Stomatal lock up following pathogenic challenge, source or symptom of costs of resistance in crops?

Luis A. J. Mur[1*], Catherine Simpson[1], Alan Gay[1], Julie A. Smith[3], Neil Paveley[3], Javier Sánchez-Martin[2], Elena Prats[2]

[1]Institute of Biological, Environmental and Rural Studies, Aberystwyth University, Aberystwyth, Ceredigion SY23 3EB, UK

[2] Spanish National Research Council (CSIC)-Institute for Sustainable Agriculture (IAS), Alameda del Obispo, Menéndez Pidal s/n, 14080 Córdoba, Spain

[3]ADAS High Mowthorpe, Duggleby, Malton, North Yorkshire, YO17 8BP

*=to whom correspondence should be addressed: lum@aber.ac.uk; +44 (0)1970 622981.

Abstract:

The cost of a plant deploying its defences against invading pathogens has often been linked to altered photosynthesis, respiration or metabolite deficiency as resources are diverted towards defence. Defence responses have been shown to alter stomatal function. During R-gene elicited resistance responses of barley and oat to powdery mildew, stomata lock in an open configuration; displaying little or no closure in response to diurnal rhythms or abscisic acid. In consequence these plants exhibit greatly compromised tolerance to drought. Conversely, in response to rust fungal pathogens, major gene resistance was linked to stomatal locking shut which will have severe impacts on gaseous exchange, photosynthetic disruption through the over-reduction of redox active components and poorer cooling via the transpirational stream. Thus, stomatal locking is likely to result in a yield cost of resistance particularly in locations with higher light and / or prone to drought, and it is therefore imperative to define their underlying mechanisms. Based on current models for stomatal regulation, we here review various - R-gene, pathogen-associated molecular patterns (PAMPs) or toxins - defence linked events and the signalling cascades that may influence guard cell function. More widely, we consider if stomatal dysfunction could be a feature of wider changes in primary metabolism where regulation of osmolytes is disrupted. This would integrate stomatal locking as one feature of wider cost of resistance phenomenon. As such, reduced stomatal lock-up could be used as a readily assessable marker for lines with lesser resistance penalty.

The plant defence buffet: Targets for agricultural exploitation.

Crop breeding programmes often seek to exploit plant disease resistance mechanisms to increase yield. These efforts have led to the definition of constitutive and inducible defences. Constitutive defences include structural components and barriers including the presence and thickness of a waxy cuticle, size of stomatal pores and cell wall components (Ferreira et al., 2006, Veronese et al., 2003). Important pre-existing antimicrobial secondary metabolites – phytoanticipins - which include saponins, phenols and cyanogenic glycosides that tend to be concentrated in the outer plant cell layers are also important components of the constitutive plant tolerance (Osbourn, 1999). However, much work has focused on induced defences (Dangl & Jones, 2001, Ferreira et al., 2006, Nurnberger et al., 2004) which are deployed only following a pathogen attack. Among these, the most well-characterised responses are the immune defences triggered by the interaction between host resistance (*R*) and pathogen encoded *avr,*genes which often leads to the elicitation of a localised programmed cell death known as the Hypersensitive Response (HR) (Mur et al., 2008). The HR is associated with cell wall reinforcement by lignification and oxidative cell-wall polymer cross-linking (Bradley et al., 1992, Dickman & Fluhr, 2013), and with the activation of a series of "defence" genes including pathogenesis-related (*PR*) genes and the production of anti-microbial secondary metabolites known as phytoalexins (Mur et al., 2008). Introgression of *R* genes into elite crop varieties is a common strategy to increase resistance to disease.

Although *R-avr* triggered resistance has been widely exploited in plant breeding programmes (McDowell & Woffenden, 2003), molecular dissection of elicitation events has revealed important roles for pathogen-associated molecular patterns (PAMPs)

(Nurnberger et al., 2004). PAMPs include diverse elicitors such as lipopolysaccharides (LPS), flagellin (a polymer of flg22) and fungal chitin whose recognition results in PAMP triggered immunity (PTI). Bacterial flagellin-receptors, which have been particularly well-characterised in Arabidopsis, are encoded by *FLS1* and *FLS2* genes expresing receptor kinases (Bauer et al., 2001, Gomez-Gomez & Boller, 2000). The treatment of flg22 induces callose deposition at the cell wall as well as *PR* gene expression (Chinchilla et al., 2007, Gomez-Gomez & Boller, 2000). Unsurprisingly, *fls2* mutant plants are more susceptible to the bacterial pathogen *Pseudomonas syringae* pathovar *tomato* (*Pst*) (Zipfel et al., 2004).

A component of PTI is the formation of papillae (Underwood & Somerville, 2013). These are cell wall appositions which provide a structural barrier against pathogen penetration. This rapidly induced defence mechanism, involves oxidative cross-linking of cell wall-bound phenolic compounds, polysaccharide chains and proteins. This highlights the role of H_2O_2-production during papillae formation (Grant & Loake, 2000). The formation of papillae is a highly complex phenomenon involving the targeted of constituents and H_2O_2 to a precise sites via vesicle trafficking (Schulze-Lefert, 2004). The importance of papillae is well illustrated by considering the role of MLO proteins. MLO perturbs vesicle targeting to reduce papillae formation and this function is maintained by fungal pathogens to aid penetration (Miklis et al., 2007, Humphry et al., 2006). Thus, varieties encoding *mlo* alleles display durable resistance to pathogens such as powdery mildew (Humphry et al., 2006).

R-avr responses do not occur in isolation from PTI and these have been elegantly integrated in the zig-zag model of immunity and susceptibility (Jones & Dangl, 2006).

Here, pathogen effectors have evolved to suppress PTI ("zig") in effector triggered susceptibly ("ETS"), however, resistance genes have evolved to interact with individual effectors (thus, becoming avr proteins) to give rise to effector-triggered immunity (ETI) ("zag").

Cost of resistance

The induced nature of many defences implies a plausible "cost for resistance". Thus, introduction of genes which trigger the constitutive expression of these defences reduce the ecological fitness of a plant (Purrington, 2000) and limit the breeding and commercial success of resistant varieties (Brown, 2002). It also may limit the exploitation of defence-inducing agrochemicals, such as for example Actigard (Acibenzolar-S-methyl) that can impair plant development (Csinos et al., 2001, Ziadi et al., 2001). Costs may be associated with a given *R* gene or to a particular linked fitness reducing allele. In this latter situation, it can take many generations of successive backcrossing to start eliminating the linkage and this may be economically viable only if losses due to disease are considerably high due to intense pathogenic pressure (Brown, 2002, Purrington, 2000). In addition, resistance costs are not only limited to *R* genes. Three independent *mlo* barley mutants exhibited a yield penalty of around 4%, possibly due to a reduction in photosynthate translocation to the developing grain as a result of necrotic leaf spotting that is particularly evident after heading (Kjaer et al., 1990). Cell necrosis has also been linked to a 5-6% reduction in grain yield in wheat carrying *Lr34* when grown in protected field trials (Singh & HuertaEspino, 1997).

Costs of resistance are typically explained in terms of metabolic demands made by defence mechanisms having a detrimental effect on host fitness (Kliebenstein & Rowe,

2008, Walters & Boyle, 2005). This conforms closely to the ecologically "growth-differentiation balance hypothesis", which assumes that resources are limiting and defence inherently represents a diversion of resources (Herms & Mattson, 1992). Alternatively, the optimal defence theory suggests that defences have evolved in a given species reflecting a balance of life-cycle, the value of protecting a given organ and the metabolic cost of defence (Rhoades & Cates, 1976). Thus, a short-lived plant such as Arabidopsis would invest little into constitutive defence and deploy defences mostly during flowering when defence would be costs-effective. Ecological costs could also result from negative effects of the interactions with microorganisms such as mycorrhizal fungi (Heil & Baldwin, 2002, Heil, 2001) or as a consequence of the activation of biotrophic defence mechanisms leading to increased susceptibility to necrotrophic pathogens (Kliebenstein & Rowe, 2008). Moving beyond these broad models, it is the task of the molecular biologist to define how signalling cascades and gene expression regulate resource allocation and how these are influenced by defence responses. Various molecular facets of how resistance costs are established are considered in various reviews within this special issue. We here consider the cost of resistance linked to stomatal lock-up during defence responses (Withers et al., 2011).

Stomatal locking in resistance responses

It is perhaps predictable that disease and the cellular disruption consequence of pathogenic infection processes involving the formation of infection structures or the production of toxins (Grimmer et al., 2012, Melotto et al., 2006) may impact on plant water relations (Ayres, 1976). However, earliest observations by, Smedegaardpetersen & Stolen, (1981) noted that barley plants inoculated with avirulent strains of powdery mildew, and hence without disease symptoms, reduced grain yield and weight by 7%

compared to uninoculated controls. This yield loss was linked to an 80% increase in respiration of resistant plants at 24 hours after inoculation, possibly to provide energy for defence reactions, (Smedegaard-Peterson and Stolen, 1981). More recently, costs associated with the barley-powdery mildew interaction have been related to photosynthesis and carbon metabolism. Thus, a reduction in photosynthesis was thought to be a result of the HR cell death itself and altered source-sink relations (Swarbrick et al., 2006).

Further insights on the underlying causes of the costs of resistance have arisen from the study of the barley – powdery mildew, *Blumeria graminis* f. sp. *hordei* (*Bgh*), pathosystem highlighting the relevance of stomata in the resistance-derived costs. Thus, we observed that inoculated barley P01 (*Mla1*, HR mediated resistance) plants were more susceptible to drought, which suggested a possible effect of pathogen resistance on stomatal function. This was confirmed by measurements of leaf water conductance and direct stomatal apertures on LTSEM micrograpghs (Prats et al., 2006). Thus, in susceptible barley Pallas lines decreased stomatal opening respect to controls was observed during successive light periods whereas closure in the dark was similar to uninoculated leaves (Fig. 1A). By contrast, in the isogenic barley P01 the stomatal conductance following pathogen challenge was similar to that of uninoculated leaves from the second light period but crucially, after the onset of epidermal HR at about 24 hours after inoculation (hai), stomata failed to close in the dark period (Fig. 1A). As the stomata of inoculated P01 failed to close in responses to exogenously applied ABA, we termed the loss of stomatal closure as 'lock-up' (Prats et al., 2006). Clearly, the resulting susceptibility to drought could contribute to costs of resistance especially where crops are fed from rain-water. Indeed, if episodes of extreme weather become

more prevalent, (Kumar, 2007) the contribution of stomatal-locking to the balance sheet of any resistance cost will be of increased significance.

Other groups have also noted that infections of pathogens of various kinds; viruses, fungal and oomycete had effects on stomatal function (reviewed in Grimmer et al., 2012). For example, in barley-infected *Rhynchosporium secalis* – stomata failed to close in the dark as symptoms developed which the authors linked to fungal production of cytokinins (Ayres & Jones, 1975). Lindsey & Gudauskas, (1975) noted decreased stomatal conductance within the chlorotic regions of maize leaves infected with *Maize dwarf mosaic virus.* Stomatal effects were also observed in bean leaves infected by *Colletotrichum lindemuthianum* (Meyer et al., 2001). In both cases, stomatal effects were linked to perturbed photosynthesis. In a key study, stomatal opening in the dark was observed in *Vicia faba* infected with *Sclerotinia sclerotiorum* and open stomata aided fungal emergence through the opposing leaf lamina. In this interaction, oxalate is a key virulence factor which appeared to antagonise ABA effects on the stomata (Guimaraes & Stotz, 2004). Other workers have also measured pathogen effects on water use efficiency (WUE) and instantaneous WUE (reviewed by Grimmer et al., 2012). Most of these studies did not consist of detailed temporal studies where resistant and susceptible reactions were compared (e.g. Fig. 1A). Thus, it is difficult to deduce whether these alterations in stomatal aperture were equivalent to the "lazy" phenotype that we observed with *Bgh* inoculated Pallas or ABA-insensitive locking that we observed in cv. P01. Whilst, mechanistically the difference between stomatal laziness and locking may be one of degree; this cannot be assumed and each may arise from qualitatively different mechanisms.

Subsequent studies of the effect of pathogen attack on stomatal function have served to provide a platform based on which the underlying mechanisms will be defined (see below). A key observation was that stomatal lock-up ocurrs more likely when adjacent epidermal cells were dead (Prats et al., 2006). This suggested that the locking effect was close-proximity dependent and certainly this effects could not be transmitted from one leaf laminar to the other (Prats, unpublished). However, in soybean infected with *Phytophthora sojae* altered stomata function was observed up to 20 mm away from the site of interaction; although this effect was transitory and stomata began to open after 8 h following challenge (McDonald & Cahill, 1999). Our findings naturally suggested the importance of cell-death to establish locking but crucially, chlorophyll fluorescence in stomata guard cells indicated that they were not themselves dead (Prats et al., 2010). Further studies in three near-isogenic Pallas barley lines carrying the single different R-genes *Mla1*, *Mla3* and *MlLa* which differed in the spatial and temporal execution of HR, confirmed the close relation between the elicitation of cell death and lock-up. In these lines, the timing and extend of the lock-up correlated with the specific HR characteristics conferred by each R gene, i.e. rapid epidermal cell death with *Mla1,* mesophyll cell death under living but penetrated epidermal cells exhibiting a delayed cell death with *Mla3* resistance, or delayed epidermal cell death coming from secondary attacks in *MlLa* resistance. The link of lock-up with cell death was further substantiated when examining *mlo*-mediated resistance mechanisms which are based on the formation of papillae. In Pallas isogenic line with *mlo*; P22, reduced stomatal closure in the dark was observed although this locking was linked to the considerable cell death observed in this genotype (Prats et al., 2006) as a consequence of uncontrolled H_2O_2 production following papilla formation (Piffanelli et al., 2002). By contrast, the *mlo* line Risø –R genotype exhibits very little cell death and it was notable that dark-associated stomatal

closure did not appear to be compromised compared to isogenic, *Bgh* susceptible, *MLO* line Risø S (Prats et al., 2006).

One question arising from the apparent close-spatial dependence between cell death and stomatal locking is the relevance of the latter in the field. It was entirely possible that stomatal lock-up is a function of the use of high inoculation densities in controlled environmental conditions. However, lock-up was observed in barley following inoculation of *Bgh* even at very low inoculum densities. Thus, dark conductance of inoculated barley P02 (*Mla*3, HR mediated resistance) at 59 hai was significantly greater than uninoculated controls with spore densities as low as 5 conidia mm^{-2} and by 155 hai even 1 conidia mm^{-2} induced a significantly higher dark conductance. In P01, inoculation with 10 to 50 conidia mm^{-2} caused significant increases in dark conductance at 59 hai although these were more obvious at 83 hai (Prats et al., 2010). Furthermore experiments conducted in the field on Pallas, P01 and P22 showed patterns of stomatal conductance effects consistent with the results from controlled conditions even though inoculation densities were low at only 3% (Smith and Paveley, unpublished).

Further studies have investigated the interactions of Brown rust (*Puccinia hordei* and *P. triticina*) in barley and wheat (Prats et al., 2007) highlighting the generality of the stomatal dysfunction following resistance responses but also the particularities derived from the specific infection/resistance processes engaged. In the cereal-rust interactions stomatal dysfunction following challenge with rust pathogens differed in extent depending on the *R* gene involved (Fig. 1B) but, whereas R gene resistance to powdery mildew caused stomatal lock-up, in the rust systems lock-shut of stomata during light periods was observed (Fig 1B, Prats et al., 2007). Such closure would perturb gaseous

exchanges and directly impact on photosynthetic CO_2 fixation which, as light reactions would be maintained, would lead to conditions of excess excitation energy and increases in oxidative stress (Osmond et al., 1997, Mateo et al., 2004). Stomatal closure would also impede the transpiration stream resulting in thermoregulatory problems. Both features would impact on yield and therefore would represent a cost of resistance. Thus, considerations of stomatal locking mechanisms should considered stomatal lock-up and lock-shut.

Opening and closing stomata.

Stomatal pores are essential players in CO_2 uptake for photosynthesis and plant cooling through the transpiration streams. The stomatal apertures are adjusted via flanking guard cell turgidity allowing modulation in response to diurnal cycles and a range of environmental cues. Before considering possible locking mechanisms, the means through which stomatal aperture are regulated must be considered. Space prevents a detailed consideration of stomatal regulation for which the reader may wish to consult some excellent reviews (Acharya & Assmann, 2009, Kim et al., 2010, Pandey et al., 2007, Schroeder et al., 2001, Outlaw, 2003, Daszkowska-Golec & Szarejko, 2013).

Plants control stomatal pore size through changes in the osmotic pressure of the guard cells influencing water import and export (Buckley, 2005) and key signalling events in the guard cell act to influence the mobilisation of osmotically-active metabolites (Fig. 2, 3). Stomatal opening starts with proton export through the activation of a plasma membrane H^+-ATPase in response to a number of stimuli including blue and red light, low CO_2 concentrations and high humidity (Kim et al., 2010). H^+ causes plasma membrane hyperpolarisation which causes a subsequent passive uptake of K^+ by inward

rectifying K^+ channels (K^+_{in}) (Outlaw, 2003, Kim et al., 2010, Fan et al., 2008). At least four, so-called shaker genes (*KAT1, KAT2, AKT1* and *AKT2*) encoding K^+_{in} channels are expressed in *Arabidopsis* guard cells (Lebaudy et al., 2008, Very & Sentenac, 2002). In order to balance K^+ uptake, guard cell take up chlorine (Cl^-) and nitrate (NO_3^-) ions from the apoplast; in the latter case by the CHL1 nitrate transporter (Outlaw et al., 2002, Guo et al., 2003). It seems likely that the accumulation of these anions is insufficient to increase in turgor pressure needed to fully open stomata which also requires the mobilisation of starch and sugar (Rob et al., 2008). In diurnal rhythms, K^+ taken up in the morning initiate starch mobilisation to form sucrose, which becomes the dominant osmotically active solute later in the day when levels of K^+ and associated anions start to decrease. Starch is also mobilised to form malate which accumulates within the vacuole and also represents an important osmotically active metabolite (Schroeder et al., 2001; and Outlaw 2002). Ion/osmolyte accumulation initiates H_2O intake via aquaporins and so open stomata (Prado & Maurel, 2013) (Fig. 2).

During stomatal closing, plasma membrane depolarisation is a key event arising from an efflux of Cl^-, NO_3^- and malate through plasma membrane anion channels. In response to blue light H^+ export is inhibited (Zhang et al., 2004) whilst activation of Ca^{2+} channels mediate both influx of Ca^{2+} in protoplasts and increases in $[Ca^{2+}]_{cyt}$ in intact guard cells (Pei et al., 2000). Internal calcium increases activates two anion channels, known as rapid (R) and slow (S) type channels. The S form plays an important role in stimulus-induced closure, and remain activated for passive anion – Cl^- and NO_3^- as well as $malate^{2-}$ efflux (Kim et al., 2010 and Outlaw, 2003). The S-type *SLOW ANION CHANNEL-ASSOCIATED 1* (*SLAC1*) gene encodes a plasma membrane protein which contains a dicarboxylate/malate transporter. In line with the model, *slac1* mutants over

accumulate anions within the guard cells and their stomata exhibit poorer closure on light-dark transition, low humidity, ABA, high CO_2 concentrations and ozone. R type channel activity was not impaired in these mutants (Vahisalu et al., 2008, Negi et al., 2008). Efflux of K^+ through outward-rectifying K^+ channels (K^+_{out}), encoded by a single shaker gene *GORK*, follows membrane depolarisation (Kim et al., 2010 and Lebaudy et al., 2008). This K^+ export along with cytosolic Ca^{2+} mediated inhibition of the H^+-ATPase and K^+_{in} channels increases (make less negative) the osmotic potential of the guard cells. The osmotic potential is also increased through the mobilisation of malate to the vacuole and cellular export. Both malate and sucrose are also diverted to form starch or in the former case, also to gluconeogenic pathways (Willmer, 1996). The subsequent increase in osmotic potential causes water to move out, lowering the turgor pressure and reducing stomatal aperture (Schroeder et al., 2001). CO_2 is a an important signal for stomatal closure and acts through its reduced form (HCO_3^-) to activate S-type channels and thus plasma membrane depolarisation (Xue et al., 2011).

The key role of ABA in regulating stomatal opening and closing is very well-characterised (Wang & Song, 2008). A number of ABA receptors such as the Pyrabactin Resistance 1 (PYR1) /PYR1-LIKE (PYL) / Regulatory component of ABA receptor 1 (RCAR) family of proteins have been reported. Under increased ABA levels, the PYR/PYL/RCAR receptors inactivate the Type 2C serine/threonine protein phosphatases (PP2Cs; Abscisic Acid Insensitive 1 and2; ABI1 and ABI2), which leads to the activation of the Sucrose-Nonfermenting1–Related Subfamily2 (SnRK2s) protein kinases (Raghavendra et al., 2010) (i.e. SRK2D (SnRK2.2), SRK2I (SnRK2.3), and SRK2E (OST1/SnRK2.6; Umezawa et al., 2009). Within the context of guard cell, OST1 is known to act as a positive regulator of stomatal closure by activating SLAC1

and inhibiting KAT1 by phosphorylation (Sato et al., 2009). A crucial point further considered below is that OST1-mediated phosphorylation influences NADPH oxidase generated H_2O_2 (Sirichandra et al., 2009, Mustilli et al., 2002). H_2O_2 is known to close stomata probably via oxidative modulation of ABI1/ABI2 (Meinhard et al., 2002, Meinhard & Grill, 2001). The NADPH oxidase encoding genes *AtrbohD* and *AtrbohF* are expressed in guard cells of Arabidopsis and double knockouts were required for a substantial reduction in ROS production and stomata closure (Kwak et al., 2003). Both ABA and ROS initiate the release of calcium from internal sources and, via Calcium Dependent Protein Kinases (CDPKs) activate anion (S and R) and K^+_{out} (GORK) channels triggering stomatal closure (Schroeder et al., 200; Neill et al., 2003). It is likely that NO and H_2O_2 work together to bring about complete stomatal closure and there is growing evidence to suggest that H_2O_2 actually stimulates NO production (Neill et al., 2008, Dubovskaya et al., 2011). ABA induced ROS and NO production lead to an increase in guard cell cytosolic calcium. H_2O_2 is thought to also increase Ca^{2+} in *Arabidopsis* guard cells by activating a plasma membrane Ca^{2+} permeable non-selective cation current (I_{Ca}) channel. Activation of these I_{Ca} channels by H_2O_2 and subsequent stomatal closure was impaired in I_{Ca} mutants although ABA still induced ROS (Pei et al., 2000)(Fig. 3).

NO is another key player in the regulation of stomatal opening albeit the mechanism of its action is only now emerging. Both ABA and ROS are thought to initiate NO production from nitrate reductase activity. NO acts through cGMP-mediated signaling (Wilson et al., 2009) as shown by inhibition of guanylate cyclase (GC); which produces cGMP, resulting in reduced ABA and NO induced stomatal closure in pea. Further, suppression of cyclic ADP ribose (cADPR) that is generated downstream of cGMP

mobilised Ca^{2+} from internal stores, also prevented stomatal closure. Other experiments have also linked NO mediated intracellular Ca^{2+} release and the regulation of guard cell K^+ and Cl^- channels to cGMP and cADPR dependent pathways (Wilson et al., 2009). The requirement of co-generation of NO and ROS to act via cGMP has recently been elegantly integrated into a signaling model based on nitrated cGMP (Joudoi et al., 2013). In this model, ROS and NO particularly O_2^-, reacts to generate peroxynitrate (O_2^- + 'NO → $^{\cdot}ONOO^-$) which nitrates NO initiated cGMP to form 8-nitro cGMP. 8-nitro cGMP acts to initiate Ca^{2+} mediated S- (and most likely R type) channel activation, leading to membrane depolarisation (Fig. 3).

Considering possible mechanisms for stomatal locking.

The apparently importance of cell death to stomatal locking could be given by an altered turgor balance of the epidermal stomatal complexes. Whereas guard cell turgor positively regulates stomatal opening, epidermal and subsidiary cell turgor provide a back pressure reducing stomatal aperture by around 50% (Buckley, 2005). The death of epidermal cells could relieve this back pressure resulting in stomatal pores to 'lock-up' (Prats et al., 2006). However, the link to cell death has been weakened in our recent work which has focused on nine oats genotypes infected with powdery mildew (*Blumeria graminis* f. sp. *avenae*) and the crown rust (*Puccinia coronata* f. sp. *avenae*). In this study, we observed that the histological pattern of cell death could not be correlated to stomatal responses which instead seemed linked to oat genotype. Instead, photosynthetic disruption –revealed by measures such as Fv/Fm – rather than HR better correlated with the patterns of stomatal locking. When light rates were increased, Fv/Fm

ratios were further reduced and stomatal dysfunction was augmented, most likely indicating a contribution from increased oxidative stress (Sanchez-Martín et al., paper in prep). Scharte et al., (2005) focusing on the interaction between tobacco (*Nicotiana tabacum*) and *Phytophthora nicotianae* demonstrated stomatal closure particularly at the interaction site linked to ROS production and a decline in photosynthesis extending farther from the infection site. To the chloroplastically generated ROS, NADPH oxidase generated ROS can be added as part of the cell death process (Torres, 2010). This elevated ROS would suppress the redox-sensitive PP2C2 phosphatase on the ABA signalling pathway to help initiate stomatal closure (Fig. 3). One means through which photosynthesis could be being affected may be via the signalling molecule NO, generated during the HR (Prats et al., 2005). Ordog et al., (2013) have recently demonstrated that applied NO caused near-immediate and dramatic loss in electron transport through PSII (as indicated by instantly decreases of photochemical fluorescence quenching coefficients [qP and qL] and ϕPSII.). This was reversed when NO was removed. Within the context of the HR, NO could be generated for many hours (Mur et al., 2005) so the effects on photosynthesis could similarly persist.

Wider comparison of events occurring following the elicitation of HR and the guard cell control reveals a series of common events. One early response in the elicitation of the HR is the activation of plasma membrane H^+ ATPases to initiate an acidification of the apoplast (Zhou et al., 2000). However, pH changes in the apoplast are dynamic so that, at least in the case of powdery-mildew interactions with the barley, the longer term response – persisting for days, appears to be sub-stomatal alkalisation (Felle et al., 2004) which could result in stomatal closure (Fig. 3). This could be via NADPH oxidase which pumps O_2^- into the apoplast which via protonation consumes H^+ ions

increasing pH (Segal et al., 1981). A direct link between pathogen–associated H^+ ATPases and guard cell control has been provided by Liu et al., (2009). The plant defence suppressor RIN4 was found to interact with H^+ATPases AHA1 and AHA2, and in *rin4* mutants proton pumping was activated to open stomata (Fig. 3). Thus, the relative kinetics of apoplastic proton pumping or alkalisation or ROS generation could influence whether stomata open or close during pathogenic interactions. Additional factor to be considered is NO, the generation of which occurs during the HR and stomatal closing (Wilson et al., 2009).

Taking all of these points together, it is entirely possible that the differential activation of H^+ fluxes and ROS and NO generation by discrete *R*–gene and PAMP triggered events could explain stomatal open and closing in certain contexts. However, the persistence of the stomatal locking phenomenon for several days (see Fig. 1) would argue against models based solely on the transitory generation of common regulatory stomatal and defence signals. The logical implication is that there are additional factors contributing to stomatal locking following pathogen attack. One that should be considered is the increased respiratory metabolism that occurs to meet the demands made by defence responses (Bolton et al., 2008, Bolton, 2009).

One metabolic consequence of the HR is the accumulation of malate as an important intermediary in the TCA cycle (Gupta et al., 2013) which integrates aerobic bioenergetic oxidation of carbohydrates, fatty acids and amino acids. Other workers have indirectly noted the pathogen-induced up-regulation of malate metabolism through increased malate dehydrogenase (NAD-malic enzyme) gene expression (Schaaf et al., 1995). This HR-linked increased accumulation of malate could be an important

component of stomatal locking. Further, transcriptional analysis of *Lr34* mediated resistance suggested that up-regulation of genes associated with the TCA, glycolysis and the GABA shunted were maintained to at least 3 days but not 7 days (Bolton et al., 2008). Increased respiration – malate accumulation would therefore be a long lasting effect influencing stomatal locking open. A key recent study has studied the role of malate removal following export from guard cell in the dark (Penfield et al., 2012). Phosphoenolpyruvate carboxykinase (PEPCK) is an enzyme involved in malate metabolism encoded by *PCK1*. *pck1* mutants failed to exhibit stomatal closure and were referred to as "jammed" in the open position; a seemingly striking parallel to defence-linked "locked" stomata. Thus, increased malate production during the HR could effectively mirror the effects of a lack of malate catabolism by PEPCK in the *pck1* mutant. This stated *pck1*-jammed stomata retained ABA responsive closure, indicating some key differences to stomatal locking (Penfield et al., 2012). The impacts of pathogen attack on primary metabolism with pathogen-challenged tissues being transformed from a sink to sources need also to be considered (Swarbrick et al., 2006)..Thus, infected tissues displayed increased sucrose mobilisation, cell wall invertase activity and some gene expression more often associated with senescence (Scharte et al., 2005, Pageau et al., 2006). Indeed, pathogen-induced glutamate dehydrogenase will reduce glutamate to α-ketoglutarate and thus represents a diversion of nitrogen assimilation in to the TCA cycle (Pageau et al., 2006). In this context, it may be relevant that stomatal-lock open in powdery mildew inoculated barley was reduced in plants grown under conditions of low nitrogen which may reflect lower malate concentrations (Simpson et al., paper in prep).

All of these models for stomatal locking under-estimate possible contributions from the interacting pathogen. The fallacy to this approach was revealed by the seminal work of Melotto et al., (2006). Focusing on Arabidopsis infected with a virulent *Pseudomonas syringae* pv. *tomato* (*Pst*) strain, it was noted that following a transient reduction at 2 h.a.i., stomatal apertures returned to be equivalent to controls by 4 hai. Detailed dissection of these responses indicated that the initial stomatal closure was a PAMPs response mediated by the FLS receptor. The transient stomatal closure resulted from the generation of NO and the effects of this were specifically countered by the *Pst* toxin coronatine (COR) which acted downstream of ABA/NO generation to relax guard cells and to aid penetration of the host (Melotto et al., 2006, Zeng & He, 2010). Other workers have identified additional components in bacterial PAMPs-induced stomatal closure including ethylene and ROS (Mersmann et al., 2010, Keinath et al., 2010). It seems to be entirely likely that in the absence of a toxin such as COR, PTI-triggered NO/ROS/ethylene production will result in the stomatal locking that has been observed by our groups. Koers et al., (2011) have examined reduced stomatal ("lazy") opening during a virulent interaction involving *Bgh* and barley. Using microelectrodes, infection was linked to S-type (slow) anion channels efflux of anions from guard cells. Crucially, both closure and K^+ extrusions could be mimicked through the application of chitosan as surrogate of the fungal PAMP chitin. Whilst these observations did not accord with the lock *open* that we observed with *Mla1* mediated interactions, this work was an important demonstration that fungal PAMPs can influence stomatal responses.

COR is not the only virulence factor which targets stomata. Oxalate is an important virulence factor produced by *Sclerotinia sclerotiorum* such that oxalate-*pac1* mutants are less able to infect their hosts (Rollins, 2003). Exogenous application of oxalate

could counter ABA-induced stomatal closure and *Arabidopsis* ABA-insensitive mutants were more resistant to *Sclerotinia sclerotiorum*. Further analysis of this interaction has demonstrated additional roles for NO and other defence signals known to influence stomata, jasmonic and salicylic acids, in resistance to *S. sclerotiorum* (Perchepied et al., 2010, Guo & Stotz, 2007, Daszkowska-Golec & Szarejko, 2013)

Other pathogen-derived factors have also been shown to perturb stomatal function. Tentoxin, produced by *Alternaria alternate* causes irreversible stomatal closure by inhibiting chloroplastic H^+ ATPases (Schadler et al., 1976). H^+ ATPases are also targeted by another toxin, fusicoccin which are glucosides of tricyclic diterpene produced by *Fusicoccum amygdali*. In line with this target, application of fusicoccin to leaves results in reduced stomatal apertures (de Boer & de Vries-van Leeuwen, 2012).

Concluding remarks

Through the efforts of several groups changes in stomatal aperture are now well-established features of plant-pathogen interactions. An underlying theme of this review has been to suggest that pathogen-associated stomatal effects could have very different underlying mechanistic causes; and here the "zig-zag" model may be useful (Dangl & Jones, 2001). With the available evidence it seems likely that plants could exhibit a PAMPs mediated stomatal closure in responses to pathogen attack. Considering this as a feature of PTI, an ETS component would be toxins such as *Pst* COR or as yet unidentified effectors which specifically target stomata to aid host penetration. Given that there is no evidence of recognition of toxin/s yet cryptic effectors within guard cell, the ETI component is that observed throughout the rest of the leaf laminar. It is clear

that the initiation ETI-linked to the HR, initiates several signaling cascades which were common to stomata signaling and these could contribute to a locking phenomenon. The persistence of the locking would also argue for the importance the photosynthetic / respiratory changes we have highlighted as being more important. This would suggest that stomatal locking is a symptom of changes in primary metabolism which have long been considered major causes of a cost of resistance (Smedegaardpetersen & Stolen, 1981). If stomata-locking is a consequence for wider costs of resistance; this phenomenon becomes an immediately useful marker for resistance costs. Thus, it is possible to envisage screening of segregating populations in a breeding trial for stomatal locking as an inexpensive assay for costs. We are still a long way from this being a viable option but further mechanistic analyses will establish if it has any validity.

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Figure Legends

Figure 1: Stomatal lock open and closed in response to fungal pathogens

(A) Time course of g_l of healthy (○) and inoculated (▲) Pallas and *Mla1* P01 barley leaves incubated under 12 h dark (shaded) / 12h light (unshaded).(A) readings taken mainly in the light. **(B)** Leaf water conductance in healthy (O) and *Puccinia. triticina*, isolate WBRS-04-02, attacked (▲) leaves of wheat lines Thatcher (susceptible), and Lr20 (resistant) in successive light (unshaded) and dark periods (grey shaded) after inoculation. Comparing within sampling times: NS = no significant difference; * = $P<0.05$; ** = $P<0.01$; *** $P< 0.001$.

Figure 2: Events linked to stomatal opening

Stomatal opening is initiated through **(I)** the activation of a plasma membrane H^+ ATPase pump resulting in apoplastic acidification. (**II**) Membrane hyperpolarisation activates inward rectifying K+ channels (K^+in) and **(III)** to balance K^+ uptake anion channels, take up chlorine (Cl^-) and nitrate (NO_3^-) ions from the apoplast; **(IV)** starch is also mobilised to form osmotically active malate and sugars. (**V**) Ion/osmolyte accumulation initiates H_2O intake via aquaporins and so open stomata. Solid black

shapes represent inward channelling events; solid white shapes outward channelling events.

Figure 3: Stomatal closing and possible interaction points with defence plant-pathogen interactions.

Abscisic acid (ABA) is a well characterised hormone initiating stomatal closure (**I**) which binds to a series of receptors of which RCAR (Regulatory component of ABA receptor 1) is shown. ABA-RCAR binding inhibits Type 2C serine/threonine protein phosphatases (PP2Cs) which otherwise act as negative regulators of ABA signalling leading to the activation of SnRK2.2, -2.3, and -2.6/OST **(II).** OST1 positively regulate stomatal closure by activating S-type (SLAC1) anion export and inhibiting (KAT1) K^+_{in} export by phosphorylation (**III**). The resulting membrane depolarisation suppress H^+ ATPase export (**IV**). OST1 also activates NADPH oxidase to generate reactive oxygen species (ROS) which can aid in inactivating the redox sensitive PP2C protein (**V**). ABA and H_2O_2 is thought to increase Ca^{2+} in *Arabidopsis* guard cells by activating a plasma membrane Ca^{2+} permeable non-selective cation current (I_{Ca}) channel and also from internal stores (**VI**). Increased in calcium will activate anion channel (S and R type) and K^+_{out} (GORK) channels to aid in membrane depolarisation to close stomata (**VII**). Both ABA and ROS are thought to initiate NO production which in turn activates the synthesis of cGMP. Co-generation of NO and ROS nitrates cGMP to form 8-nitro cGMP (**VIII**) initiating Ca^{2+} mediated S- (and most likely R type) channel activation, leading to membrane depolarisation.(not shown on the diagram). Osmotic changes can come about the mobilisation of malate to the vacuole or cellular export and subsequent metabolism **(IX)**. Malate can also feed into gluconeogenic pathways to pass into the

tricarboxylic acid cycle (TCA cycle) (**X**). Both malate and sucrose can be used in the biosynthesis of starch **(XI)**.

Pathogen and defence responses could influence stomatal function at several points. (**i**) ROS generated from pathogen-elicited NADPH oxidase or photosynthetic disruption could affect PP2C and increase Ca^{2+} in the guard cells contributing to closure of stomata. (**ii**) NO generated during R-gene ,(most likely linked to cell death) or pathogen-associated molecular patterns (PAMP) elicited responses, will cross-talk with stomatal regulation. Although NO acts on stomata differently according to concentration (Wilson et al. 2009); these events are likely to close stomata. (**iii**) Very early H^+ ATPase activation following the elicitation of the HR initiated transient apoplastic acidification which would open stomata but longer term alkalinisation (Felle et al. 2004) would result in closing. (**iv**) Guard cell H^+ ATPases could be targeted by RIN4 interacting effectors to suppress activity and thus closing stomata or H^+ ATPase activating effectors / toxins would promote stomatal opening. (**v**) Increased malate accumulation during the HR (Gupta et al., 2013) could affect the malate metabolism and export required to close stomata. Background image, electron micrograph of *Blumeria graminis* f. sp. *hordei*-elicited single cell death in Pallas *Mla1* line P01 occurring adjacent to a stomata.