The role of *Arabidopsis* ABA receptors from the PYR/PYL/RCAR family in stomatal acclimation and closure signal integration

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Stomata are microscopic pores found on the surfaces of leaves that act to control CO₂ uptake and water loss. By integrating information derived from endogenous signals with cues from the surrounding environment, the guard cells, which surround the pore, ‘set’ the stomatal aperture to suit the prevailing conditions. Much research has concentrated on understanding the rapid intracellular changes that result in immediate changes to the stomatal aperture. In this study, we look instead at how stomata acclimate to longer timescale variations in their environment. We show that the closure-inducing signals abscisic acid (ABA), increased CO₂, decreased relative air humidity and darkness each access a unique gene network made up of clusters (or modules) of common cellular processes. However, within these networks some gene clusters are shared amongst all four stimuli. All stimuli modulate the expression of members of the PYR/PYL/RCAR family of ABA receptors. However, they are modulated differentially in a stimulus-specific manner. Of the six members of the PYR/PYL/RCAR family expressed in guard cells, **PYL2 is sufficient for guard cell ABA-induced responses, whereas in the **responses to CO₂**, **PYL4** and **PYL5** are essential. Overall, our work shows the importance of ABA as a central regulator and integrator of long-term changes in stomatal behaviour, including sensitivity, elicited by external signals. Understanding this architecture may aid in breeding crops with improved water and nutrient efficiency.**

Stomata are pores on the leaf surface that are bounded by two guard cells. Their role is to optimize gas exchange in changing environmental conditions^{1–3}. In the light, stomata open, allowing CO₂ to enter the leaf for photosynthesis. In contrast, darkness, high concentrations of CO₂, reductions in relative air humidity (RH) that are equivalent to an increase in the water vapour pressure deficit (VPD) and exposure to the hormone abscisic acid (ABA) all promote **stomatal closure^{4–8}. This is a rapid process typically lasting from 20 to 40 min.** Reductions in stomatal aperture are the result of turgor and volume reduction in the respective guard cell pair. The key player in ABA- and CO₂-dependent guard cell deflation is the guard cell anion channel **SLAC1**, together with the **SLAC1-activating protein kinase, OST1** (ref. ⁹). Under low, subthreshold concentrations of ABA, the protein phosphatase ABI1 keeps the guard cell anion channel kinases dephosphorylated and SLAC1 inactive¹⁰. When the concentration of ABA increases it binds to receptors of the PYL/PYL/RCAR family^{11–13}. ABA receptor interaction prevents the ABI1-mediated inactivation of the SLAC1 kinase. This results in anion channel opening, which in turn brings about depolarization of the plasma membrane. The change in membrane

potential results in the release of osmotically active K⁺ and anions. This is followed by osmotic water release causing guard cell deflation and stomatal closure^{4,9,14,15}.

Over the last 40 years most investigations have concentrated on identifying and understanding how individual guard cell proteins and intracellular second messengers interact to bring about these rapid changes in stomatal aperture. In contrast, we know much less about the processes that underlie stomatal acclimation to longer term environmental changes¹⁶. These involve alterations in gene expression, which result in **changes in stomatal development and function.** In the former category it is recognized that plants acclimate to increases in the concentration of atmospheric carbon dioxide and light intensity by decreasing the number or size of stomata that develop on the surface of the plant leaves^{17–21}. Reducing stomatal density is known to increase plant water-use efficiency²². Stomata also acclimate to changing environments by **altering their sensitivity to ABA.** In *Arabidopsis* this happens during development, with the youngest guard cells being ABA insensitive²³. Guard cell sensitivity to ABA is modulated by water stress or exposure to increased VPD^{23,24}. In these cases, ‘switching on’

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ABA responsiveness provides the plant with the ability to control its water relations.

Understanding how stomata acclimate to changes in their environment has clear relevance to the global challenge of producing crops that are more resilient to changes in the environment. Here we identify changes in guard cell gene expression that are likely to contribute to the mechanisms that allow stomatal aperture responses to adapt in the longer term to changes in closure-inducing environmental signals. We reveal that these responses are characterized by the expression of both stimulus-specific suites of genes and core genetic modules that are regulated by all closure-inducing stimuli. We also suggest that part of stomatal acclimation to all these signals is achieved by **controlling the sensitivity of the guard cell to ABA**. This is achieved by differentially regulating the expressions of members of the PYR/PYL/RCAR family of ABA receptors. Moreover, we show, in the cases of ABA and elevated CO₂, that response specificity is achieved by these signals accessing different members of the PYR/PYL/RCAR family of receptors.

Stimulus-specific modulation of guard cell gene expression. To investigate the effects of ABA, low air humidity/increased VPD, darkness and elevated CO₂ on guard cell gene expression, we used a microarray-based approach. We reanalysed two microarray datasets (ABA and RH/VPD treatment) from a previous study⁶ and combined them with two new microarray studies where we used darkness and high CO₂ as stomatal closing signals (for detailed analysis of the differentially expressed genes see Supplementary Table 1a). We first analysed the resulting dataset using classical pathway analyses and this revealed that each closure-inducing signal addressed a defined, unique cluster of gene pathways (Fig. 1, Supplementary Fig. 1a and Supplementary Information 1). We also found that gene clusters are shared among two, three or all four stimuli. For example, the shared clusters for ABA and **lowered RH** (equivalent to increased VPD) are dominated by the ABA response (Supplementary Fig. 1b) and the **late embryogenesis abundant pathways** and are upregulated by both treatments. To gain further information about the interrelationship of these pathways we analysed the coexpression patterns of the genes from each pathway using the public ATTED II database²⁵. This analysis revealed that both pathways are highly interconnected (Supplementary Fig. 1c–e). Notably, **RAB18**, **DAA1** and **MYB74**, which we had previously identified as being **core to RH- (VPD)** and ABA-induced guard cell responses²⁶, were coexpressed with several other genes common to both responses, such as the dehydrins and members of the *HVA22* family (Supplementary Fig. 1c–e).

The shared gene cluster of darkness and low RH (high VPD) is dominated by abiotic stress and glucosinolate pathway genes. Both clusters contain **flavin monooxygenases/glucosinolate oxygenases (FMOGS)**, which are responsible for the last step of side-chain modification of aliphatic glucosinolates. *FMOGS* are involved in biotic and abiotic stress responses in general and most of them are induced by ABA²⁷. The shared clusters for darkness/CO₂, ABA/darkness, ABA/CO₂ and RH/CO₂ are dominated by more general metabolic pathways, receptor kinases, cell wall degradation, major carbohydrate metabolism, glucosidases and transport (Supplementary Fig. 1a). To directly model these complex interconnections we performed an integrative network analysis on the basis of a rigorous mathematical approach (see Methods).

Specifically, we incorporated the set of protein–protein interactions from the STRING database, which primarily represents physical interactions between proteins, into our analysis. By integrating these data we added a new dimension into the expression data. In contrast to the coexpression network, this allows the detection of functional signalling links at the posttranslational protein level. We analysed the network on the basis of the measured expression levels into functionally coherent modules. These can be regarded as the building blocks of cellular processes, such as basic signalling cascades. The resulting

Fig. 1 | Guard cell signalling pathway analysis. MapMan pathways with a significant enrichment in at least one stimulus are visualized in a heat map. Pathways are listed in rows and stimuli in columns. Darker shades (corresponding to the negative decadic logarithm of the enrichment *P* value) depict more significant enrichments. For each treatment and the respective controls, four microarrays were analysed, except darkness (*n* = 3). All replicates represent biologically independent samples. A competitive pathway enrichment test was performed as implemented in the camera function from the limma package based on the gene-expression data.

optimal solutions represent maximally responsive network modules with respect to the specific experimental stimuli.

This approach is hypothesis-free and allows an unbiased exploration of stimulus-triggered network responses, and, in particular, can integrate novel, hitherto unknown, genes and connections into a pathway. Based on this algorithm we were able to expose clusters and connections that cannot be easily detected by classical methods. This revealed stimulus-specific gene modules responsive to different stomata-closing signals (Fig. 2a–d) (for details see Supplementary Information 2, Supplementary Table 1b and Supplementary Fig. 2a–d).

Fig. 2 | Integrative networks analysis identifies distinct stomatal closing signal modules. **a–d**, Stimulus-specific modules: optimal responsive subnetworks of regulated genes in the entire network for ABA (**a**), RH (**b**), darkness (**c**) and CO₂ (**d**) stimulation. Circles denote positive-scoring genes (with highly significant expression changes), whereas rectangles denote negative-scoring nodes (for example, additional nodes of modest regulation), which are implicated by the algorithm on the basis of their functional network context. Red indicates upregulation and green represents downregulation, where darker colours correspond to higher fold change values. Gene clusters of common cellular processes are enclosed by shaded lines highlighting the functional substructure of the modules. Only ABA-responsive genes are named (for details see Supplementary Fig. 2). **e**, ABA receptors are the main components in adaptation to stomatal closing signals. Each module contains a cluster of typical ABA-responsive genes. In all cases, ABA receptors are affected. Among the closing signals, the kind of receptors as well as the direction of regulation differ markedly, indicating an altered sensitivity towards ABA in the adaptation process to the individual signals. Blue text indicates genes known to be involved in ABA signalling. For each treatment, and the respective controls, four microarrays were analysed, except darkness ($n = 3$). All replicates represent biologically independent samples. The network analysis is based on the statistics and P values described in Supplementary Table 1a.

Closure-inducing signals differentially modulate PYR/PYL/RCAR receptor expression. Another feature to emerge from our investigations was that the different closure-inducing signals led to differential expression of ABA receptor family members in guard cells. At the level of the whole plant, and at the tissue level, it has

been documented that ABA application regulates the expression of certain PYR/PYL/RCAR receptors^{13,28}. In our investigations we found that six guard cell-localized PYR/PYL/RCARs were affected (Fig. 2e). In the case of ABA, three receptors (*PYL2*, *PYL4* and *PYL5*) were downregulated, while darkness upregulated *PYL1*.

Exposure to dry air (increased VPD) resulted in the downregulation of *PYR1*, *PYL2*, *PYL4* and *PYL8*. In high CO₂, *PYL8* is upregulated while *PYL2* is downregulated. These data suggest that ABA and receptors of the *PYR/PYL/RCAR* family are involved in the response to all these closure-inducing stimuli. One possible interpretation of these results is that, in the long term, acclimation to these signals is achieved through modulating the transcript abundance of individual *PYR/PYL/RCAR* family members. The net result of this would be to fine-tune guard cell sensitivity to ABA. In this context it is interesting that we found that the transcript abundance for these receptors was differentially sensitive to ABA concentration. Specifically, we observed that 250 nM ABA induced a reduction in *PYL4* abundance, while changes in the abundance of *PYL2*, *PYL4*, *PYL5*, *PYL8* and *PYR1* were only observed on treatment with ten times this concentration of ABA (2.5 µM). In this context, it should be mentioned that the products of ABA metabolism might interact with ABA receptors. This is indeed the case for phaseic acid, which is capable of binding to *PYL2* (ref. ²⁹). It is therefore possible that these metabolic products could also regulate transcriptional changes of the receptor genes. We next investigated whether other guard cell ABA-regulated genes were, like the *PYR/PYL* genes, differentially regulated in a concentration-dependent manner. As is apparent from the data in Table 1 and Supplementary Fig. 3 (see Supplementary Fig. 3 and Methods for details and statistics), *ABR*, *HAI1* and *MYB60* responded to ABA stimulation in a concentration-dependent manner. Together, these observations suggest a framework in which ABA regulates specific receptor family member abundance in a concentration-dependent manner. This would potentially provide a high degree of control over receptor and response sensitivity.

Differential guard cell gene expression regulated by *PYR/PYL* family members. We next decided to investigate the possibility that different members of the *PYR/PYL/RCAR* receptor family differentially regulate gene expression. For this we used *Arabidopsis* *PYR/PYL/RCAR* receptor mutants. The *Arabidopsis* genome contains 14 *PYR/PYL/RCAR* genes and previous studies have shown that generation of quadruple, pentuple and sextuple mutants is required to obtain robust ABA-insensitive phenotypes^{12,28,30}. However, both biochemical analyses of different receptor-phosphatase complexes and of receptor gene-expression patterns suggest that the function of ABA receptors is not completely redundant^{28,31,32}. Analysis of the single *pyl8* mutant, for example, has revealed a non-redundant role of *PYL8* in root sensitivity to ABA^{33,34}, while the *PYL9/RCAR1* receptor specifically regulates the protein phosphatase AHG1 (ref. ³²) and promotes leaf senescence³⁵. Additionally, a non-redundant function of the dimeric receptor BdPYL1 has been reported in *Brachypodium*³⁶. Functional diversification follows the evolutionary expansion of a gene family and, therefore, we investigated the role of different ABA receptors in guard cells.

Our transcriptomic data confirmed that guard cells express the six ABA receptors *PYR1*, *PYL1*, *PYL2*, *PYL4*, *PYL5* and *PYL8* that have previously been shown to be relevant for stomatal closure^{7,28}. We therefore asked whether, in guard cell ABA signalling, the six different receptors are redundant, or if each *PYR/PYL* has individual functions. To answer this question, we used the *pyr/pyl* quintuple mutant known as 12458 (ref. ²⁸), where the receptors *PYR1* and *PYL2*, *PYL4*, *PYL5* and *PYL8* are knocked out, and the 11458 (ref. ³³) loss-of-function mutant in which *PYR1* and *PYL1*, *PYL4*, *PYL5* and *PYL8* are knocked out. In addition, we took advantage of the availability of quintuple mutant complementation lines. These lines, under the control of the guard cell-specific promoter GC1 (*At1g22690*)³⁷, express single receptors individually (see details in Supplementary Fig. 4).

To exclude the possibility that the following results were influenced by overexpression of the individual receptors in the

Table 1 | ABA dose-response of the transcription of guard cell ABA-receptor and ABA-regulated genes

Gene	Locus	ABA concentration				
		25 nM	100 nM	250 nM	2.5 µM	25 µM
<i>PYR1</i>	At4g17870					64 ^a
<i>PYL1</i>	At5g46790					
<i>PYL2</i>	At2g26040				39 ^a	18 ^a
<i>PYL4</i>	At2g38310			66 ^a	36 ^a	19 ^a
<i>PYL5</i>	At5g05440				33 ^a	16 ^a
<i>PYL8</i>	At5g53160				77 ^a	54 ^a
<i>MYB60</i>	At1g08810					42 ^a
<i>ABR</i>	At3g02480				1,331 ^b	5,679 ^b
<i>HAI1</i>	At5g59220		173 ^{b,c}		594 ^b	1,535 ^b

On ABA spray application, transcription was either induced, reduced or remained unchanged. Numbers represent the expression changes presented in Supplementary Fig. 3 (in percentages) relative to untreated control. Blank entry, no significant change in expression; ^adownregulation; ^bupregulation; ^cdenotes that the value has weak significance and represents only 11% of the maximum induction (for sample sizes and statistics, see related Supplementary Fig. 3 and Methods).

complementation lines, we measured the expression levels of *PYR1*, *PYL1*, *PYL2*, *PYL4*, *PYL5* and *PYL8* in guard cells of wild type, both quintuple mutants and the complementation lines in the 12458 background (Supplementary Fig. 5a–f). These results showed that neither *PYL1* (present in 12458) nor *PYL2* (present in 11458) were upregulated in the quintuple mutants to compensate for the lack of the ABA receptors that were knocked out. Furthermore, the complementation lines showed wild type-like expression of the respective receptors (maximally varying about 2–3-fold). To confirm that ABA receptor RNA abundance is mirrored at the protein level, we selected *PYL2* as a test case for a mass spectrometry-based proteomic approach, because *PYL2* is the most relevant for ABA-induced stomatal closure (see ‘Loss of *PYL2* gene renders stomata ABA insensitive’). As expected *PYL2* receptor peptides were neither found to be present in the 12458 mutant, nor in the associated *PYR1*, *PYL4* and *PYL5* complementation lines. In wild-type guard cells and those of the *PYL2*-complemented 12458 mutant, however, *PYL2* peptides were detected at comparable levels (Supplementary Fig. 5g). These findings underline the suitability of the GC1 promoter for driving almost physiological guard cell ABA receptor expression.

To resolve whether different members of the *PYL/PYR* receptor family regulated expression of guard cell genes in a differential fashion we concentrated on the ability of ABA to regulate the following known guard cell ABA-responsive genes: *MYB60*, *ABI1*, *ABI2*, *CIPK25* and *KCR2* (ref. ⁶) (see Table 2 and Supplementary Fig. 6 for details and statistics). We observed *MYB60* downregulation in wild type and the 11458 (*PYL2* present) mutant, but not in the 12458 (*PYL1* present) mutant. Complementation of the latter mutant with *PYR1* or *PYL2* restored the wild-type phenotype, indicating that ABA-dependent downregulation of *MYB60* is under the control of these receptors. Upregulation of *ABI2* expression was abolished in 11458 but it was restored by *PYR1* or *PYL1* complementation, indicating that *PYR1* and *PYL1* are sufficient for *ABI2* expression. Likewise, upregulation of *CIPK25* was abolished in 11458 but restored by *PYL1* or *PYL8*, indicating that they are sufficient for *CIPK25* expression. Complex regulation of *KCR2* expression seems to occur, since ABA-induced upregulation was abolished in 11458 (*PYL2* present) and could not be restored in complementation lines. Finally, although *RAB18* appeared to be induced by ABA always, the presence of *PYL1* in the 12458 mutant ($P=0.00018$) or combined with *PYR1* ($P=0.025$) led to a 12- to 14-fold induction (more

Table 2 | Individual ABA receptor family members control the expression of downstream genes

	MYB60	ABI2	CIPK25	KCR2	RAB18
Wild type	26 ^a	268 ^b	268 ^b	195 ^b	400 ^b
12458 (PYL1 present)		298 ^b	269 ^b	285 ^b	1,218 ^b
PYR1::12458	40 ^a		303 ^b	194 ^b	1,436 ^b
PYL2::12458	38 ^a	251 ^b	228 ^b	200 ^b	731 ^b
PYL4::12458		307 ^b	331 ^b	244 ^b	491 ^b
PYL5::12458		314 ^b			381 ^b
11458 (PYL2 present)	31 ^a				649 ^b
PYR1::11458	22 ^a	203 ^b			956 ^b
PYL1::11458	14 ^a	160 ^b	178 ^b		433 ^b
PYL4::11458	25 ^a				421 ^b
PYL5::11458	25 ^a				301 ^b
PYL8::11458	43 ^a		215 ^b		493 ^b

ABA-spraying (25 μ M) led to up- or downregulation of genes in wild-type plants. Quintuple knockout and complementation lines revealed the receptors necessary for ABA-induced gene regulation. Numbers represent the expression changes presented in Supplementary Fig. 6 (in percentages) relative to untreated control. ^aDownregulation; ^bupregulation; blank cell, no significant change in expression (for sample sizes and statistics see related Supplementary Fig. 6 and Methods).

than three times that of the wild type), but, when just one of the receptors PYL2, PYL4 or PYL5 was expressed, this overinduction was suppressed. Since we found no strong differences in the basal levels of the examined genes in the different genetic backgrounds (Supplementary Table 1c), these findings indicate that transcriptional guard cell ABA responses are mediated by individual receptors or distinct *PYR/PYL* combinations.

Loss of *PYL2* gene renders stomata ABA insensitive. The above experiments reveal the role of individual *PYR/PYL* family ABA receptors in the control of guard cell gene expression. We next turned our attention to investigating whether individual receptor family members have distinct roles to play in the control of stomatal aperture and gas exchange. To do this we first used infrared gas analysis (IRGA)³⁸. In this approach we used excised leaves and applied ABA via the petioles to exclude any effect of the roots on transpiration. Under these conditions wild-type leaves opened their stomata on illumination and adjusted their aperture to the environmental settings before application of ABA³⁹. We found that, in the light, the 12458 quintuple mutant (*PYL1* present), in contrast to the 11458 mutant (*PYL2* present), exhibited a severe open-stomata phenotype that manifested itself in rapid wilting on exposure to light. To overcome this experimental difficulty, we preincubated plants in the dark with 25 μ M ABA for 1 h before applying the light stimulus. Using this protocol, on illumination, wild-type plants remained in a non-transpiring state (that is, ABA inhibited light-induced stomatal opening). In contrast, transpirational water loss of the ABA-insensitive 12458 mutant increased over time (Fig. 3a), whereas the 11458 quintuple mutant, which expressed wild-type *PYL2*, behaved almost like wild type (Fig. 3a). Thus, we tested all available complementation lines in the 12458 background. The 12458 mutant, when complemented by *PYR1*, exhibited reduced transpiration, but the presence of *PYR1* did not fully complement the ABA-insensitive phenotype of the quintuple mutant that had lost *PYL2* (Fig. 3b). These data suggest a key role for *PYL2* in the guard cell ABA-signalling pathway that leads to stomatal closure. This was confirmed by complementing *PYL2* in the 12458 mutant, which resulted in a plant that showed the same ABA suppression of

Fig. 3 | *PYL2* is sufficient for stomatal ABA sensitivity. Excised leaves, in darkness, were preincubated with ABA fed via the petiole to prevent stomatal opening in the light. The stomatal aperture was measured by gas exchange as a function of relative transpiration. **a**, Stomata of wild type and the 11458 mutant (*PYL2* present) remained almost closed, while those of the 12458 mutant appeared to be ABA insensitive. **b**, *PYL2* in the 12458 background fully restored the wild-type phenotype, while *PYR1* reacted with incomplete ABA sensitivity. **c**, *PYL4* and *PYL5* were unable to restore the ABA sensitivity of the 12458 quintuple background line. Values are normalized to time point 0. $n = 28$ for Col 0, $n = 6$ for 12458, $n = 9$ for 11458, $n = 5$ for *PYR1*::12458, $n = 5$ for *PYL2*::12458, $n = 6$ for *PYL4*::12458, $n = 7$ for *PYL5*::12458. All replicate numbers represent biologically independent samples, mean $\pm$ s.e.m.

stomatal transpiration as the wild type (Fig. 3b). In contrast, guard cell expression of *PYL4* and *PYL5* did not complement the rapidly wilting 12458 mutant phenotype (Fig. 3c).

In addition to IRGA measurements, which in this case provide data on the effects of manipulating gene expression on stomatal transpirational control at the level of the whole leaf, we followed the response of individual stomata in their natural environment of

intact leaves. This was achieved by applying 50 μM ($\pm$)-ABA (corresponding to 25 μM biologically active ABA) to single stoma via micropipette-assisted electro-infusion. By introducing ABA directly to the guard cells we aimed to exclude any indirect hormone effects, via, for example, the mesophyll cells and/or vasculature, on the control of transpiration by stomata. Using this protocol we observed that stomata of wild-type *Arabidopsis* closed within 10 min of exposure to ABA (Fig. 4a,b and Supplementary Videos 1 and 2). In contrast, stomata of the 12458 mutant (*PYL1* present) did not respond to the hormone and remained open. This mutant was also the only one with substantially wider open stomata under control conditions (Fig. 4c). In the quintuple mutant (12458) complemented by *PYL2* and the 11458 mutant (*PYL2* present), we observed stomatal closure that was identical to wild type. We also observed that *PYL5* was not able to complement the ABA-insensitive phenotype (Fig. 4a,b and Supplementary Videos 1 and 2). This demonstrates that *PYL2* is sufficient to fully rescue the guard cell ABA signalling and fast stomatal closure. To confirm our findings that *PYL2* and, partially, *PYR1* are able to complement the ABA insensitivity of the 12458 mutant, we repeated the ABA preincubation experiment with the *pyl2-1* loss-of-function mutant (seed strain GT2864)¹² and the corresponding *Ler* wild type strain. Although *PYR1*, *PYL1*, *PYL4*, *PYL5* and *PYL8* are present in this mutant, *pyl2-1* behaved almost like the *PYR1* complementation line in the 12458 background (Supplementary Fig. 7), supporting our hypothesis that *PYL2* and *PYR1* are the key receptors for proper ABA-induced stomatal closure.

PYL4 and PYL5 are sufficient for CO₂-induced stomatal closure. Since Raschke's research in the 1970s we have known that stomata require ABA to sense elevated CO₂ (refs. 7,8,40,41). However, the molecular basis for this interaction has not been clear. We took advantage of the panel of ABA receptor mutants previously described to investigate the basis of this interaction using IRGA. Stomata that had been opened in the light in the presence of 400 ppm CO₂ (ambient) were exposed to 1,000 ppm CO₂. As expected, this resulted in a decrease in transpiration consistent with a reduction in stomatal aperture (Fig. 5a). Next, we used the 11458 mutant, in which *PYL2* is expressed naturally. We had previously shown that this *PYL2*-expressing mutant showed a wild type-like ABA-induced closure response (Fig. 3b). However, in contrast to ABA, CO₂-induced closure was impaired in this mutant, as well as in the 12458 mutant (Fig. 5a). Stomata of complementation lines in the 12458 background (with *PYL1* present) expressing *PYR1* or *PYL2* also remained impaired in their response to high CO₂ (Fig. 5b). However, lines that express *PYL4* or *PYL5* in guard cells regained stomatal CO₂ sensitivity that was similar to wild-type plants (Fig. 5c). These data show that *PYL4* or *PYL5* are sufficient for the induction of closure by increased CO₂. Interestingly, except for the *PYL5* complementation line, all other mutants showed remarkably higher ground levels of transpiration in the light and under ambient CO₂ atmosphere compared with wild type (Supplementary Fig. 8). Only the *PYL4* complementation line started to reopen their stomata after about 2 h of high CO₂ treatment. Future studies will investigate whether this behaviour represents a phenomenon associated with adaptation (Supplementary Fig. 8).

Discussion

The primary objective of the work described in this paper has been to explore how stomata adapt to changed environmental conditions. Our focus has not been on changes in stomatal development that are induced by changes in the environment. Rather, we have concentrated on examining the mechanisms likely to underpin changes in, for example, the sensitivity of the stomatal aperture response to closure-inducing stimuli. Specifically, when we looked at four different closure-inducing stimuli, we found that they regulated a diverse set of intracellular pathways. While there was some overlap,

Fig. 4 | ABA-induced movements of individual stoma. Electro-infusion of ABA to single stoma. **a**, Stomata of wild type, 11458 mutants (*PYL2* present) and the *PYL2*::12458 complementation line closed within 10 min following an ABA pulse. In contrast, stomata of the 12458 mutant (without *PYL2*) and the *PYL5*::12458 complementation line remained fully open. **b**, Amplitudes of the changes in stomatal pore width from fully open to maximum closed after ABA infusion revealed that the presence of *PYL2* is sufficient to close stomata to wild-type levels. Stomata of the 12458 mutant remained open. In contrast to all other samples, the width of *PYL5*::12458 stomata showed little but ABA-independent closure over time. **c**, Stomatal width of wild type and mutants after 20 min illumination. Only 12458 mutants showed substantially wider stomatal pores before ABA application. $n = 9$, all replicate numbers represent biologically independent samples, mean $\pm$ s.e.m.

there were also marked differences, and this must reflect the specific effects of these signals on guard cell properties. However, markedly, the integrated network analysis (see Supplementary Information 3) revealed that all closure-inducing signals induced changes in the expression of the *PYR/PYL/RCAR* ABA receptor family. This provides a mechanism for all stimuli to modify stomatal function,

Fig. 5 | PYL4 and PYL5 render guard cells CO_2 sensitive. Whole plants were placed in gas exchange cuvettes in the dark at 400 ppm CO_2 and stomatal aperture was measured as a function of the relative transpiration, by gas exchange. Following stomatal opening in the light, the CO_2 concentration was increased to 1,000 ppm. **a**, Stomata of wild-type plants closed about 60%, while both quintuple mutants did not react. **b**, The PYL2-complemented 12458 mutant did not react to elevated CO_2 and the PYR1-complemented stomata also remained impaired. **c**, 12458 mutants complemented with either PYL4 or PYL5 closed their stomata in a wild-type manner. Values are normalized to time point 0. $n=20$ for Col 0, $n=12$ for 12458, $n=6$ for 11458, $n=4$ for PYR1::12458, $n=6$ for PYL2::12458 and $n=7$ for PYL5::12458. All replicate numbers represent biologically independent samples, mean $\pm$ s.e.m.

through modulating the sensitivity of the ABA-signalling pathway. We investigated the control of guard cell gene expression by ABA in more detail and found that individual genes, including members of the ABA receptor family, responded to ABA in a concentration-dependent manner. This conclusion was strengthened when we

found that the expression of PYL4 or PYL5 in guard cells was sufficient for CO_2 -induced stomatal closure, but was not sufficient to restore the ability of ABA to inhibit light-induced stomatal opening in the 12458 mutant, whereas PYL2 was sufficient for this response. Our data on the ABA response of the complemented lines agree with a previous publication⁴², which showed that *pyr1/pyl1/pyl2/pyl4* quadruple mutant plants did not close their stomata following ABA application, and that ABA preincubation could not prevent stomatal opening by light. In contrast, in another study⁴³ using the same mutant, ABA application to open stomata did not induce stomatal closure, but ABA preincubation prevented stomatal opening. In the latter paper the authors used guard cells in epidermal peels (obtained by macerating leaves in a Waring blender) floating on ABA solutions. Future studies will have to investigate whether, and how, mechanical stress feeds back onto the ABA biology of guard cells. It should be noted that the *pyl2-1* single mutant¹² behaved like the PYR1-complemented 12458 plants, indicating that PYR1 and PYL2 are the most important receptors for the fast ABA response of guard cells. However, one might not be able to exclude the possibility that either PYL8 or heterodimers involving receptors other than PYL2 are also competent to rescue the ABA-insensitive phenotype of the 12458 mutant.

In terms of stomatal CO_2 responsiveness, a previous report agrees that CO_2 -induced stomatal closure is disrupted in ABA receptor mutant backgrounds⁸. In contrast, another paper concludes that 'rapid CO_2 signal transduction leading to stomatal closure via an ABA-independent pathway' occurs⁴⁴. How might these radically different conclusions be reconciled? It is, of course, only possible to speculate. However, as documented earlier²³ stomatal responsiveness to ABA varies from insensitivity to sensitivity, and this is regulated by relative air humidity. Modulation of stomatal sensitivity by plant growth conditions, such as relative humidity, could provide a framework to explain the disparity in the results. The veracity of such a possibility requires further experimentation. Nevertheless, it is worth pointing out that examination of the data in Hsu et al.⁴⁴, as in Merilo et al.⁷, reveals that the quadruple receptor mutants *pyr1/pyl4/pyl5/pyl8* and hexuple *pyr1/pyl1/pyl2/pyl4/pyl5/pyl8* display reduced CO_2 responsiveness compared with wild type. These data do indicate a role for ABA receptors in the response to CO_2 . This was also the conclusion reached when stomatal conductance in response to CO_2 was analysed in wild type and *pyr1/pyl1/pyl2/pyl4/pyl5/pyl8* (ref. 7). Finally, the long-term exposures of the in-gel kinase assays done by Hsu et al.⁴⁴ showed a basal activity of OST1 in guard cells, which, according to the authors, is probably necessary for rapid CO_2 -induced stomatal closure. Such a basal level of phosphorylation would be very sensitive to phosphatase and PYR/PYL/RCAR activity, which can be modified by nanomolar changes in ABA concentration (K_d for ternary complexes in the 20–40 nM range^{11,13}). However, to return to the current investigation, our data, including its in-depth analysis of receptor mutants, are strongly supportive of a role for ABA receptors, in particular PYL4 and PYL5, in the stomatal response to increased concentrations of CO_2 .

Taken together our investigations reveal not only the complexity of the underlying cellular mechanisms responsible for controlling stomatal responses to closure-inducing signals, and how this might contribute towards stomatal response acclimation, but also a plausible mechanism for how stomata might integrate multiple signals. The results here are likely to be of benefit to plant breeders who are interested in engineering crops to respond to the complex multiple environmental stresses that are likely to be associated with future climates.

Methods

Plant material and growth conditions. *Arabidopsis thaliana* plants were grown in soil that was semi-sterilized for 20 min at 100°C and cultivated in climate chambers (Binder KBWF 720; www.binder-world.com) in a 12-h day–night

rhythm (22/16°C, 60% RH) and were illuminated with 125 $\mu\text{mol m}^{-2} \text{s}^{-1}$ white light. The gas exchange measurements and the expression studies were performed with 6–8-week-old, adult, non-flowering plants.

Preparation of the ABA receptor mutants. Using column genomic DNA as template we amplified the promoter region of Atlg22690 located 1,716 base pairs (bp) upstream of the start codon, named GC1 (ref. ³⁷), by PCR, using the primers GC1 forward 5'-ACCATGGAGTAAAGATTTCAGTAACCC and GC1 reverse 5'-ATCCATGGTATTCTTGAGTAGTGATTTGAAG. Next, GC1 was cloned in pCR8/GW, excised as a NcoI fragment and cloned in front of either PYR1, PYL2, PYL4, PYL5 or PYL8. The resulting pGC1-PYR/PYL construct was recombined by LR reaction into modified pAlligator2 (ref. ⁴⁵), which has been previously doubly digested with HindIII-EcoRV, treated with Klenow fragment and religated to obtain a construct lacking the 35S promoter. Each modified pAlligator2-GC1:PYR/PYL construct was transferred to *Agrobacterium tumefaciens* C58C1 (pGV2260)⁴⁶ by electroporation and used to transform 12458 and 11458 mutant plants^{28,33} by the floral dip method⁴⁷. T1 transgenic seeds were selected on the basis of seed GFP fluorescence and sown in soil to obtain the T2 generation. Homozygous T3 progeny were used for further studies. Several PYL8::12458 complementation lines were also tested, but did not grow properly. They showed a severe phenotype and excised leaves wilted immediately. These mutants could not be used in any of our physiological experiments and were thus excluded from our analyses.

IRGA. Stomatal closing assays. Application of ABA and low air humidity were performed as described⁶. In brief, ABA solution (50 μM ($\pm$)-ABA) was sprayed onto the plants until the surface was fully covered. The low-humidity treatment was performed by reducing the RH from 80% to 20% at 22°C, which equals a VPD change from 0.529 to 2.116 kPa. High CO₂ (1,000 ppm) was applied to plants in airtight boxes after 2 h illumination at ambient air to close stomata. For the samples in darkness, plants were fully shaded following 2 h of illumination. Each closing signal persisted for 4 h. To minimize diurnal effects on gene expression, all procedures were started in the morning 2 h after onset of illumination.

Leaf gas exchange measurements. Transpiration rates were measured with a custom-made system as described elsewhere³⁸. Carbon dioxide response measurements were performed with intact plants at 20°C and 52.5 $\pm$ 3% RH (VPD = 1.123 Pa). The soil surface of the pots was tightly covered with plastic foil to avoid water evaporation. After the transpiration rates had stabilized in the dark, plants were illuminated with 125 $\mu\text{mol m}^{-2} \text{s}^{-1}$ white light. After 1 h of illumination the CO₂ concentration of the air stream was increased from 400 ppm to 1,000 ppm.

The effect of ABA on the light-induced transpiration of *Arabidopsis* was measured with detached leaves and the same conditions as described for the CO₂ measurements. To avoid xylem embolism, the leaves were cut under water. The petioles were directly transferred into 50 μM $\pm$ ABA solution and incubated in darkness. After 1 h ABA incubation leaves were illuminated with 125 $\mu\text{mol m}^{-2} \text{s}^{-1}$ white light and the transpirational water loss was recorded.

ABA electro-infusion. This method was performed according to ref. ⁴⁸. Excised leaves from 5–6-week-old plants were attached to a Petri dish using double-sided tape and submerged in a bath solution (1 mM KCl, 1 mM CaCl₂, 5 mM MES/BTP, pH 6). The Petri dish was mounted to an upright microscope (Axioskop 2FS, Zeiss) and the leaves were left for incubation while being exposed to 125 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light. Stomata on the abaxial side of the leaves were visualized with a water immersion objective (Achromplan x63/0.9W, Zeiss) and images were recorded with a camera (CoolSNAP HQ, VisiTron Systems) in 30-s intervals using VisiView (VisiTron Systems) imaging software. Electrodes from borosilicate capillaries (inner/outer diameter = 0.56/1.0 mm; Hilgenberg) were pulled on a horizontal laser puller (P2000, Sutter Instrument) to achieve resistances ranging from 60 to 100 M Ω . The electrodes were tip-loaded with 50 μM ($\pm$)-ABA, backfilled with 300 mM KCl and connected by Ag/Ag half-cells to the head stage (HS-2A $\times$ 0.01; Axon Instruments, Molecular Devices) of a TEVC amplifier (GeneClamp 500; Axon Instruments). Reference electrodes were prepared using capillaries filled with 300 mM KCl, sealed with an agar plug (2% w/v agarose, 300 mM KCl and connected to ground with an Ag/Ag half-cell). Microelectrodes were driven with a piezoelectric micromanipulator (MM3A; Kleindiek). Before electro-infusion the electrode was brought into close proximity with an open stoma and left for 10 min to ensure that no mechanical closing stimulus was applied. ABA or control solution was released from the capillary by a negative, 10-min, 1-nA current pulse. After an additional 10 min the image recording was stopped.

ABA spray experiments. *Arabidopsis* plants were treated with ABA via spraying until the leaves were moist. To investigate the dose dependency of the ABA receptor expression in wild-type plants, ABA solutions with 0 (control plants), 0.025, 0.1, 0.25, 2.5 and 25 μM ABA in deionized water containing 1:10,000 Triton X-100 were used. To investigate ABA-regulated guard cell expression patterns, plants were sprayed with 25 μM ABA solution or deionized water with Triton X-100. After 4 h of incubation, guard cells were sampled and RNA was extracted.

CO₂ treatment for microarrays. Single, potted 6–7-week-old *Arabidopsis thaliana* (Col 0) plants were subjected to an air stream containing ambient CO₂ (380 ppm) and 50 $\pm$ 5% RH (VPD = 1.322 kPa) in climate chambers at 22 $\pm$ 2°C and 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ white light. After 1 h half of the plants were treated with 1,000 ppm CO₂ for 4 h. Guard cells were sampled and RNA was extracted for microarray hybridization.

Darkness treatment for microarrays. Single potted 6–7-week-old *Arabidopsis thaliana* (Col 0) plants were subjected to an air stream containing ambient CO₂ (400 ppm) and 50 $\pm$ 5% RH (VPD = 1.322 kPa) in climate chambers at 22 $\pm$ 2°C and 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ white light. After 2 h half of the plants were kept in darkness for 2 h. Guard cells were sampled and RNA was extracted for microarray hybridization.

ABA and low RH (increased VPD) treatment. These microarray data come from a previous study⁶ in which the ABA and low-humidity (increased VPD) signals persisted over 4 h.

Sampling and RNA extraction. Guard cell sampling and RNA extraction were performed as described⁶. Guard cell samples were frequently tested via vital staining to confirm that contamination by other cell types remained below 5–10%.

Quantitative real-time PCR. Quantitative real-time PCR (qPCR) was performed as described⁴⁹. All transcripts were normalized to 10,000 molecules of actin2/8 and thus denoted as relative expression. All indicated replicate numbers refer to biological replicates. Primer sequences used: PYR1 fwd 5'-GCTGACGAATTACA AATCCGTT-3', PYR1 rev 5'-ACCGTCGCGAGTTTCTG-3', PYL1 fwd 5'-CGTG AACGTGATAAGTGG-3', PYL1 rev 5'-TGAACCGTCGTAACCGAT-3', PYL2 fwd 5'-CATAACCCAACGCATCCA-3', PYL2 rev 5'-AACTCAAGCCGCTCGGTA-3', PYL4 fwd 5'-CCGCTCGTTTTCACACAC-3', PYL2 rev 5'-GTGTTGCCTGG AGGAACATC-3', PYL5 fwd 5'-TGGTGAATGATGACACAG-3', PYL1 rev 5'-AG ACTGAAGGTTGCACCG-3', PYL8 fwd 5'-TGTGTGGTCACTTGTGAG-3', PYL8 rev 5'-TGAACCGCAAGACGTTCA-3', KCR2 fwd 5'-ATGTGGATGCACTATC A-3', KCR2 rev 5'-AAGGTTATCCGGTACAA-3', ABI2 fwd 5'-GGACTTAGAG GCTATTG-3', ABI2 rev 5'-AGGATTAAATCCATTAGT-3', MYB60 fwd 5'-ATG CTGTGACAAGATAGG-3', MYB60 rev 5'-AAAGTTTCCACGTTTAAAT-3' CIPK25 fwd 5'-AGATCCAAAACGTAGAAG-3', CIPK25 rev 5'-CTTACACAACCTCAAC GAC-3', HAI1 fwd 5'-GTTGAATAGTTTTCACGA-3', HAI1 rev 5'-GCCGTATT TAGGATAAG-3', ABR fwd 5'-GGTGAATGATGACCAAG-3', ABR rev 5'-AT AAAGATCCAAATGGACG-3', RAB18 fwd 5'-AGAAGGAATAACACAAA-3', RAB18 rev 5'-CAATACAACGACCGAA-3'.

PYL2 protein identification. For the identification and quantification of PYL2, parallel reaction monitoring (PRM) via mass spectrometry was used. Four or five biological replicates from each experimental group were examined. Proteins were extracted from the ground guard cells following the phenol extraction/ammonium acetate precipitation method described⁵⁰. Briefly, 150 mg starting material was processed. For tryptic digestion, an aliquot of 20 μg protein was digested with 0.2 μg trypsin. Samples were cleaned using C18 solid phase extraction according to the manufacturer (Pierce C18 spin columns, ThermoFisher Scientific) and dissolved in 5% acetonitrile (ACN) and 0.1% formic acid (FA). Peptides from the candidate proteins were designed using Skyline (v.4.2) and ordered from ThermoFisher Scientific (PEPotec Grade 1). The ultra performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) analysis was performed on an Ultimate 3000 UPLC system (Dionex, Thermo Scientific) equipped with a C18 PepMap100 precolumn (5 μm , 300 $\mu\text{m} \times$ 5 mm, Thermo Scientific) and an EasySpray C18 column (3 μm , 75 $\mu\text{m} \times$ 15 cm, Thermo Scientific) using a gradient of 5% to 20% ACN in 0.1% FA for 10 min followed by a gradient of 10% to 35% ACN in 0.1% FA for 4 min and a final gradient from 35% to 95% ACN in 0.1% FA for 2.5 min and a Q Exactive Orbitrap mass spectrometer (Thermo Scientific). The flow-rate was set at 250 $\mu\text{l min}^{-1}$. The Q Exactive was operated in positive-ion mode with a nanospray voltage of 1.5 kV and a source temperature of 250°C. ProteoMass LTQ/FT-Hybrid ESI Positive Mode CalMix (MSCAL5-1EA SUPELCO, Sigma-Aldrich) was used as an external calibrant and the lock mass 445.12003 as an internal calibrant. For the characterization of the standard peptide library, the instrument was operated in a data-dependent acquisition mode with a survey MS scan at a resolution of 70,000 (full width at half maximum at m/z 200) for the mass range of m/z 350–1,800 for precursor ions, followed by MS/MS scans of the top ten most intense peaks with +2, +3 and +4 charged ions above a threshold ion count of 16,000 at a 35,000 resolution using a normalized collision energy of 28 eV with an isolation window of 3.0 m/z and dynamic exclusion of 10 s. All data were acquired with Xcalibur v.2.2 software (Thermo Scientific). The most suitable peptide per protein was chosen to set the PRM analysis. To avoid overlapping time windows the samples were analysed on the Q Exactive in PRM mode in two separate runs (see Table 3).

PRM analysis was performed in Skyline v.4.2. The dicyano-1,4-benzoquinone analysis of the peptide library was done with proteome discoverer and the library was loaded in Skyline as an msf file. The NCBI *Arabidopsis* fasta file was loaded and the results of the PRM run were loaded as raw files with the following settings: MS1 orbitrap detection 70,000 resolution, MS2 orbitrap detection 20,000 resolution. Only peptides showing three accurate transitions were accepted.

Table 3 | Specification of PRM conditions

Peptide name	Sequence	Peptide length	m/z	Z	Start	End	Run
PYL2	LISGDDGVGSVR	12	587.8068	2	25.9	28.9	1

'Start' and 'End' refer to the start and end times that the quadrupole window was open for the specified peptide, respectively.

Microarray hybridization. For transcriptome profiling, samples were hybridized on an Agilent Platform using the Agilent *Arabidopsis* v4 microarray chips (catalogue no. 021169) (<http://www.agilent.com>). RNA quantity was measured with an ND-100 Spectrophotometer v3.3.0 (NanoDrop Technologies). RNA integrity was confirmed using an Agilent RNA 6000 Nano Chip on an Agilent 2100 BioAnalyzer (vB.02.03 BSI307). Total RNA (500 ng) was used for each sample labelling. Labelling and preparation of samples for hybridization was performed as described in the one-colour microarray-based gene-expression analysis protocol provided by Agilent, including the one-colour RNA spike-in kit (v5.0.1, 2006; Agilent Technologies). Slides were scanned on the Agilent Microarray Scanner with extended dynamic range at high resolution (5 µm). Datasets were extracted by propriety software package (v9.5.3.1, Agilent Technologies) using a standard protocol.

Array analysis. Data preprocessing was performed using Bioconductor software⁵¹ with the statistical programming environment R⁵². Normalization has been performed using negative control probes and quantile normalization using negative and positive control probes as implemented in the *neqc* function⁵³ of the *limma* package⁵⁴. Differential gene expression for all stimuli was calculated using the moderated *t*-statistic approach as implemented in the R package *limma*⁵⁴, which has been specifically developed for the analysis of small sample size experiments. The *P* values of all results were corrected for multiple testing by using the false discovery rate (FDR)⁵⁵. To identify guard cell-specific gene regulation, we used a meta-analysis approach based on the order statistic of the stimulus *P* values. Therefore, *P* values from a one-sided moderated *t*-test of differentially expressed genes between guard cells and leaf tissue have been derived from the *limma* model. Subsequently, for each stimulus a combined *P* value has been calculated using the second-order statistics of the stimulus and the guard cell versus leaf *P* value. This results in a set of genewise *P* values for guard cell-specific stimulus effects.

Pathway enrichment analyses. Analysis was carried out by a model-based approach, which can be integrated directly into the *limma* analysis of differential expression. A competitive gene-set test has been applied to all stimulation contrasts, as implemented in the function *camera*⁵⁶. This function tests whether a set of genes is highly ranked relative to other genes, in terms of differential expression, accounting for potential intergene correlation. Pathways for the analysis have been obtained from the MapMan catalogue⁵⁷ based on TAIR v.10 (2012), from which all gene sets with more than 5 genes and fewer than 500 genes have been included in the analysis.

Integrative network analysis. Network data has been obtained from the STRING database (v9.0, <http://string-db.org>)⁵⁸. All interactions of the genome of *Arabidopsis thaliana* have been extracted from the database, yielding a total of 717,810 interactions between 16,465 genes. For the analysis we used the high confidence network (combined score >0.7) resulting in 69,603 interactions between 7,090 genes. After mapping all genes on the array to the network, the resulting largest connected component comprising 69,329 interactions between 6,724 genes has been extracted. This constitutes the base network used in all subsequent network analyses.

An optimal algorithm has been established to decompose the large intricate network structure into functionally coherent network modules⁵⁹. These modules can be viewed as building blocks of cellular processes, such as basic metabolic pathways or stimuli-specific signalling. To identify these signalling subnetworks the genes (network nodes) need to be scored according to their measured regulation, and responsive modules can be identified using an exact search algorithm.

Here, a statistical model to derive functional scores of closing signal responsiveness in guard cells has been used. Therefore, all stimuli *P* values have been derived from the *limma* analysis as detailed above. Integrated network analysis node (gene) scores have been computed based on these *P* values as detailed in ref.⁵⁹ using the routines implemented in the R package *BioNet*⁶⁰. Briefly, a beta-uniform mixture model has been fitted to the *P* value distribution, thereby decomposing the signal and noise components of the distribution. Based on this, network scores have been calculated as the log likelihood ratio of the signal to the noise component. Thus, positive scores reflect signal content (low *P* values), whereas the negative scores reflect non-significant genes (noise). The signal to noise threshold has been adjusted by multiple testing using the FDR. For the different stimuli, appropriate FDR values have been selected according to the different effect strengths controlling the size of the resulting modules. These mainly contain genes with a maximal response, but also a few tightly associated genes that show no differential regulation on the transcriptomic level (represented

as squares in the network). Subsequently, an exhaustive network search has been performed on the entire node-scored network to identify the maximum-scoring subnetworks using an exact algorithm⁵⁹. The resulting optimal solutions represent maximally significant, differentially regulated modules. On the basis of functional scoring of stimulation, this constitutes the optimal responsive modules within the entire network. This also means that no other module shows a stronger guard cell-specific closing signal response.

Statistics. To test significances on qPCR (Tables 1 and 2 and Supplementary Figs. 3 and 6) we used a multivariable linear model on log-transformed expression values (relative to actin), adjusting additionally for the day of experiment. Heteroscedasticity-consistent standard errors for regression coefficients have been calculated as implemented in the sandwich package using the default setting⁶¹. For dose-response experiments (Supplementary Fig. 3) comparisons of different concentrations to the unstimulated control have been multiple-testing corrected on the basis of Dunnett's post hoc test procedure for many-to-one comparisons. We used the step-down adjustment (method = 'free') as implemented in the 'multcomp' package in R⁶². Reported *P* values are based on two-sided tests, *P* values <0.05 are regarded as significant. Levels of significance were **P* ≤ 0.05, ***P* ≤ 0.01, ****P* ≤ 0.001.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The data that support the findings of this study are available from the corresponding authors upon reasonable request. Microarray data of the ABA and low air humidity treatments that were taken from ref.⁶ were deposited in the Gene Expression Omnibus (GEO) database <http://www.ncbi.nlm.nih.gov/geo> with accession no. GSE41054. The microarray data from CO₂ and darkness experiments were deposited in the same database under GSE118520.

Code availability

Algorithms and statistics used in the analyses are based on published approaches available in R packages (mainly Bioconductor framework) and other cited publicly available repositories.

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Author contributions

M.D. and T.M. conceived and conducted bioinformatics. H.M.M., H.B. and P.A. conceived, performed and analysed the expression studies. H.M.M. and E.M. conducted and analysed gas exchange measurements. M.P.-L. and P.L.R. conceived and conducted the generation of transgenic plants. C.-M.G. and S.C.C. conceived and conducted proteomic analyses. J.H. conceived, conducted and analysed electro-infusion experiments. P.A., P.L.R., H.K., K.A.S.A.-R., T.M., A.M.H. and R.H. designed and conceived the study. M.D., T.M., P.A., A.M.H. and R.H. wrote the manuscript. All authors discussed the results and commented on the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Data collection

We used for guard cell imaging VisiView v2.1.4 (Visitron Systems), for proteomics Xcalibur v2.2 (Thermo Fisher Scientific), network data have been obtained from the STRING database v9.0

Data analysis

We used Excel 2016-2018, for proteomics Skyline v4.2 (MacCoss Lab Software), for microarray analyses and the modules R v3.2.2 and the packages limma v3.26.9, BioNet v1.30.0, multcomp v1.4-6, and MapMan vTAIR10

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Microarray data were deposited in the Gene Expression Omnibus (GEO) database with accession no. GSE41054 for ABA and low air humidity treatments, and under

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Sample size	Sample size was not calculated but determined based on previous studies and experiences. Sample size of all experiments was sufficient to result in statistical significance and reproducibility. All replicates represent biologically independent samples. As is common practice, the design of the microarray analysis is based on four biological replicates for each condition.
Data exclusions	Density plots of raw signal intensities indicated two microarray outliers which have been removed prior subsequent analyses.
Replication	All experiments were conducted at least three times and gave similar results. For each type of reproduced experiment, plants grown under identical conditions, of the same age, and always at the same time of day were used to minimize batch effects. Microarray data have been replicated and validated by qPCR as described in Bauer et al. (2013), Curr Biol, 23, 53-57 and Bauer et al. (2013), Mol Plant, 6, 1703–1706.
Randomization	Plants for each experiment were always chosen randomly
Blinding	Blinding was performed in gas exchange and electro-infusion experiments. All other experimental data were calculated unbiased.

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