Cell PRESS

New checkpoints in stomatal defense

Jean-Luc Montillet[1,2,3] and Heribert Hirt[4]

[1] Commissariat à l'Energie Atomique, IBEB, SBVME, Laboratoire d'Ecophysiologie Moléculaire des Plantes, 13108 Saint-Paul-lez-Durance, France
[2] CNRS, UMR 7265 Biologie Végétale et Microbiologie Environnementales, 13108 Saint-Paul-lez-Durance, France
[3] Université Aix-Marseille, 13108 Saint-Paul-lez-Durance, France
[4] Unité de Recherche en Génomique Végétale (URGV), Plant Genomics, INRA/CNRS/University of Evry/Saclay Plant Sciences, 2 Rue Gaston Cremieux, F-91057 Evry, France

Recent reports have revealed new guard cell signaling elements that function in stomatal defense in *Arabidopsis thaliana* (*Arabidopsis*). We discuss here the role of oxylipins, salicylic acid (SA), and abscisic acid (ABA) in stomatal immunity in response to the bacterial pathogen *Pseudomonas syringae*.

Guard cell closure: one pathway for abiotic and biotic stress signaling

Stomata are microscopic pores in the epidermis of aerial organs of plants which ensure the gas exchanges of CO_2 and water vapor both of which are required for photosynthesis and water homeostasis. Plants control the size of stomata by regulating the osmotic pressure of the two guard cells that flank stomatal openings and ABA has been attributed a predominant role in this stomatal regulation in response to variations of the physical environmental conditions [1].

In 2006, Maeli Melotto *et al*. [2] also assigned stomata a function in the early phases of innate immunity. This pioneering work established that stomata close 1 to 2 h after bacterial recognition, thereby preventing the entry of microbes and host tissue colonization. This response is referred to as the stomatal defense [3] and can be triggered by surface inoculation of plants with not only plant, but also human bacterial pathogens such as *Escherichia coli*. It was further demonstrated that microbial-associated molecular patterns (MAMPs), such as bacterial flagellin, flg22, a biologically active 22 amino acid long peptide of flagellin, and lipopolysaccharide (LPS), caused stomatal closure in *Arabidopsis*. Moreover, it was found that flg22-induced stomatal closure depended on the plant FLAGELLIN-SENSING2 (FLS2) receptor. Based on the observations that *Arabidopsis* mutants defective in ABA synthesis (*aba3-1*) or ABA signaling (*ost1-2*) were more susceptible to *Pseudomonas syringae* p.v. *tomato* (*Pst*) upon surface inoculation and that *Pst* or MAMPs were no longer able to trigger significant stomatal closure in these mutants, ABA was attributed a central role in stomatal defense [2]. SA has also been put forward as a key regulator in this signaling cascade [3,4]. SA induced stomatal closure and plants with reduced SA levels, such as *eds5-1*, *eds16/sid2*, or nahG lines, or plants affected in SA signaling, such as *npr1*, were compromised in their ability to close stomata in response to either bacteria or MAMPs and displayed lower stomatal defense [2,4]. A causal link between SA and ABA has been further suggested based on the observation that the ABA-deficient mutant line *aba2-1* no longer closed stomata in response to exogenously applied SA, whereas guard cells of SA-deficient *sid2* and nahG plants responded normally to ABA [4]. Together, these data suggested that both ABA and SA were required for stomatal closure and that SA action was upstream of that of ABA. Finally, it has been shown that the phytotoxin coronatine (COR) secreted by virulent strains of *Pst*, enables these strains to reopen stomata, thereby circumventing host stomatal defense and enabling the bacteria to colonize plant tissues [2].

The biotic stress-signaling cascade in guard cells: new pieces of the jigsaw puzzle

Recent work has unraveled several new components of guard cell signaling in response to biotic stress signals. The analysis of the cell type-specific leaf transcriptome of *Arabidopsis* allowed the identification of the guard cell 9-specific lipoxygenase LOX1 [5]. LOX1 was found to participate in stomatal defense and was required to trigger stomatal closure in response to both *Pst* and flg22 [6]. 13- and 9-lipoxygenases use polyunsaturated fatty acids as substrates to produce a variety of oxylipins. The best known product of 13-LOX enzymes is jasmonic acid, but the biological action of 9-LOX products is less documented. Interestingly, among various candidates, fatty acid hydroperoxides and reactive electrophile species oxylipins (RES oxylipins) were found as likely products of LOX1 and could induce stomatal closure at nanomolar concentrations [6]. It was further shown that the mitogen-activated protein kinases MPK3 and MPK6 function upstream and SA acted downstream of these LOX1-derived oxylipins. Finally, COR blocked the biological activity of oxylipins in a COI1 (CORONATINE INSENSITIVE1)-dependent manner (Figure 1). Together, these findings reveal the functioning of a guard cell-specific oxylipin pathway in the plant immune response.

Does ABA regulate or modulate the biotic stress signaling in guard cells?

Recently, evidence has been provided that questions the central function of ABA in the biotic stress signaling cascade of *Arabidopsis* guard cells [6]. The inability of the ABA mutants (*ost1-2* or *aba3-1*) to close stomata in

Corresponding author: Hirt, H. (hirt@evry.inra.fr).
Keywords: abscisic acid; lipoxygenase; mitogen-activated protein kinase; oxylipins; *Pseudomonas*; salicylic acid.

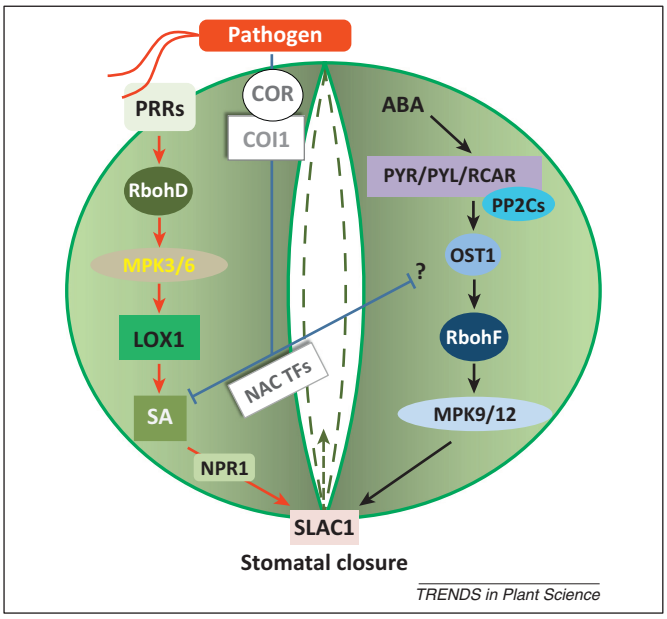

Figure 1. Signaling steps that mediate stomatal movements upon pathogen inoculation. Upon pathogen attack, pathogen-associated molecular patterns (PAMPs) activate host pattern recognition receptors (PRRs) leading to reactive oxygen species (ROS) production through the NADPH oxidase isoform RbohD. Downstream, the two mitogen-activated protein kinases (MAPKs) MPK3 and MPK6 are both required to activate the guard cell lipoxygenase LOX1 which catalyzes the peroxidation of poly unsaturated fatty acids into fatty acid hydroperoxides and reactive electrophile species (RES) oxylipins, both of which are potent inducers of stomatal closure. Salicylic acid (SA) accumulation is controlled by this oxylipin production and is required to convey downstream signals via the regulatory protein NPR1 (NONEXPRESSOR of PR GENES1) to the activation of the anion channel SLAC1. This final step contributes to stomatal closure. The ABA-mediated pathway requires the soluble PYR/PYL/RCAR (PYRABACTIN RESISTANCE/PYRABACTIN-LIKE/REGULATORY COMPONENTS OF ABA RECEPTOR) receptors which bind ABA and subsequently sequester the inhibitory protein phosphatase 2Cs (PP2Cs), thereby liberating the active form of the protein kinase OST1 (OPEN STOMATA 1). The direct interaction of OST1 with the NADPH oxidase RbohF leads to ROS production. MPK9 and MPK12 function as positive regulators of ROS-mediated ABA signaling and their activity is enhanced by H_2O_2. Finally, SLAC1 function is required to trigger stomatal closure in response to both, ABA and biotic signals. Recent data indicate that instead of regulating the biotic stress signal, ABA modulates this pathway likely by controlling the synthesis of the ion channels and H^+-ATPases which are required to adjust the osmotic pressure of guard cells. Coronatine (COR) produced by virulent *Pseudomonas* strains binds to the plant receptor COI1 (CORONATINE INSENSITIVE 1) and contributes to stomatal reopening through the three (NAM and ATAF1, ATAF2 and CUC2) NAC transcription factors [10]. This step represses both SA accumulation and ABA signaling suggesting that a block of this mechanism is required for complete stomatal reopening. These findings allow to infer that ABA should be produced in the long term upon plant pathogen inoculation.

response to MAMPs, flg22, or LPS along with their lower stomatal defense upon surface inoculation with the COR-deficient mutant bacterium *Pst* DC3118 [2] are both observations indicating that the ABA signaling pathway does regulate stomatal defense. However, a detailed analysis of stomatal responses to increasing doses of ABA, flg22, or the oxylipin 13-oxo-octadecadienoic acid (13-KODE) revealed that *ost1-2* stomata were only partially impaired in their response to flg22 or 13-KODE. Similarly, *aba2-1*, affected in ABA synthesis, displayed significant stomatal closure to flg22 and 13-KODE [6]. These data indicate that, in guard cells, an ABA-independent mechanism is still able to convey biotic stress signals. In line with these data, it has been also shown that flg22 or oxylipins were unable to activate the protein kinase OPEN STOMATA1 (OST1) which is a key component of the ABA signaling cascade. Because OST1 activation requires the presence of ABA [1] it can be assumed that ABA was unlikely produced immediately (10 min) after flg22 or oxylipin treatments. Strengthening the assumption that ABA is not produced very early after biotic stress, it was also demonstrated that over a period corresponding to the pathogen-induced stomatal closure (0.5–1 h), ABA-specific transcripts, such as *RD29b*, *ABI1*, and *ABI2*, were not produced following surface inoculation of plants with *Pst*. Even if ABA is not produced early after inoculation or MAMP treatment, ABA is essential in order to optimize stomatal closure in response to biotic stress and to enable plants to raise an efficient stomatal defense. To understand ABA-mediated modulation of stomatal response to biotic stresses, it must be considered that guard cell movements require the regulation of plasma membrane-located ion channels and H^+-ATPases to modify the osmotic pressure of these cells. The synthesis of these proteins is known to be ABA-dependent [1] and in ABA-defective mutants, the basal amounts of these proteins are likely too low to maintain normal responses. This situation is illustrated by the fact that ABA induces lower stomatal closure in the *aba2-1* mutant than in wild type plants and this is also observed in response to flg22 or 13-KODE [6]. Therefore, ABA appears to prime the efficiency of both its own and that of other stimuli, such as biotic signals. Further evidence for an ABA-independent biotic stress signaling pathway comes from the analysis of the reactive oxygen species (ROS)-dependent step required for stomatal closure upon biotic and abiotic stimuli. Interestingly the response to biotic stress exclusively depends on the isoform RbohD [9], whereas the ABA-mediated stomatal closure requires the OST1-dependent activation of the NADPH oxidase RbohF [7,8].

Concluding remarks and open questions

In summary, it appears that guard cell closure in response to abiotic and biotic stresses is mediated by largely ABA-dependent and oxylipin pathways, respectively. Nevertheless, the mechanisms whereby biotic signals autonomously regulate these processes in guard cells will require further investigations. The present model envisions that downstream of biotic stress signal perception, the two MAPKs, MPK3 and MPK6, are activated. Because these protein kinases display a large substrate range, it will be interesting to investigate an involvement of phosphorylation in the mechanism of the LOX1-dependent production of oxylipins. Structure–function analyses suggested that these oxylipins might covalently bind or alkylate thiol-containing target(s) to convey the signal towards SA accumulation. Downstream of this step, SA-mediated regulation of ABA synthesis could enable clarification of the function of ABA in the activation of stomatal defense. In this respect, it would be interesting to test whether genes involved in ABA synthesis or signaling are up-regulated in response to SA treatment. Apart from RES oxylipins, other naturally occurring thiol-reagents such as phenolics or isothiocyanates have been shown to display a potent activity on guard cells likely via covalent reactions with the RES oxylipin targets [6]. Hence, identification of these guard cell receptors of RES will undoubtedly help to understand how these cells sense and respond to different environmental stimuli. A recent approach, based on a random genetic

screen, identified *Arabidopsis* mutants that could rescue the virulence of COR-deficient mutant bacteria [3]. This work has contributed to characterize new genes essential for stomatal defense and should provide more insight into the key steps required for the plant immune response at the guard cell level.

One important conclusion that can be drawn from the latest findings is the existence of two guard cell pathways to mediate abiotic and biotic stress signaling. This offers independent strategies to improve plant stress resistance to pathogens without compromising tolerance to abiotic stresses, such as drought or heat, which belong to the most important economic challenges in agriculture. However, considering that other hormones and metabolites could also affect guard cell closure, our current model (Figure 1) is probably still far too simple to account for all regulatory mechanisms of guard cell behavior.

References

1 Joshi-Saha, A. *et al.* (2011) A brand new start: abscisic acid perception and transduction in the guard cell. *Sci. Signal.* 4, re4
2 Melotto, M. *et al.* (2006) Plant stomata function in innate immunity against bacterial invasion. *Cell* 126, 969–980
3 Zeng, W. *et al.* (2011) A genetic screen reveals *Arabidopsis* stomatal and/or apoplastic defenses against *Pseudomonas syringae* pv. *tomato* DC3000. *PLoS Pathog.* 7, e1002291
4 Zeng, W. and He, S.Y. (2010) A prominent role of the flagellin receptor FLAGELLIN-SENSING2 in mediating stomatal response to *Pseudomonas syringae* pv. *tomato* DC3000 in *Arabidopsis*. *Plant Physiol.* 153, 1188–1198
5 Leonhardt, N. *et al.* (2004) Microarray expression analyses of Arabidopsis guard cells and isolation of a recessive abscisic acid hypersensitive protein phosphatase 2C mutant. *Plant Cell* 16, 596–615
6 Montillet, J.L. *et al.* (2013) An abscisic acid-independent oxylipin pathway controls stomatal closure and immune defense in Arabidopsis. *PLoS Biol.* 11, e1001513
7 Kwak, J.M. *et al.* (2003) NADPH oxidase AtrbohD and AtrbohF genes function in ROS-dependent ABA signaling in Arabidopsis. *EMBO J.* 22, 2623–2633
8 Sirichandra, C. *et al.* (2009) Phosphorylation of the *Arabidopsis* AtrbohF NADPH oxidase by OST1 protein kinase. *FEBS Lett.* 583, 2982–2986
9 Mersmann, S. *et al.* (2010) Ethylene signaling regulates accumulation of the FLS2 receptor and is required for the oxidative burst contributing to plant immunity. *Plant Physiol.* 154, 391–400
10 Zheng, X.Y. *et al.* (2012) Coronatine promotes *Pseudomonas syringae* virulence in plants by activating a signaling cascade that inhibits salicylic acid accumulation. *Cell Host Microbe* 11, 587–596

1360-1385/$ – see front matter
http://dx.doi.org/10.1016/j.tplants.2013.03.007 Trends in Plant Science, June 2013, Vol. 18, No. 6

Plant Science Conferences in 2013

International Symposium on Plant Photobiology (ISPP)
3–6 June, 2013
Edinburgh, UK
http://hallidaylab.bio.ed.ac.uk/ISPP.html

24th International Conference on Arabidopsis Research (ICAR)
24–28 June, 2013
Sydney, Australia
http://www.sallyjayconferences.com.au/icar2013/

SEB Valencia 2013
3–6 July, 2013
Valencia, Spain
http://www.sebiology.org/meetings/Valencia/Valencia.html

Plant Metabolic Engineering
The Interface of Plant Metabolism and Humanity
7–12 July, 2013
Waterville Valley, NH
http://www.grc.org/meetings.aspx?year=2013

The Annual Meeting of the Society of Plant Signaling and Behavior
8–10 July, 2013
Vancouver, Canada
http://plantsignaling2013.com/