Plant Signaling & Behavior 6:12, 1921-1925; December 2011; ©2011 Landes Bioscience

Serine/threonine protein phosphatases
Multi-purpose enzymes in control of defense mechanisms

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Key words: cantharidin, crosstalk, jasmonic acid, phytohormone signaling, protein phosphatase

Depending on the threat to a plant, different pattern recognition receptors, such as receptor-like kinases, identify the stress and trigger action by appropriate defense response development.[1,2] The plant immunity system primary response to these challenges is rapid accumulation of phytohormones, such as ethylene (ET), salicylic acid (SA) and jasmonic acid (JA) and its derivatives. These phytohormones induce further signal transduction and appropriate defenses against biotic threats.[3,4] Phytohormones play crucial roles not only in the initiation of diverse downstream signaling events in plant defense but also in the activation of effective defenses through an essential process called signaling pathway crosstalk, a mechanism involved in transduction signals between two or more distinct, "linear signal transduction pathways simultaneously activated in the same cell."[5]

Specific inhibitors of serine/threonine protein phosphatases (PPP) such as okadaic acid and cantharidin and mutations of PPP have been used to probe the role of PPP in various processes connected to different types of stress such as pathogens, wounding, cold and drought.[6-11] However, until the publication of our recent paper on the effects of cantharidin on the entire transcriptome of Arabidopsis,[12] there were no studies demonstrating the full capacity of a PPP inhibitor to impact gene transcription. The microarray experiment described in this paper revealed the breadth of the role of PPP in responses to biotic and abiotic stresses. Arabidopsis response to cantharidin mimicked the response to a pathogen infection. Gene sets, which are recognized as key players in the regulation of the phytohormone signaling pathways, were regulated by PPPs.

Cantharidin Treatments Affect the Jasmonate Biosynthetic Pathway

The JA response is induced by herbivores and various types of necrotrophic pathogens.[4,13] PPPs are proposed to be involved in the signal transduction pathway of this response.[10,11] Our microarray analysis by the MIPS FunCat database supported this hypothesis by revealing that 34 cantharidin-affected genes of Arabidopsis belong to wounding-response genes. Fourteen additional genes were directly or indirectly involved in JA biosynthesis: two *AOC* (allene oxide cyclase), two *ACX* (acyl-CoA oxidase), and two *PEX14* (peroxisome defective 2), seven genes of the *JAZ* family (jasmonate-ZIM-domain protein), and *PDF1.2* (plant defensin 1.2). The pool of affected genes also includes genes (hormone-responsive) which are expressed in the presence of JA and genes that are JA-independent wounding markers (**Table 1**). In addition, up to 86% of these genes were strongly upregulated by cantharidin at one or more of the time points after treatment (**Fig. 1**). This observation lends additional support to the idea that PPPs might act as a negative regulator of JA signaling.

JA biosynthesis occurs in two cellular compartments: chloroplasts and peroxisomes. Genes encoding lipoxygenase (LOX) and AOC, which are involved in the synthesis of cis-(+)-12-oxophytodienoic acid from α-linolenic acid (C18:3) in the chloroplast, are either up or downregulated at one or more of the time points after cantharidin treatment (**Table 1**). In the *LOX* gene family, the upregulated *LOX1* (AT1G55020) and *LOX3* (AT1G17420) encode lipoxygenase enzymes involved in biosynthesis of oxylipins.[14] LOX3 belongs to 13-lipoxygenases and is responsible for the biosynthesis of JA while LOX1 has 9-lipoxygenase activity, and its role is still unclear.[15] Four *AOC* homologues encoding AOC1, 2, 3 and 4 were identified in the Arabidopsis genome.[16] Two of them were affected by cantharidin treatment with *AOC3* upregulated (2.4-fold at 2 h) and *AOC4* downregulated (-1.4-fold at 10 h). The functional role of PPP on the regulation of these *AOC* genes remains to be elucidated. Recent research on four genes of the JA biosynthetic pathway (*LOX2*, *AOS*, *AOC2* and *OPR3*) revealed that, like most JA responsive genes, they are downregulated by SA.[17]

Regulation of the Expression of JA-responsive Genes

The jasmonate ZIM-domain repressor family (JAZ) consists of 12 members that control the expression of JA-responsive genes by interacting with transcription factors such as AtMYC2.[18,19] Not all JAZ proteins have empirically confirmed repressor

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Submitted: 08/27/11; Accepted: 09/19/11
DOI: 10.4161/psb.6.12.18146

functions (Chico et al. 2008).[18] JA accumulation induces JAZ repressor interactions with ubiquitin ligase SCF^{COI1} and subsequent 26S proteosome degradation.[3,20,21] It is worth mentioning that JAZ and ligase SCF^{COI1} interaction depends on bioactive JA derivatives, particularly JA-isoleucine (JA-Ile) conjugate.[3,22] The JAZ proteins encoded by JA-responsive genes are involved in control of the JA pathway by negative feedback mechanisms.[18] Their expression takes place within minutes after wounding at both local and systemic levels.[3,21] Chung et al. observed significant differences in timing of JAZ expression in response to herbivory compared to mechanical wounding.[21] JAZ-response to feeding by the insect *Spodoptera exigua* was rapid and long lasting, while the response to mechanical wounding was immediate and short-lived. Their experiment elegantly shows how fine tuned the timing of the plant immune system is for different challenges. In our experiment, the expression of up to seven *JAZ* genes increased after 24 h (**Fig. 1 and Table 1**). Only *JAZ1* was upregulated at all time points, and *JAZ8* had the highest expression among all induced *JAZ* genes. In our experiment, the transcription pattern of particular *JAZ* members was similar to that caused by herbivory feeding.

After 24 h exposure to cantharidin, the expression of JA marker genes such as *PDF1.2* and *VSP* (vegetative storage protein) increased 2.3- and 1.8-fold, respectively (**Table 1**). We observed an upregulation of *ATPEP1/PROPEP1* and *PEPR1* which encode proteins responsible for amplification of JA-dependent responses. The protein PROPEP1 is a precursor of the elicitor peptide 1 (ATPEP1), and PEPR1 is its receptor.[23] ATPEP1 belongs to the so called Damage Associated Molecular Patterns (DAMPS), and its presence induces *PDF1.2* expression.[23] Also, exogenous SA or induction of SA-dependent responses causes downregulation of *PDF1.2* and *VSP2* genes.[24] Interestingly, Rojo et al.[11] showed that

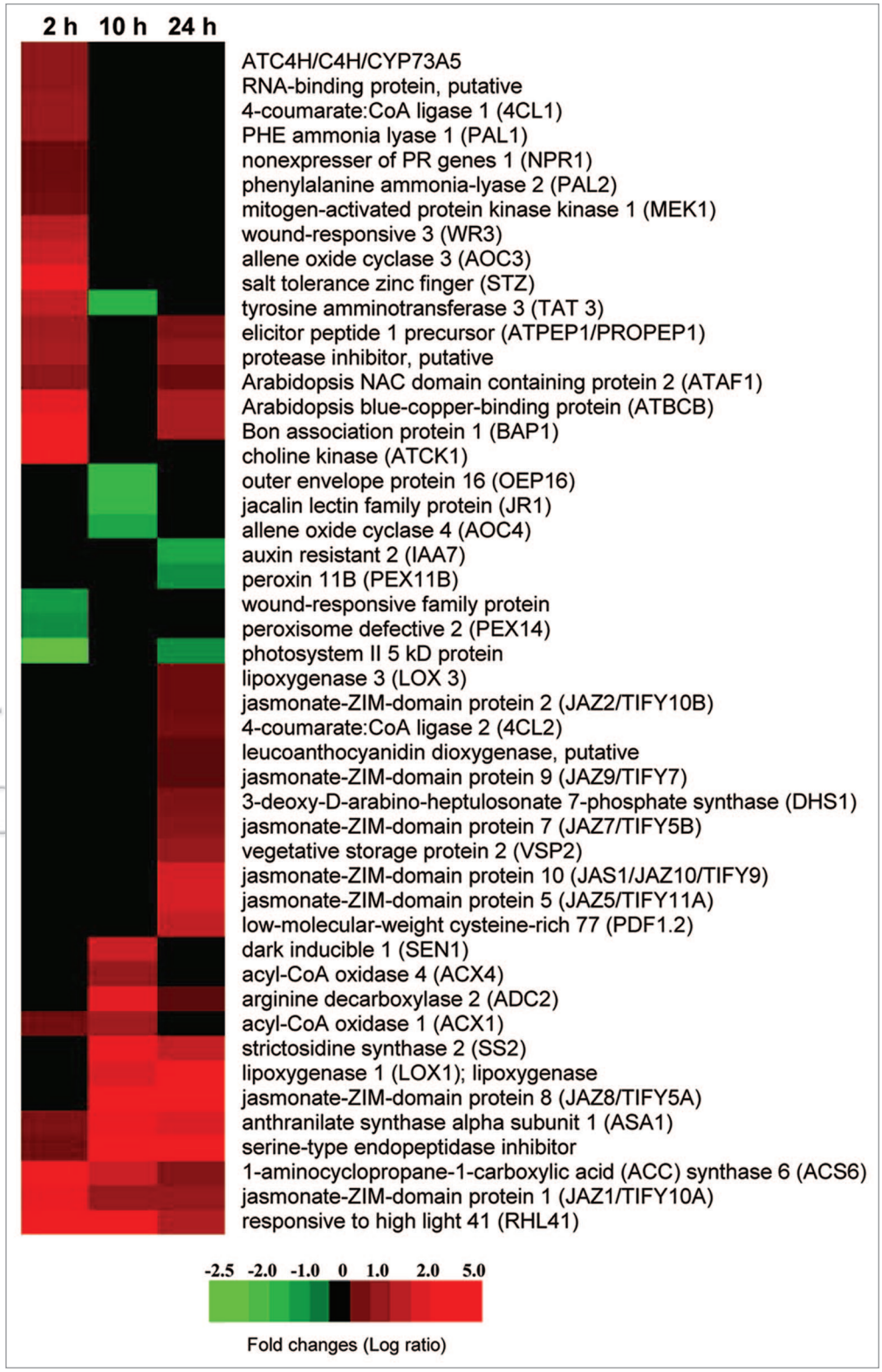

Figure 1. Heat-map showing expression profiles of wounding-responsive genes of Arabidopsis at three time points after treatment with 200 μM cantharidin.

Table 1. Selected genes involved in signaling pathways in response to cantharidin treatment

Array Element	Locus Identifier	Signal Log Ratio 2 h	Signal Log Ratio 10 h	Signal Log Ratio 24 h	Annotation
257769_at	AT3G23050			-1.4	Auxin resistant 2 (IAA7)
265149_at	AT1G51400	-2.2		-1.2	Photosystem II 5 kD protein
253203_at	AT4G34710		2.7	1.1	Arginine carboxylase 2 (ADC2)
254283_s_at	AT4G22870; AT4G22880			1.1	Leucoanthocyanidin dioxygenase, putative
261564_at	AT1G01720	1.7		1.3	Arabidopsis NAC domain containing protein 2 (ATAF1)
261037_at	AT1G17420			1.3	Lipoxygenase 3 (LOX3)
258047_at	AT3G21240			1.4	4-coumarate:CoA ligase 2 (4CL2)
247213_at	AT5G64900	1.9		1.5	Elicitor peptide 1 precursor (ATPEP1/PROPEP1)
252831_at	AT4G39980			1.5	3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DHS1)
254926_at	AT4G11280	3.4	2.4	1.6	1-aminocyclopropane-1-carboxylic acid (ACC) synthase 6 (ACS6)
266168_at	AT2G38870	2		1.7	Protease inhibitor, putative
245928_s_at	AT5G24770; AT5G24780			1.8	Vegetative storage protein 2 (VSP2)
246099_at	AT5G20230	2.7		2	Arabidopsis blue-copper-binding protein (ATBCB)
251336_at	AT3G61190	3.4		2	Bon association protein 1 (BAP1)
247655_at	AT5G59820	4.3	4.2	2.1	Responsive to high light 41 (RHL41)
260391_at	AT1G74020		2.8	2.3	Strictosidine synthase 2 (SS2)
250738_at	AT5G05730	1.5	2.8	2.6	Anthranilate synthase alpha subunit 1 (ASA1)
256321_at	AT1G55020		2.6	3.1	Lipoxygenase 1 (LOX1); lipoxygenase
249101_at	AT5G43580	1.4	3.3	3.5	Serine-type endopeptidase inhibitor
266225_at	AT2G28900		-1.7		Outer envelope protein 16 (OEP16)
259383_at	AT3G16470		-1.7		Jacalin lectin family protein (JR1)
263539_at	AT2G24850	2.3	-1.6		Tyrosine aminotransferase 3 (TAT3)
253161_at	AT4G35770		2.4		Dark inducible 1 (SEN1)
261144_s_at	AT1G19660; AT1G75380	-1.3			Wound-responsive family protein
259764_at	AT1G64280	1.3			Nonexpresser of PR genes 1 (NPR1)
251984_at	AT3G53260	1.3			Phenylalanine ammonia-lyase 2 (PAL2)
253993_at	AT4G26070	1.4			Mitogen-activated protein kinase kinase 1 (MEK1)
267470_at	AT2G30490	1.7			Cinnamate 4-hydroxylase (ATC4H/C4H/CYP73A5)
256770_at	AT3G13790	1.7			RNA-binding protein, putative
256186_at	AT1G51680	1.8			4-coumarate: CoA ligase 1 (4CL1)
263845_at	AT2G37040	1.8			PHE ammonia lyase 1 (PAL1)
248551_at	AT5G50200	2.2			Wound-responsive 3 (WR3)
261648_at	AT1G27730	2.8			Salt tolerance zinc finger (STZ)
261506_at	AT1G71697	3.8			Choline kinase (ATCK1)
257644_at	AT3G25780	2.4			Allene oxide cyclase 3 (AOC3)
259366_at	AT1G13280		-1.4		Allene oxide cyclase 4 (AOC4)
245249_at	AT4G16760	1.4	1.9		Acyl-CoA oxidase 1 (ACX1)
246304_at	AT3G51840		1.8		Acyl-CoA oxidase 4 (ACX4)
252411_at	AT3G47430			-1.2	Peroxin 11B (PEX11B)
247422_at	AT5G62810	-1.2			Peroxisome defective 2 (PEX14)
250292_at	AT5G13220			2.5	Jasmonate-ZIM-domain protein 10 (JAS1/JAZ10/TIFY9)
256017_at	AT1G19180	2.8	1.8	1.8	Jasmonate-ZIM-domain protein 1 (JAZ1/TIFY10A)
262171_at	AT1G74950			1.3	Jasmonate-ZIM-domain protein 2 (JAZ2/TIFY10B)
261033_at	AT1G17380			2.5	Jasmonate-ZIM-domain protein 5 (JAZ5/TIFY11A)
266901_at	AT2G34600			1.6	Jasmonate-ZIM-domain protein 7 (JAZ7/TIFY5B)

Table 1. Selected genes involved in signaling pathways in response to cantharidin treatment (continued)

Array Element	Locus Identifier	Signal Log Ratio 2 h	Signal Log Ratio 10 h	Signal Log Ratio 24 h	Annotation
256159_at	AT1G30135		3.4	3.7	Jasmonate-ZIM-domain protein 8 (JAZ8/TIFY5A)
260205_at	AT1G70700			1.1	Jasmonate-ZIM-domain protein 9 (JAZ9/TIFY7)
249052_at	AT5G44420			2.3	Low-molecular-weight cysteine-rich 77 (PDF1.2)

the protein phosphatase inhibitor okadaic acid does not induce *VSP* gene expression, while JA and wounding do. Although genes *JR1* (jacalin lectin family protein), *WR3* (wound responsive gene 3), and *ATCK* (choline kinase) were regulated in the same way in our and Rojo's experiments, the expression of both *WR3* and *ATCK* is wounding but not JA-dependent.

Crosstalk of Signaling Pathways in Response to Cantharidin Treatments

Plants activate a response system upon pathogen or insect attack by producing phytohormonal signals such as SA, JA and ET. The interactions between these induced defense-signaling pathways are documented as positive and negative crosstalk.[25,26] Mediators of this extraordinarily complicated network and signal markers which facilitate the identification of the dominating signal pathway are both important aspects of research on crosstalk. Several such genes were affected by cantharidin treatment.[12] For example, gene *NPR1* (Nonexpressor of PR genes 1) slightly increased expression at the 2-h time point (1.3) (**Table 1**). NPR1 functions as a sensor of redox status and controls SA and JA signal transduction pathways by negatively regulating SA signaling, and positively modulating the expression of JA-dependent genes during herbivore attack.[25] The situation became even more complicated when Leon-Reyes et al. discovered that NPR1-mediated SA/JA crosstalk is modulated by one more ET factor.[26] Also, the role of NPR1 varies, depending on its cellular localization, nuclear or cytosolic. Two other proteins located in the crosstalk downstream of NPR1 were proposed as SA/JA interaction regulators: glutaredoxin (GRX480) and WRKY70, a transcription factor of SA-dependent genes. The roles of GRX480 were discovered because of its strong interaction with TGA transcription factors.[27] In our experiment, *GRX480* was strongly induced by cantharidin (2.4 at 10 h time point and 1.3 at 24 h time point) while the transcript level of *WRKY70* decreased (-1.3 at 10 h and -1.2 at 24 h).[12] The role of this transcription factor in regulation of SA-dependent signaling was described in a study where its accumulation caused a decrease of MeJA-dependent *PDF1.2* expression.[28] This indirectly agreed with our data: downregulation of *WRKY70* and upregulation of *PDF1.2*. Results presented above might suggest PPPs' critical role in negative regulation of plant defenses, where PPP inhibition prevents the establishment of appropriate crosstalk between signaling pathways. Moreover, cantharidin seems to have major effect on the JA signaling pathway. The most characteristic signal markers (*PDF1.2* and *VSP)* showed strong expression changes (**Table 1**). The expression of the very commonly used SA signal marker gene *PR1* (*Pathogenesis Related gene 1*), as well as other members of Pathogenesis-Related family (such as *PR2*), were downregulated and only one (*PR5*) upregulated at the 24 h time point.[12] Taken together, these data suggest that cantharidin treatment might favor both JA-dependent and -independent wound responses. This hypothesis will need additional empirical confirmation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

1. Boller T, He SY. Innate immunity in plants: an arms race between pattern recognition receptors in plants and effectors in microbial pathogens. Science 2009; 324:742-4.
2. Albert M, Jehle AK, Lipschis M, Mueller K, Zeng Y, Felix G. Regulation of cell behaviour by plant receptor kinases: Pattern recognition receptors as prototypical models. Eur J Cell Biol 2010; 89:200-7.
3. Koo AJ, Gao X, Jones AD, Howe GA. A rapid wound signal activates the systemic synthesis of bioactive jasmonates in Arabidopsis. Plant J 2009; 59:974-86.
4. De Vos M, Van Oosten VR, Van Poecke RM, Van Pelt JA, Pozo MJ, Mueller MJ, et al. Signal signature and transcriptome changes of Arabidopsis during pathogen and insect attack. Mol Plant Microbe Interact 2005; 18:923-37.
5. Wang KL, Li H, Ecker JR. Ethylene biosynthesis and signaling networks. Plant Cell 2002; 14:131-51.
6. MacKintosh C, Lyon GD, MacKintosh RW. Protein phosphatase inhibitors activate anti-fungal defence responses of soybean cotyledons and cell cultures. Plant J 1994; 5:137-47.
7. Facchini PJ, Johnson AG, Poupart J, de Luca V. Uncoupled defense gene expression and antimicrobial alkaloid accumulation in elicited opium poppy cell cultures. Plant Physiol 1996; 111:687-97.
8. Shin H, Shin HS, Guo Z, Blancaflor EB, Masson PH, Chen R. Complex regulation of Arabidopsis AGR1/PIN2-mediated root gravitropic response and basipetal auxin transport by cantharidin-sensitive protein phosphatases. Plant J 2005; 42:188-200.
9. País SM, González MA, Téllez-Iñón MT, Capiati DA. Characterization of potato (*Solanum tuberosum*) and tomato (*Solanum lycopersicum*) protein phosphatases type 2A catalytic subunits and their involvement in stress responses. Planta 2009; 230:13-25.
10. Dammann C, Rojo E, Sánchez-Serrano JJ. Abscisic acid and jasmonic acid activate wound-inducible genes in potato through separate, organ-specific signal transduction pathways. Plant J 1997; 11:773-82.
11. Rojo E, Titarenko E, León J, Berger S, Vancanneyt G, Sánchez-Serrano JJ. Reversible protein phosphorylation regulates jasmonic acid-dependent and -independent wound signal transduction pathways in *Arabidopsis thaliana*. Plant J 1998; 13:153-65.
12. Bajsa J, Pan Z, Duke SO. Transcriptional responses to cantharidin, a protein phosphatase inhibitor, in *Arabidopsis thaliana* reveal the involvement of multiple signal transduction pathways. Physiol Plant 2011; 143:188-205.
13. Mithöfer A, Boland W. Recognition of herbivory-associated molecular patterns. Plant Physiol 2008; 146:825-31.
14. Liavonchanka A, Feussner I, Lipoxygenases: occurrence, functions and catalysis. J. Plant Physiol 2006; 163:348-57.
15. Kilaru A, Herrfurth C, Keereetaweep J, Hornung E, Venables BJ, Feussner I, et al. Lipoxygenase-mediated oxidation of polyunsaturated *N*-acylethanolamines in Arabidopsis. J Biol Chem 2011; 286:15205-14.
16. Stenzel I, Hause B, Miersch O, Kurz T, Maucher H, Weichert H, et al. Jasmonate biosynthesis and the allene oxide cyclase family of *Arabidopsis thaliana*. Plant Mol Biol 2003; 51:895-911.
17. Leon-Reyes A, Van der Does D, De Lange ES, Delker C, Wasternack C, Van Wees SC, et al. Salicylate-mediated suppression of jasmonate-responsive gene expression in Arabidopsis is targeted downstream of the jasmonate biosynthesis pathway. Planta 2010; 232:1423-32.
18. Chico JM, Chini A, Fonseca S, Solano R. JAZ repressors set the rhythm in jasmonate signaling. Curr Opin Plant Biol 2008; 11:486-94.
19. Chini A, Fonseca S, Fernandez G, Adie B, Chico JM, Lorenzo O, et al. The JAZ family of repressors is the missing link in jasmonate signalling. Nature 2007; 448:666-71.
20. Chini A, Fonseca S, Chico JM, Fernández-Calvo P, Solano R. The ZIM domain mediates homo- and heteromeric interactions between Arabidopsis JAZ proteins. Plant J 2009; 59:77-87.

21. Chung HS, Koo AJ, Gao X, Jayanty S, Thines B, Jones AD, et al. Regulation and function of Arabidopsis JASMONATE ZIM-domain genes in response to wounding and herbivory. Plant Physiol 2008; 146:952-64.
22. Katsir L, Chung HS, Koo AJ, Howe GA. Jasmonate signaling: a conserved mechanism of hormone sensing. Curr Opin Plant Biol 2008; 11:428-35.
23. Krol E, Mentzel T, Chinchilla D, Boller T, Felix G, Kemmerling B, et al. Perception of the Arabidopsis danger signal peptide 1 involves the pattern recognition receptor AtPEPR1 and its close homologue AtPEPR2. J Biol Chem 2010; 285:13471-9.
24. Spoel SH, Koornneef A, Claessens SM, Korzelius JP, Van Pelt JA, Mueller MJ, et al. NPR1 modulates cross-talk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytosol. Plant Cell 2003; 15:760-70.
25. Koornneef A, Pieterse CM. Cross talk in defense signaling. Plant Physiol 2008; 146:839-44.
26. Leon-Reyes A, Spoel SH, De Lange ES, Abe H, Kobayashi M, Tsuda S, et al. Ethylene modulates the role of NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 in cross talk between salicylate and jasmonate signaling. Plant Physiol 2009; 149:1797-809.
27. Ndamukong I, Abdallat AA, Thurow C, Fode B, Zander M, Weigel R, et al. SA-inducible Arabidopsis glutaredoxin interacts with TGA factors and suppresses JA-responsive PDF1.2 transcription. Plant J 2007; 50:128-39.
28. Li J, Brader G, Kariola T, Palva ET. WRKY70 modulates the selection of signaling pathways in plant defense. Plant J 2006; 46:477-91.