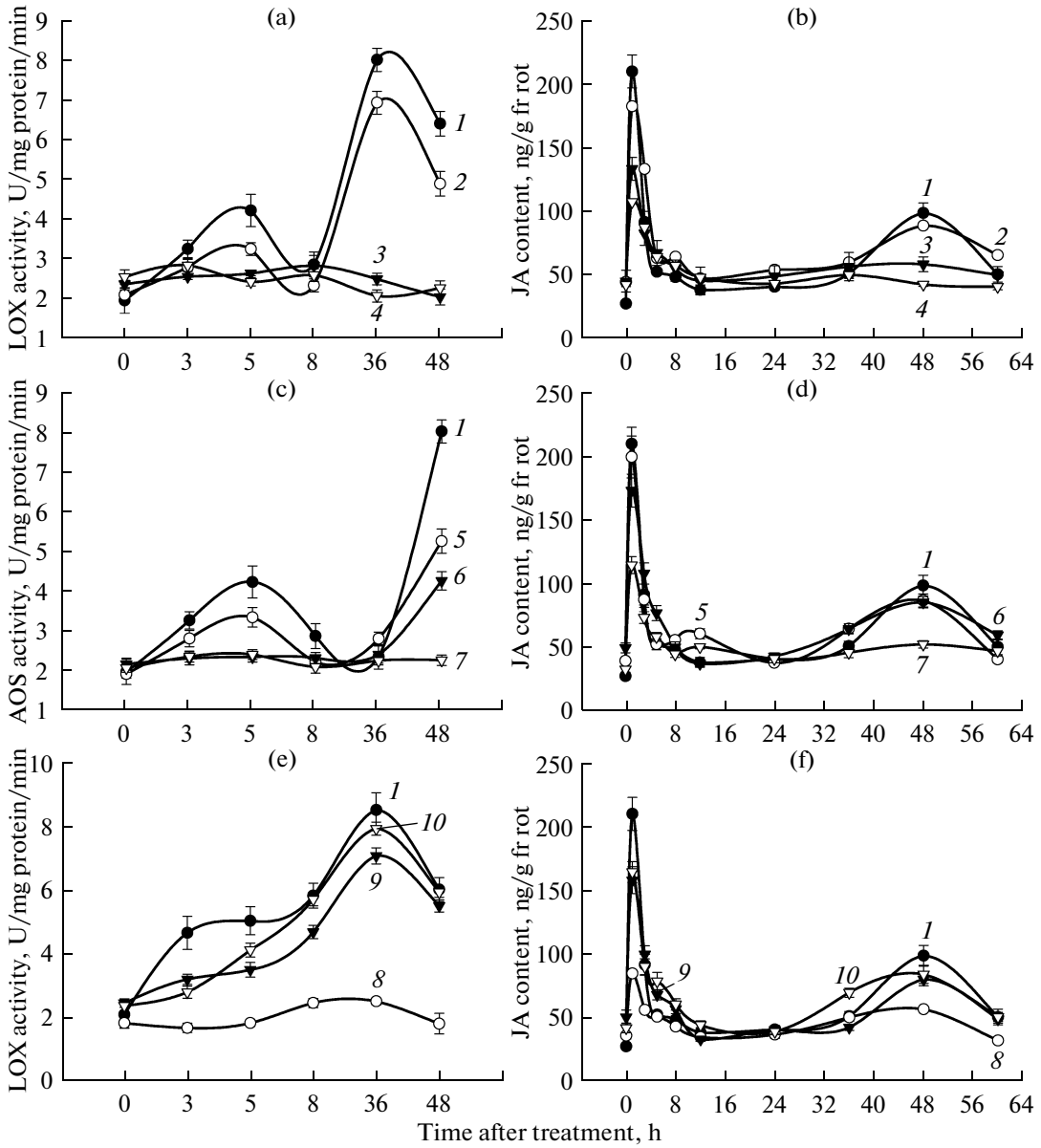

Fig. 4. Effect of various concentrations of NDGA, ASA, and LA on LOX/AOS activity and JA content induced by wounding. (*1*) Wound; (*2*) 0.1 mM NDGA + wound; (*3*) 0.2 mM NDGA + wound; (*4*) 0.5 mM NDGA + wound; (*5*) 0.5 mM ASA + wound; (*6*) 1.0 mM ASA + wound; (*7*) 2.5 mM ASA + wound; (*8*) 25 μM LA + wound; (*9*) 75 μM LA + wound; (*10*) 100 μM LA + wound.

wounding, being about twice higher than in control (Fig. 5). However, pretreatment with 200 μM NDGA before wounding could totally block the H_2O_2 burst, and pretreatment with 2.5 mM ASA also declined the H_2O_2 content to 60% of that induced by wounding (Figs. 5a, 5b). LA (75 μM) could stimulate H_2O_2 accumulation quickly, and its value was about 80% of that induced by wounding (Fig. 5c). These results showed that LOX and AOS played important roles in wound-induced oxidative stress and were closely involved to the wound-inducible accumulation of JA and H_2O_2.

PAL is the key enzyme of the phenylpropanoid biosynthesis pathway, and its activity is an important indicator of plant tolerance to wounding. PAL activity was effectively activated by wounding, reached its maximum at 48 h, and then declined (Fig. 6). But pretreatment with 200 μM NDGA and 2.5 mM ASA inhibited wound-induced PAL activity intensely. At application of 75 μM LA, PAL activity exhibited a similar change curve as wounding treatment or even more fierce; its highest value was 18% higher than after wounding treatment.

MDA is the result of peroxidation of membrane lipids, and its level reflects the degree of the membrane injury. As shown in Fig. 7a, MDA content increased along with increasing NDGA concentration. After

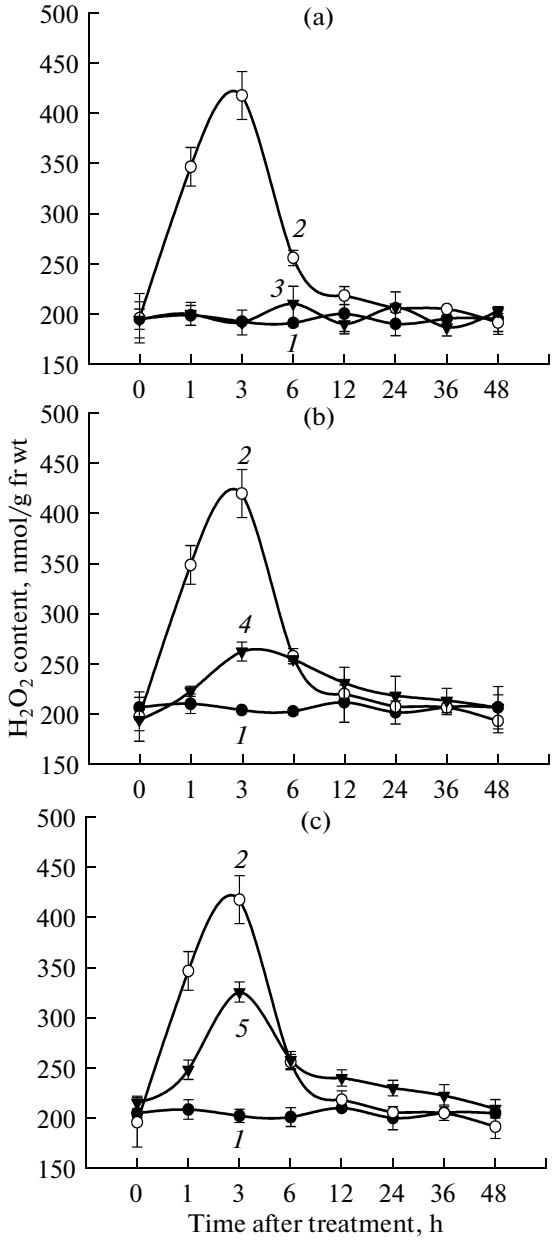

Fig. 5. Effect of NDGA (a), ASA (b), and LA (c) on wound-inducible H_2O_2 content.
Intact pea seedling leaves were used as a control. (*1*) Control; (*2*) wound; (*3*) 0.2 mM NDGA + wound; (*4*) 25 mM ASA + wound; (*5*) 75 μM LA + wound.

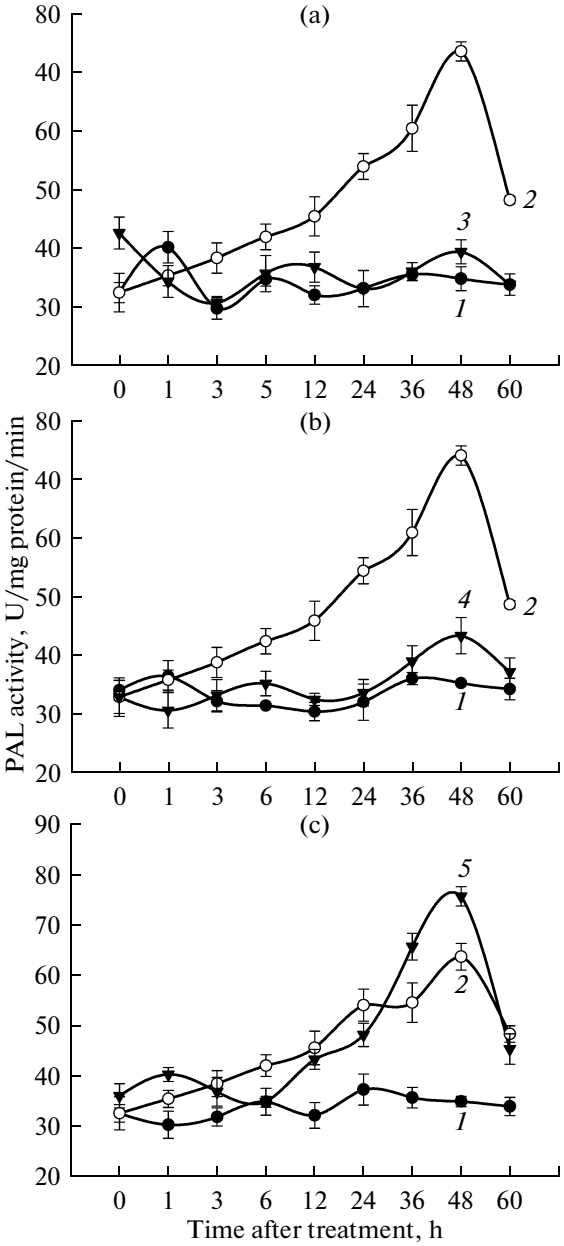

Fig. 6. Effect of NDGA (a), ASA (b), and LA (c) on wound-inducible PAL activity.
Intact pea seedling leaves were used as a control.
(*1*) Control; (*2*) wound; (*3*) 0.2 mM NDGA + wound; (*4*) 25 mM ASA + wound; (*5*) 75 μM LA + wound.

pretreatment with 200 and 500 μM NDGA, wound-induced MDA content increased obviously, about 60% higher than wounding treatment. But there was no significant difference between these two treatments. Similar as after NDGA pretreatments, MDA content increased evidently after pretreatments with high concentrations of ASA (1 and 2.5 mM), and no significant difference existed between these two treatments (Fig. 7b). Additionally, the higher concentration of LA stimulated the MDA level in pea seedlings (Fig. 7c).

DISCUSSION

LOX and AOS Were Essential to JA Accumulation Induced by Wounding

Plants encounter various inevitable environmental stresses during their life and have evolved diverse defense mechanisms against stresses. One of the most known ones includes the signal transduction pathways of phytohormones, such as JA, salicylic acid, ethylene, and ABA. Endogenous JA level is extremely low

[22], which is accordance to the characteristic of plant hormones. A body of researches demonstrated that JAs employed broad-spectrum physiological functions during plant development, including inhibiting root growth, seed germination, tuber formation, trichome formation, promoting senescence, and inducing flower development [1]. In addition, there were also many studies focusing on JA-mediated wound defense response.

JA accumulated rapidly in wounded pea seedlings and varied in a biphasic manner: "JA burst" was observed in the earlier phase and another lower accumulation in the later phase [22]. This wound-induced JA accumulation was accompanied by the activation of LOX and AOS (Figs. 2, 3). Applied JA could also stimulate the LOX/AOS activity and their mRNA expression (Figs. 2, 3). Other studies also indicated that all enzymes related to JA biosynthesis could be induced by JA [4]. But wound-induced JA rose instantaneously, which occurred before the expression of LOX and AOS (Figs. 2, 3). This may suggest that the wound-induced JA accumulation stimulated the de novo biosynthesis of LOX/AOS, which accumulation in its turn promoted the increase in JA.

Whether the expression of LOX and AOS was related to the accumulation of JA induced by wounding, the endogenous level of LOX and AOS should be regulated to investigate the change in wound-induced JA. The traditional methods are transgenes, mutant screening, and inhibitor application. We inhibited the enzyme activities by spraying with its inhibitors. Therefore, in order to identify the role of LOX and AOS in the course of JA accumulation induced by wounding, NDGA and ASA, the inhibitors of LOX and AOS, respectively, were used for studying the interaction between LOX, AOS, and JA induced by wounding. The appropriate inhibitor concentration not only inhibited the LOX/AOS activity but also inhibited effectively the JA level induced by wounding (Figs. 4a–4d). It was noted that the inhibitory action of NDGA and ASA on wound-induced JA was selective; these inhibitors could totally inhibit the JA accumulation in the later phase but only partially in the earlier phase. The effect of applied LA on wound-induced LOX/AOS activity and JA content was concentration-dependent (Figs. 4e, 4f). In sum, the wound-induced JA was both regulated at the transcriptional and enzymic level of LOX/AOS.

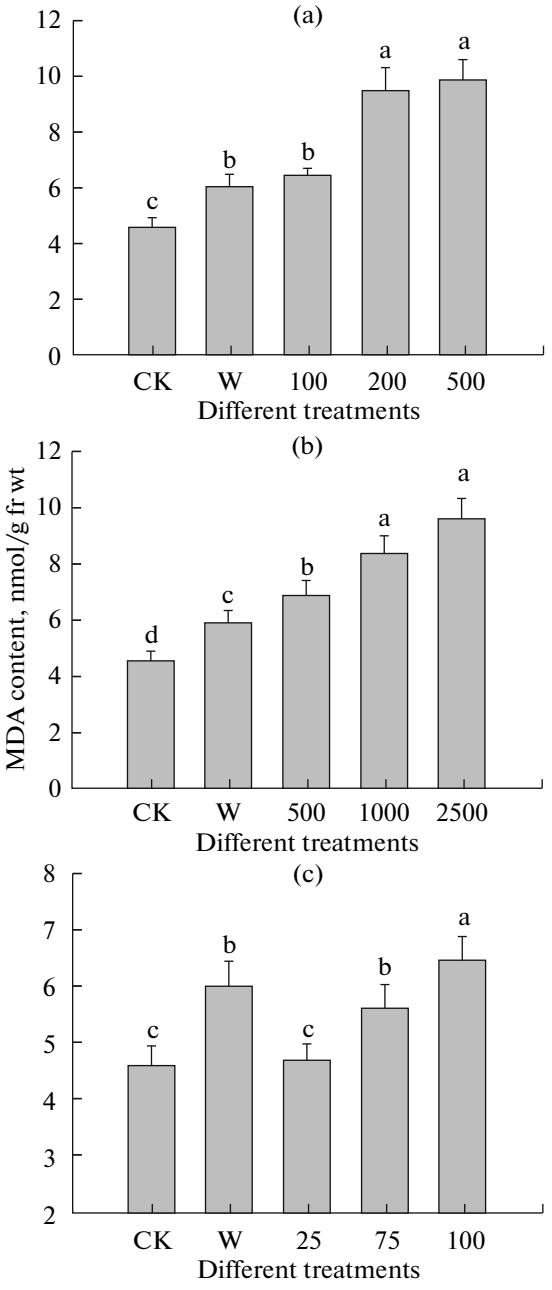

Fig. 7. Effect of NDGA, ASA, and LA on MDA content induced by wounding.

Intact pea seedling leaves were used as a control (CK). W—wounding. (a) 100, 200, and 500 indicated that pea leaves were sprayed with 100, 200, and 500 μM NDGA before wounding; (b) 500, 1000, and 2500 indicate that pea leaves were sprayed with 500, 1000, and 2500 μM ASA before wounding; (c) 25, 75, and 100 indicated that pea leaves were treated with 25, 75, and 100 μM LA. Different letters indicate a statistical difference at $P \leq 0.05$ among different treatments according to Duncan's multiple range test.

LOX/AOS Played Important Roles in Defense Response of Pea Seedlings Induced by Wounding

The results above indicated that the activation of LOX/AOS was closely related to the JA accumulation induced by wounding. Lots of reports demonstrated that wounding and applied JA could induce oxidative stress [23], which was also observed in the wounded pea seedlings (Fig. 5). But wound-induced H_2O_2 burst was inhibited by the pretreatment with NDGA and ASA and, in contrast, was stimulated by applied LA. ROS could possess direct defense function in plants, inducing lipid peroxidation, protein and DNA oxidation [24]. However, a large number of ROS attack the

biomembranes, leading to peroxidation of membrane lipids. MDA is produced by peroxidation of lipids, and its level represents the degree of membrane injury [25]. Our experiments revealed that LOX/AOS activities were inhibited by pretreatment with NDGA and ASA, and in contrast the MDA level enhanced along with the inrease in the applied concentrations. In other words, the ability of resistance to wounding of pea seedlings declined gradually due to the inhibition of LOX/AOS activity. Reversely, after application of LA, MDA level increase was accompanied by the activation of LOX/AOS, inducing the more intensely damage to pea seedlings (Fig. 7). This implies that LOX and AOS took part in the wound-induced oxidative burst course, and it was suggested that the activation of LOX/AOS contributed to the reduction of ROS toxicity.

Additionally, a series of secondary metabolites could be synthesized when plants were subjected to wounding, for example, phenylpropanes, flavonoids, and terpenoids. These compounds mainly gathered near the wound, participated in the wound recovery responses and blocked the insect attack. Secondary metabolites were synthesized along the phenylpropanes metabolic pathway, whose key enzyme is PAL [26]. Therefore, PAL activity was used for indicating the pea seedling ability of resistance to wounding. In wounded pea seedlings, PAL activity enhanced obviously, indicating that wound defense response could be induced efficiently by wounding (Fig. 6). Furthermore, *PAL* mRNA could be also stimulated by wounding [3, 26]. On the other hand, the inhibition of *PAL* gene transcription and protein synthesis decreased the enhanced PAL activity induced by wounding, which suggests that de novo synthesis of PAL was essential to these defense responses [27]. And wound-induced increase in the PAL activity was inhibited by pretreatment with NDGA and ASA (Fig. 6). This may imply that the activation of LOX/AOS was necessary in the wound-mediated defense response.

Consequently, when pea seedlings were subjected to wounding, LOX and AOS, the key enzymes of JA biosynthesis, showed a biphasic response curve like JA, but the times of the accumulation peaks occurring were ahead of that for JA. It was suggested that the wound-induced JA was regulated at both the transcriptional and enzymic levels of LOX/AOS. The application of LOX and AOS inhibitors revealed that the activation of LOX and AOS played essential roles in the wound-mediated defense response of pea seedlings.

ACKNOWLEDGMENTS

This work was supported by National Natural Science Foundation of China (no. 30611468) and a major program of Beijing Municipal Science and Technology Commission (no. D07060500160701).

REFERENCES

1. Wasternack, C., Jasmonates: An Update on Biosynthesis, Signal Transduction and Action in Plant Stress Response, Growth and Development, *Ann. Bot.,* 2007, vol. 100, pp. 681–697.
2. Creelman, R.A. and Mullet, J.E., Biosynthesis and Action of Jasmonates in Plants, *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 1997, vol. 48, pp. 355–381.
3. McConn, M., Creelman, R.A., Bell, E., Mullet, J.E., and Browse, J., Jasmonate Is Essential for Insect Defense in *Arabidopsis, Proc. Natl. Acad. Sci. USA,* 1997, vol. 94, pp. 5473–5477.
4. Wasternack, C., Oxilipins: Biosynthesis, Signal Transduction and Action, *Plant Hormone Signaling,* Hedden, P., and Thomas, S., Eds., Oxford: Blackwell, 2006, pp. 185–228.
5. Porta, H. and Rocha-Sosa, M., Plant Lipoxygenases Physiological and Molecular Features, *Plant Physiol.*, 2002, vol. 130, pp. 15–21.
6. Bell, E., Creelman, R.A., and Mullet, J.E., A Chloroplast Lipoxygenase Is Required for Wound-Induced Jasmonic Acid Accumulation in *Arabidopsis, Proc. Natl. Acad. Sci. USA,* 1995, vol. 92, pp. 8675–8679.
7. Halitschke, R. and Baldwin, I.T., Antisense LOX Expression Increases Herbivore Performance by Decreasing Defense Response and Inhibiting Growth-Related Transcriptional Reorganization in *Nicotiana attenuate, Plant J.*, 2003, vol. 36, pp. 794–807.
8. Heitz, T., Bergey, D.R., and Ryan, C.A., A Gene Encoding a Chloroplast Targeted Lipoxygenase in Tomato Leaves Is Transiently Induced by Wounding, Systemin, and Methyl Jasmonate, *Plant Physiol.,* 1997, vol. 114, pp. 1085–1093.
9. Bohland, C., Balkenhohl, T., Loers, G., Feussner, I., and Grambow, H.J., Differential Induction of Lipoxygenase Isoforms in Wheat upon Treatment with Rust Fungus Elicitor, Chitin Oligosaccharides, Chitosan, and Methyl Jasmonate, *Plant Physiol.,* 1997, vol. 114, pp. 679–685.
10. Nemchenko, A., Kunze, S., Feussner, I., and Kolomiets, M., Duplicate Maize 13-Lipoxygenase Genes Are Differentially Regulated by Circadian Rhythm, Cold Stress, Wounding, Pathogen Infection, and Hormonal Treatments, *J. Exp. Bot.*, 2006, vol. 57, pp. 3767–3779.
11. Xu, C.C. and Zou, Q., Effect of Drought on Lipoxygenase Activity, Ethylene and Ethane Production in Leaves of Soybean Plants, *J. Integr. Plant Biol.*, 1993, vol. 35, Suppl., pp. 31–37.
12. Yang, H.Q., Jia, W.S., and Zhang, D.P., Relationship between Lipoxygenase Activity and ABA Accumulation in *Malus hupehensis* Roots under Water Stresses, *J. Integr. Plant Biol.*, 2000, vol. 42, pp. 244-248.
13. Laudert, D., Pfannschmidt, U., Lottspeich, F., Holländer-Czytko, H., and Weiler, E.W., Cloning, Molecular and Functional Characterization of *Arabidopsis thaliana* Allene Oxide Synthase (CYP 74), the First Enzyme of the Octadecanoid Pathway to Jasmonates, *Plant Mol. Biol.,* 1996, vol. 31, pp. 323–335.
14. Laudert, D. and Weiler, E.W., Allene Oxide Synthase: A Major Control Point in *Arabidopsis thaliana* Octadecanoid Signaling, *Plant J.*, 1998, vol. 15, pp. 675–684.

15. Heath, R.L. and Packer, L., Photoperoxidation in Isolated Chloroplasts. I. Kinetics and Stoichiometry of Fatty Acid Peroxidation, *Arch. Biochem. Biophys.,* 1968, vol. 125, pp. 189–198.

16. Deng, A., Tan, W., He, S., Liu, W., Nan, T., Li, Z., Wang, B., and Li, Q.X., Monoclonal Antibody-Based Enzyme Linked Immunosorbent Assay for the Analysis of Jasmonates in Plants, *J. Integr. Plant Biol.*, 2008, vol. 50, pp. 1–7.

17. Axelrod, B., Cheesbrough, T.M., and Leakso, S., Lipoxyginase from Soybeans, *Methods Enzymol.,* 1981, vol. 7, pp. 443–451.

18. Zimmeraman, D.C. and Vick, B.A., Hydroperoxide Isomerase: A New Enzyme of Lipid Metabolism, *Plant Physiol.,* 1970, vol. 46, pp. 445–450.

19. Solecka, D. and Kacperska, A., Phenylpropanoid Deficiency Affects the Course of Plant Acclimation to Cold, *Physiol. Plant.*, 2003, vol. 119, pp. 253–262.

20. Patterson, B.D., Mackae, E.A., and Ferguson, I.B., Estimation of Hydrogen Peroxide in Plant Extracts Using Titanium(IV), *Analyt. Biochem.*, 1984, vol. 139, pp. 487–492.

21. Logemann, J., Schell, J., and Willmitzer, L., Improved Method for the Isolation of RNA from Plant Tissues, *Analyt. Biochem.*, 1987, vol. 163, pp. 16–20.

22. Yang, H.R., Tang, K., Liu, H.T., Pan, Q.H., and Huang, W.D., Jasmonic Acid Is Induced in a Biphasic Manner in Response of Pea Seedlings to Wounding, *J. Integr. Plant Biol.*, 2009, vol. 51, pp. 562–573.

23. Musetti, R., di Toppi, L.S., Martinin, M., Ferrini, F., Loschi, A., Favali, M.A., and Osler, R., Hydrogen Peroxide Localization and Antioxidant Status in the Recovery of Apricot Plants from European Stone Fruit Yellows, *Eur. J. Plant Pathol.*, 2005, vol. 112, pp. 53–61.

24. Wu, G., Shortt, B.J., Lawrence, E.B., Elaine, E.B., Fitzsimmons, K.C., and Shah, D.M., Disease Resistance Conferred by Expression of a Gene Encoding H_2O_2-Generating Glucose Oxidase in Transgenic Potato Plants, *Plant Cell*, 1995, vol. 7, pp. 1357–1368.

25. Wang, Y., Meny, Y.L., Ishidawa, H., Hibino, T., Tanaka, Y., Nii, N., Takabe, T., and Meng, Y.L., Photosynthetic Adaptation to Salt Stress in Three-Color Leaves of a C4 Plant (*Amaranthus tricolor*), *Plant Cell Physiol.*, 1999, vol. 40, pp. 6680–6745.

26. Diallinas, G. and Kanellis, A., A Phenylalanine Ammonia-Lyase Gene from Melon Fruit: cDNA Cloning, Sequence and Expression in Response to Development and Wounding, *Plant Mol. Biol.*, 1994, vol. 26, pp. 473–479.

27. Ke, D. and Saltveit, M.E., Wound-Induced Ethylene Production, Phenolic Metabolism and Susceptibility to Russet Spotting in Iceberg Lettuce, *Physiol. Plant.*, 1989, vol. 76, pp. 412–418.