A viral RNA silencing suppressor interferes with abscisic acid-mediated signalling and induces drought tolerance in *Arabidopsis thaliana*

JACK H. WESTWOOD¹, LUCY McCANN^{1,†}, MATTHEW NAISH¹, HEATHER DIXON^{1,‡}, ALEX M. MURPHY¹, MATTHEW A. STANCOMBE¹, MARK H. BENNETT², GLEN POWELL², ALEX A. R. WEBB¹ AND JOHN P. CARR^{1,*}

¹Department of Plant Sciences, University of Cambridge, Downing Street, Cambridge, CB2 3EA, UK

²Imperial College London, South Kensington Campus, London, SW7 2AZ, UK

SUMMARY

Cucumber mosaic virus (CMV) encodes the 2b protein, which plays a role in local and systemic virus movement, symptom induction and suppression of RNA silencing. It also disrupts signalling regulated by salicylic acid and jasmonic acid. CMV induced an increase in tolerance to drought in *Arabidopsis thaliana*. This was caused by the 2b protein, as transgenic plants expressing this viral factor showed increased drought tolerance, but plants infected with CMV Δ 2b, a viral mutant lacking the 2b gene, did not. The silencing effector ARGONAUTE1 (AGO1) controls a microRNA-mediated drought tolerance mechanism and, in this study, we noted that plants (*dcl2/3/4* triple mutants) lacking functional short-interfering RNA-mediated silencing were also drought tolerant. However, drought tolerance engendered by CMV may be independent of the silencing suppressor activity of the 2b protein. Although CMV infection did not alter the accumulation of the drought response hormone abscisic acid (ABA), 2b-transgenic and *ago1*-mutant seeds were hypersensitive to ABA-mediated inhibition of germination. However, the induction of ABA-regulated genes in 2b-transgenic and CMV-infected plants was inhibited more strongly than in *ago1*-mutant plants. The virus engenders drought tolerance by altering the characteristics of the roots and not of the aerial tissues as, compared with the leaves of silencing mutants, leaves excised from CMV-infected or 2b-transgenic plants showed greater stomatal permeability and lost water more rapidly. This further indicates that CMV-induced drought tolerance is not mediated via a change in the silencing-regulated drought response mechanism. Under natural conditions, virus-induced drought tolerance may serve viruses by aiding susceptible hosts to survive periods of environmental stress.

INTRODUCTION

Viruses induce varied physiological, metabolic and developmental changes in susceptible hosts (Handford and Carr, 2006). These changes are often detrimental to host fitness. However, viruses are obligate intracellular parasites and are therefore absolutely dependent on their hosts for replication, transmission and survival (Hull, 2002). Thus, it can be reasoned that, as viruses and their hosts co-evolve, there should arise trade-offs between the pathogen and its host to balance viral virulence against the need for sufficient hosts to survive to support the continued existence of the virus (Fraile and García-Arenal, 2010). Consistent with this idea, several recent studies have found positive effects of virus infection on host fitness (reviewed by Roossinck, 2011). Examples of this include the following: a densovirus that was found to be essential for wing formation in the rosy apple aphid (Ryabov *et al.*, 2009); a virus of a mutualistic fungal endophyte that conferred thermal tolerance to the host plant (Marquez *et al.*, 2007); and several plant viruses that have been found to confer drought tolerance on a number of susceptible hosts (Xu *et al.*, 2008).

Virus infection induces changes in host transcript abundance (Rodrigo *et al.*, 2012; Whitham *et al.*, 2003). Some of these changes can be attributed to the ability of these pathogens to disrupt RNA silencing through the expression of viral suppressors of RNA silencing (VSRs; Díaz-Pendón and Ding, 2008). RNA silencing mediated by short-interfering RNAs (siRNAs) is a potent antiviral defence mechanism, and many plant viruses encode VSRs, although there is great diversity in the mode of action of these factors (Csorba *et al.*, 2009). The 2b protein encoded by cucumber mosaic virus (CMV) is a VSR and virulence determinant (Brigneti *et al.*, 1998; Mochizuki and Ohki, 2012). The silencing suppressor activity of 2b is primarily caused by its ability to bind siRNAs (Duan *et al.*, 2012; González *et al.*, 2010, 2012; Goto *et al.*, 2007). However, this VSR also binds ARGONAUTE1 (AGO1) (González *et al.*, 2010; Zhang *et al.*, 2006), one of two RNA 'slicer' enzymes known to be involved in plant antiviral RNA silencing (Baumberger and Baulcombe, 2005; Harvey *et al.*, 2011).

VSRs can cause disease symptoms because RNA silencing pathways play important roles in the regulation of plant development

*Correspondence: Email: jpc1005@hermes.cam.ac.uk

Present addresses:

†University of East Anglia, Norwich Research Park, Norwich, NR4 7TJ, UK;

‡University of Nottingham, Jubilee Campus, Nottingham, NG8 1BB, UK.

(Guo *et al.*, 2005; Mallory *et al.*, 2005; Reyes and Chua, 2007; Schommer *et al.*, 2008). For example, through its interactions with small RNAs and AGO proteins, the 2b protein disrupts microRNA (miRNA)-mediated regulation of host mRNA abundance (González *et al.*, 2010, 2012; Lewsey *et al.*, 2007; Zhang *et al.*, 2006). This leads to abnormal plant development patterns, resulting in disease symptoms, such as stunting and altered leaf morphology (Lewsey *et al.*, 2007, 2009; Zhang *et al.*, 2006). The 2b protein also interferes with hormone signalling pathways, including those mediated by the defensive signals salicylic acid (SA) and jasmonic acid (JA) (Ji and Ding, 2001; Lewsey *et al.*, 2010), which may explain some of its effects on plant–aphid interactions (Ziebell *et al.*, 2011).

Xu *et al.* (2008) reported that CMV infection induced drought tolerance in a range of hosts, including *Beta vulgaris*, *Cucurbita pepo*, *Solanum lycopersicum*, *Nicotiana benthamiana* and *N. tabacum*, as well as freezing tolerance in *B. vulgaris*. Here, we report that CMV infection induces drought tolerance in *Arabidopsis thaliana*, which is not only a well-studied model plant species, but also one of the natural hosts for this virus (Pagán *et al.*, 2010). In *A. thaliana* ecotype Col-0, the 2b proteins of CMV strains Fny and LS differ in their ability to bind and inhibit AGO1, which participates in silencing mediated by miRNAs and siRNAs (Baumberger and Baulcombe, 2005). In Col-0, although Fny2b has the ability to bind AGO1 and, consequently, to inhibit endogenous miRNA pathways, LS2b does not (Lewsey *et al.*, 2007). In this study, we investigate whether the 2b protein facilitates drought tolerance through its known effects on miRNA and siRNA-mediated silencing or on other systems, including signalling mediated by abscisic acid (ABA), an important phytohormone controlling drought responses (Raghavendra *et al.*, 2010; Robertson *et al.*, 2009).

RESULTS

The 2b protein is responsible for CMV-induced drought tolerance

Following 8 days without water, Fny-CMV-infected *Arabidopsis* plants showed significantly higher water contents than mock-inoculated plants and exhibited no obvious wilting or other drought-related symptoms (Tukey's test, $P = 0.001$; Fig. 1A,B). Virus infection had no effect on the hydration of plants in well-watered conditions (Fig. 1B). Plants infected with the Fny-CMV 2b gene deletion mutant, Fny-CMV Δ 2b, were wilted and their water content did not differ significantly from that of mock-inoculated plants following drought (Tukey's test, $P = 0.65$; Fig. 1B). This indicated that the 2b protein may be responsible for conferring drought tolerance to Fny-CMV-infected plants. Therefore, we assessed drought tolerance in transgenic plants that constitutively expressed the Fny2b protein. Following 8 days without water, the water content of nontransgenic plants was reduced from 90% to approximately 20% (Fig. 1D). Two independent transgenic lines, 2.30F and

3.13F (Lewsey *et al.*, 2007), that constitutively express the Fny2b protein, lost around half of their water content (Fig. 1D), which was significantly less than the proportion of water lost by nontransgenic plants (Tukey's test; $P < 0.01$), and they were obviously less wilted in appearance than nontransgenic plants (Fig. 1C). Plants of line 2.30F expressed a lower level of the 2b protein than plants of line 3.13F and showed a milder symptom-mimicking phenotype (Fig. 1A; Lewsey *et al.*, 2007). However, there was no difference in the extent of drought tolerance between these lines.

Given these results and the known effectiveness of the Fny2b protein, both in the suppression of siRNA-directed antiviral RNA silencing and disruption of the miRNA-mediated regulation of endogenous gene expression, we decided to assess the drought tolerance of mutant plants impaired in the machinery of these different RNA silencing pathways. Mutant plants deficient in *DICER-LIKE1* (*dcl1*) are compromised in the production of miRNAs, but retain functional siRNA biogenesis machinery (Blevins *et al.*, 2006; Deleris *et al.*, 2006; Schauer *et al.*, 2002). We found that *dcl1-9* mutant plants appeared healthy and suffered no significant reduction in water content following 8 days of water withdrawal (Tukey's test, $P = 0.99$; Fig. 2A,B). Triple *dcl2/3/4* mutants, which are compromised in siRNA biogenesis but possess an intact miRNA pathway (Deleris *et al.*, 2006), also exhibited no significant loss of water following 8 days of drought (Tukey's test, $P = 0.41$; Fig. 2B).

In *Arabidopsis* ecotype Col-0, the Fny2b protein strongly inhibits miRNA pathways and siRNA-mediated antiviral silencing. However, in this background, the 2b protein from the LS strain of CMV only interferes with siRNA-mediated silencing (Lewsey *et al.*, 2007). To disentangle the effects of the 2b protein on miRNA and siRNA pathways, we assessed the drought tolerance of transgenic plants expressing the LS 2b protein. Consistent with the finding that *dcl2/3/4* plants were more tolerant of drought (Fig. 2B), LS2b-transgenic plants also lost less water following 8 days of drought stress (Fig. 2A), demonstrating again that interference with the siRNA pathway promoted drought tolerance.

The Fny2b protein disturbs miRNA-mediated RNA silencing partly through its interaction with AGO1, a silencing factor that participates in the miRNA-mediated regulation of host mRNAs, as well as in antiviral siRNA-mediated silencing (Baumberger and Baulcombe, 2005; González *et al.*, 2010; Zhang *et al.*, 2006). Plants of the mutant line *ago1-25* (Morel *et al.*, 2002) were significantly more tolerant of drought than wild-type plants (Tukey's test, $P < 0.05$; Fig. 2D) and, remarkably, when infected with Fny-CMV were completely resistant to water loss following 8 days of drought treatment (Fig. 2C,D) (Tukey's test, $P = 1.00$). Although we found that mock-inoculated *ago1-25* mutants were more tolerant than wild-type plants to drought stress, Fny-CMV infection of *ago1* mutants caused them to resist all water loss during the 8 days of the experiment. This may be because *ago1-25* plants possess a residual AGO1 activity (Morel *et al.*, 2002) which is further suppressed by the Fny2b protein when the plants are

Fig. 1 Comparison of the drought tolerance of mock-inoculated, Fny-CMV-infected and Fny-CMVΔ2b-infected (A,B) and nontransgenic and *Fny2b*-transgenic (C,D) plants. Eight days after the start of drought treatment, representative plants were photographed next to their watered counterparts. Analysis of variance (ANOVA) with *post-hoc* Tukey tests was used to assess the statistical significance of the data. Asterisks indicate the statistical significance of drought treatment within each plant group: ** $P < 0.01$; *** $P < 0.001$; ns, not significant. '+' indicates the statistical significance of drought treatment between groups compared with drought-treated mock-inoculated (B) or nontransgenic (D) plants: ++ $P < 0.01$; ns, not significant. Error bars represent standard error of the mean. Scale bars represent 2.5 cm. $n \geq 10$ for each treatment group. CMV, cucumber mosaic virus.

infected with CMV. However, it may also be the result of a cumulative effect of 2b inducing drought tolerance through an additional, AGO1-independent mechanism(s).

The effects of the 2b protein on ABA signalling are not dependent on the inhibition of AGO1

ABA is a critical component of the signalling network regulating plant responses to stress (Raghavendra *et al.*, 2010; Robertson *et al.*, 2009). Among the processes most sensitive to the action of ABA is the inhibition of germination, which provides a straightforward assay for alterations in ABA perception or signalling (Cutler *et al.*, 2010; Sirichandra *et al.*, 2009). We examined the effect of ABA treatment on the germination of 2b-transgenic and *ago1-25*

mutant plants. Germination of wild-type, mutant and *Fny2b*-transgenic plants was inhibited by the presence of ABA, particularly at concentrations of 0.3 μM and above (Fig. 3). At these concentrations, only 32% of *Fny2b*-transgenic seeds were able to germinate relative to 78%, 69% and 68% for wild-type, transgenic control and *LS2b*-transgenic plants, respectively (Fig. 3A). *ago1-25* mutants were also more sensitive to ABA-mediated inhibition of germination, consistent with earlier observations (Earley *et al.*, 2010) (Fig. 3B). Transgenic control plants expressing an untranslatable version of the *Fny2b* transcript (Lewsey *et al.*, 2007) did not differ significantly from nontransgenic plants in their sensitivity to ABA (Tukey's test, $P > 0.05$; Fig. 3A).

We also assessed the accumulation of the ABA-responsive transcripts *RD29A* and *NCED3* in 2b-transgenic and *ago1-25*

Fig. 2 Comparison of the drought tolerance of plants compromised in the silencing pathways mediated by microRNAs (miRNAs) (*dcl1-9*) or short-interfering RNAs (siRNAs) (*dcl2/3/4*) and *Fny2b*- (line 2.30F) and *LS2b*-transgenic (line 4.3B) plants (A,B) and plants of a line compromised in ARGONAUTE1 (AGO1) activity (*ago1-25*), a target of the 2b protein, with and without cucumber mosaic virus (CMV) infection (C,D). Eight days after the start of drought treatment, representative plants were photographed next to their watered counterparts. Analysis of variance (ANOVA) with *post-hoc* Tukey tests was used to assess the statistical significance of the data. Asterisks indicate the statistical significance of drought treatment within each plant line: ** $P < 0.01$; *** $P < 0.001$; ns, not significant. The statistical significance of drought treatment between groups compared with drought-treated wild-type (WT) plants is indicated by: + $P < 0.05$; ++ $P < 0.01$; ns, not significant. Error bars represent standard error of the mean. Scale bars represent 2.5 cm. $n \geq 10$ for each group.

mutant plants. In response to 10 μ M ABA, the expression of both the ABA- and desiccation-responsive gene, *RD29A*, and the ABA biosynthetic gene, *NCED3* (Xiong and Zhu, 2003), was induced by approximately 2.5-fold in wild-type plants and in *ago1-25* mutant plants (Fig. 4A). However, in *Fny2b*- and *LS2b*-transgenic plants, the induction of *RD29A* in response to exogenous ABA was attenuated (Fig. 4A). *RD29A* expression was also measured in *Fny*-CMV-infected plants that had been drought stressed for 8 days. There was a large induction (approximately 12-fold) of *RD29A* following drought in mock-inoculated plants relative to well-watered, mock-inoculated plants. In comparison, the induc-

tion of *RD29A* in drought-stressed *Fny*-CMV-infected plants was almost entirely suppressed (Fig. 4B).

To determine whether *Fny*-CMV infection or the presence of its 2b protein altered ABA biosynthesis, we measured basal ABA levels in nontransgenic and *Fny2b*-transgenic plants with and without *Fny*-CMV and *Fny*-CMV Δ 2b infection (Fig. 5). No statistically significant differences in ABA levels were observed between the treatment groups, indicating that neither *Fny*-CMV infection nor transgenic expression of its 2b protein altered ABA accumulation.

Taken together, the results indicate that CMV and its 2b protein do not affect ABA accumulation, but do affect ABA signalling and/or

Fig. 3 Germination of *Fny2b*-transgenic plants (line 3.13F) (A) and *argonaute1* (*ago1-25*) mutants (B) was hypersensitive to abscisic acid (ABA). *LS2b*-transgenic (line 4.3B) and control transgenic plants expressing an untranslatable version of the 2b protein did not differ from nontransgenic plants. Germination was scored after 5 days and expressed relative to the total number of germinating seeds for each line in the absence of ABA. Analysis of variance (ANOVA) with *post-hoc* Tukey tests was performed at each time point. Asterisks indicate the significance of *Fny2b*-transgenic (A) or *ago1-25* (B) germination rates compared with nontransgenic or wild-type plants, respectively: * $P < 0.05$; *** $P < 0.001$. Error bars represent the standard error of the mean. Data points are the mean of at least three independent experiments.

perception. However, the results of the gene expression analyses and seed germination assays show that the aspects of ABA signalling affected by CMV are distinct from those regulated by AGO1.

Enhanced drought tolerance in 2b-transgenic plants is not caused by altered stomatal density or conductance

We found no significant differences in stomatal density between leaves from nontransgenic and 2b-transgenic plants (Student's *t*-test, $P > 0.05$; Fig. 6A). In addition, we measured water loss from rosettes excised from well-watered wild-type, *Fny2b*- and *LS2b*-transgenic, *ago1-25* mutant, *dcl2/3/4* mutant and control transgenic plants. Rosette mass was measured over 4 h following the separation of rosettes from roots. Paradoxically, at every time point over this period, *Fny2b*-transgenic rosettes lost significantly more water than rosettes of other plant types tested (Fig. 6B). Water loss in all rosettes was greatest in the first 30 min following excision (Fig. 6B; Table 1), but rosettes of plants expressing the *Fny2b* protein dried out at a significantly faster rate (Tukey's test, $P < 0.001$; Table 1). Between the 1- and 4-h post-excision time points, the rate of water loss slowed in all plants, although 2b-transgenic plants continued to lose water faster than the other plant lines (Table 1, $P < 0.001$). The rate of water loss was also greater for rosettes excised from *Fny*-CMV-infected plants (Fig. 6C). These results suggest that stomata on leaves of 2b-transgenic and CMV-infected plants have an increased conductance. Indeed, measurements of stomatal conductance using infrared gas analysis (IRGA) showed that constitutive transgenic expression of the *Fny2b* protein caused a three-fold increase in stomatal conductance (Student's *t*-test, $P < 0.05$; Fig. 6D).

Our results with rosettes excised from CMV-infected and 2b-transgenic plants, and the analyses of stomatal density and conductance, showed that 2b-mediated drought tolerance could not be explained by physiological or developmental changes in the leaves (Fig. 6). Therefore, it seems more likely that changes in the roots can explain the increased drought tolerance seen in whole plants expressing the *Fny* or *LS 2b* proteins in plants infected with the virus (Figs 1 and 2). It is known that CMV infection and transgenic expression of 2b proteins derived from both *Fny* and *LS* strains of CMV can increase the growth of lateral roots (Lewsey *et al.*, 2007), and we wondered whether this was the underlying cause of 2b-induced drought tolerance. As expected from the previous study, lateral root numbers and the length of lateral roots were increased on transgenic plants expressing the *Fny2b* protein (Fig. 7). Interestingly, both the number and length of lateral roots were decreased markedly (by approximately 75%) on *ago1-25* mutant plants and, for mutants compromised in siRNA-mediated silencing (*dcl3/3/4* triple mutants), there was a trend towards decreased lateral root number and length (Fig. 7). We found that, in *ago1-25* mutants, lateral root growth was reduced by up to 75%

Fig. 4 The 2b protein disrupts abscisic acid (ABA)- and drought-responsive gene expression. (A) Analysis of *RD29A* and *NCED3* transcript abundance by quantitative polymerase chain reaction (Q-PCR) in *Fny2b*- (line 3.13F) and *LS2b*-transgenic (line 4.31B) seedlings and *ago1-25* mutant seedlings exposed to 1 and 10 μ M ABA for 3 h. (B) Q-PCR analysis of *RD29A* expression in *Fny*-CMV-infected plants exposed to drought for 8 days. Error bars represent the standard error of the mean. CMV, cucumber mosaic virus; WT, wild-type.

relative to wild-type plants (Fig. 7), whereas *dcl2/3/4* plants exhibited a lateral root density intermediate between *ago1-25* and the wild-type. Although transgenic plants expressing *LS2b* can exhibit increased lateral root length, the *LS2b*-transgenic line used in this study (line 4.3B; Lewsey *et al.*, 2007) is a low expressor line that does not show a statistically significant increase in this parameter (Fig. 7). Thus, there is no clear correlation between alterations in root development and increased whole plant drought tolerance in either *2b*-transgenic plants or mutant plants compromised in either AGO1 function or siRNA-mediated silencing.

DISCUSSION

The CMV 2b protein confers drought tolerance and interferes with ABA-mediated gene expression in Arabidopsis

In line with work using other hosts (Xu *et al.*, 2008), CMV infection induced tolerance to drought in Arabidopsis. This was attributable to disruption by the 2b protein of gene expression mediated by ABA, a phytohormone with a central role in the regulation of responses to drought (Raghavendra *et al.*, 2010; Robertson *et al.*, 2009). Thus, in addition to its effects on SA- and JA-mediated

signalling, which condition many aspects of resistance to biotic attack (Ji and Ding, 2001; Lewsey *et al.*, 2010), the 2b protein also interferes with ABA-mediated signalling, a key component of resistance to abiotic stress. Intriguingly, ABA is also increasingly being recognized as a factor determining the outcome of plant-microbe interactions (De Torres Zabala *et al.*, 2009).

AGO1 regulates an miRNA- and ABA-dependent drought tolerance mechanism described by Li *et al.* (2012). By exploiting strain-specific differences between CMV 2b proteins, and using *ago1*, *dcl1* and *dd2/3/4* mutant plants, we uncovered the existence of another drought tolerance mechanism that is siRNA dependent but miRNA independent. However, although CMV and its 2b protein affect ABA-mediated gene expression, the drought tolerance they engender is unlikely to result from the perturbation of either of the two silencing-mediated drought tolerance systems. All three of these drought tolerance mechanisms appear to operate in the roots.

Comparing the effects of silencing mutants and CMV and its VSR on drought tolerance and responses to ABA

Small RNAs have been implicated in the regulation of responses to abiotic stresses (Khraiwesh *et al.*, 2012; Sunkar *et al.*, 2007).

Fig. 5 Cucumber mosaic virus (CMV) and its 2b protein do not alter the accumulation of abscisic acid (ABA). Basal ABA levels, measured by reverse-phase liquid chromatography coupled ionization/tandem mass spectrometry, were not altered significantly [analysis of variance (ANOVA), $P = 0.101$] in nontransgenic or *Fny2b*-transgenic plants at 14 days post-inoculation with either Fny-CMV or Fny-CMV Δ 2b. DW, dry weight.

Plants belonging to *ago1* mutant lines exhibit enhanced tolerance to drought, hypersensitivity to ABA-mediated germination inhibition and alterations in ABA-regulated gene expression (Earley *et al.*, 2010; Li *et al.*, 2012). The close relationship of ABA signalling with AGO1 was demonstrated by Li *et al.* (2012), who showed that the up-regulation of *MIR168a*, a negative regulator of AGO1 that contains ABA-responsive elements within its promoter, conferred drought tolerance. Our observation that plants of the mutant lines *dcl1-9* and *dcl2/3/4* were tolerant of water limitation not only confirms a role in drought tolerance for miRNAs, which are generated by DCL1, but also suggests one for siRNAs, which are generated by DCLs 2, 3 and 4 (Deleris *et al.*, 2006). AGO1 utilizes siRNAs and miRNAs to direct its silencing, meaning that it can regulate drought tolerance mechanisms mediated by either small RNA class. Importantly, miRNAs are critical to the production of mature *trans*-acting siRNAs (ta-siRNAs), so that mutants deficient in miRNA processing will also be deficient in ta-siRNA-mediated regulation of gene expression. The miRNAs *MIR159*, *MIR168* and *MIR169* have been implicated as direct positive regulators of ABA signalling (Li *et al.*, 2008, 2012; Reyes and Chua, 2007). As well as being regulated by miRNAs, AGO1 is also negatively regulated by DCL2- and DCL4-derived siRNAs in an AGO1-dependent manner (Mallory and Vaucheret, 2009; Martínez de Alba *et al.*, 2011), which suggests that lesions in the siRNA pathway could feed through to affect AGO1-regulated ABA and drought responses. However, no specific siRNAs have been

reported to regulate ABA signalling or drought responses in Arabidopsis. One study found that the *dcl2/3/4* triple mutant was hypersensitive to the effects of ABA on seed germination (Zhang *et al.*, 2008), but we did not see ABA hypersensitivity in drought-tolerant *LS2b*-transgenic plants, an indication that 2b-induced drought tolerance may not be mediated through effects on the siRNA pathway.

There were other lines of evidence suggesting that drought tolerance induced by CMV and its 2b protein may not be entirely explained by direct effects on silencing pathways. AGO1 is a binding target of the Fny2b protein (Duan *et al.*, 2012; González *et al.*, 2010; Zhang *et al.*, 2006), but this protein also binds small RNAs (González *et al.*, 2010, 2012). In contrast, *LS2b* does not bind AGO1 and presumably can only disrupt siRNA pathways by binding small RNAs (González *et al.*, 2012; Lewsey *et al.*, 2007; Zhang *et al.*, 2006), yet plants expressing *LS2b* were also drought tolerant. Furthermore, although *ago1-25* mutant seeds were hypersensitive to ABA-mediated inhibition of germination, *Fny2b*-transgenic seeds were less sensitive to ABA, and *LS2b*-transgenic seed germination was no more sensitive to exogenous ABA than the germination of control seed. It was also found that the expression of the ABA-responsive transcripts *RD29A* and *NCED3* was attenuated in *Fny2b*- and *LS2b*-transgenic seedlings following exogenous ABA application, whereas *ago1-25* exhibited no difference from the wild-type. These results do not entirely rule out the idea that 2b-mediated effects on siRNA- or miRNA-mediated drought responses could help to engender drought tolerance in CMV-infected plants. However, they indicate that interference with AGO1 is not a vital factor in virus-induced drought tolerance and suggest that this drought tolerance does not depend on the VSR activity of 2b. The results indicate that interference with ABA signalling can occur at several different points in the pathway, resulting in increased drought tolerance.

Although cucumoviral 2b proteins interact with host silencing components, such as AGO proteins 1 and 4, as well as with miRNAs and siRNAs (Duan *et al.*, 2012; González *et al.*, 2010, 2012; Goto *et al.*, 2007), they can interact with other cellular factors that are not likely to be silencing components (Ham *et al.*, 1999; Inaba *et al.*, 2011; Nakahara *et al.*, 2012). Transgenic plants expressing a VSR derived from a geminivirus were tolerant of drought, although this was thought to be caused by disruption of the cellular protein degradation machinery, rather than through effects on silencing (Lozano-Duran *et al.*, 2011). Perhaps it is through a similar interaction that the 2b protein affects ABA signalling and induces drought tolerance.

Alterations in root function are involved in drought tolerance

Aerial tissues separated from roots of *ago1-25* or *dcl2/3/4* mutant plants, or of *LS2b*-transgenic plants, dried out at rates

Fig. 6 Well-watered transgenic plants expressing the *Fny2b* viral suppressor of RNA silencing (VSR) lost more water and had a higher stomatal conductance despite having an unaltered number of stomata. (A) The number of stomata was not altered by transgenic expression of the *Fny2b* protein. Epidermal peels taken from nontransgenic and *Fny2b*-transgenic plants were examined under a light microscope. Images were taken at 20 $\times$ magnification and the numbers of stomata were counted ($n = 10$ fields). Scale bars represent 20 μm . (B,C) Rosettes were cut from their roots and weighed at various intervals over a period of 4 h. Loss of mass was equated to water loss and calculated relative to the mass of the leaf at 0 h. At every time point, *Fny2b*-transgenic and cucumber mosaic virus (CMV)-infected plants had lost significantly more water than every other plant line tested (Tukey's tests, $P < 0.05$). Asterisks indicate statistical significance of *Fny2b*-transgenic plant water content compared with that of wild-type (WT) plants (Tukey's tests): *** $P < 0.001$ ($n \geq 10$ plants for each data point). (D) Infrared gas analysis (IRGA) measurements showed that *Fny2b*-transgenic plants had a significantly higher stomatal conductance than nontransgenic plants (***Student's t -test, $P < 0.001$; $n \geq 10$ per plant line). Error bars represent standard error of the mean.

similar to those excised from control plants. Strikingly, aerial parts of *Fny2b*-transgenic plants exhibited markedly greater rates of water loss, which could be explained by the greater conductivity of the stomata on their leaves. Thus, improved resistance of the leaves to water loss cannot explain the enhanced resilience to drought stress exhibited by intact plants belonging to either silencing mutant or *2b*-transgenic lines, or by CMV-infected plants. Our results contrast with observations of *B. vulgaris*, where leaves removed from CMV-infected plants showed greater tolerance to drying (Xu *et al.*, 2008).

The elaboration of lateral roots is highly responsive to the environment and the increased root surface they provide might enhance the water uptake efficiency of plants or their resilience

in the face of other abiotic stresses (Casimiro *et al.*, 2003; Malamy and Ryan, 2001; Nibau *et al.*, 2008). Small RNAs regulate lateral root initiation. For example, *miR390* and *TAS3* regulate lateral root growth (Marin *et al.*, 2010) in a manner dependent on auxin, a critical regulator of lateral root formation (Eckardt, 2005). Perhaps not surprisingly, the *dcl1-9* mutation and transgenic expression of the potyviral VSR, P1/HC-Pro, increased lateral root growth (Guo *et al.*, 2005), and Lewsey *et al.* (2007) found that CMV infection or expression of either *Fny2b* or *LS2b* in transgenic Arabidopsis plants could enhance lateral root growth.

However, the effects of *2b* protein expression on lateral root development were dependent on the expression level of the

	Water loss rate (%/h): $t = 0$ h to $t = 0.5$ h		Water loss rate (%/h): $t = 1$ h to $t = 4$ h	
	Mean	<i>P</i> value	Mean	<i>P</i> value
Wild-type	-17.38 ± 0.71	1	-4.63 ± 0.15	1
<i>Fny2b</i> -transgenic	-26.91 ± 2.12	<0.001***	-7.86 ± 0.43	<0.001***
<i>LS2b</i> -transgenic	-17.79 ± 1.04	1	-5.55 ± 0.24	0.55
<i>ago1-25</i>	-17.79 ± 0.51	1	-5.09 ± 0.12	0.97
<i>dcl2/3/4</i>	-17.64 ± 0.66	1	-4.96 ± 0.59	0.97
Transgenic control	-20.27 ± 1.51	0.73	-5.51 ± 0.40	0.55
Mock-inoculated	-5.73	1	-3.68	1
<i>Fny</i> -CMV-infected	-9.96	0.009**	-5.24	0.003**

Water loss was calculated from the reduction in weight over a 4-h period following the removal of rosettes from soil, and rates were calculated from the slopes of the graphs in Fig. 4. For all plants, water loss was greatest during the first 30 min and was lower in the subsequent 3 h. In both periods, *Fny2b*-transgenic and *Fny*-CMV-infected plants lost water more rapidly than all other lines. Mean water loss rates are given $\pm$ standard error of the mean. The significance (*P*) values reported result from *post-hoc* Tukey comparisons between plants of each line and their relevant control (wild-type or mock-inoculated). CMV, cucumber mosaic virus.

***P* < 0.01.

****P* < 0.001.

transgene (Lewsey *et al.*, 2007). Indeed, the *LS2b*-transgenic line used in this study did not display any statistically significant effects on lateral root phenotype, yet plants of this line possessed significantly enhanced tolerance to drought. Furthermore, for the silencing mutants, in particular for the *ago1-25* plants, we found that mutation actually decreased lateral root growth, even though drought tolerance was increased. Therefore, we believe that it is unlikely that changes in root architecture can explain the greater drought tolerance seen in CMV-infected *Arabidopsis* and in *2b*-transgenic plants, or in *ago1-25* or *dcl2/3/4* mutant plants. We conclude that the root physiology of these plants has been altered so that they take up or retain water more efficiently, or that roots produce signals (Nibau *et al.*, 2008) that may control water loss from other parts of the plant.

Providing its hosts with a conditional advantage may help CMV to ensure its own long-term survival

Arabidopsis is a natural host for CMV (Pagán *et al.*, 2010). We speculate that, under natural conditions, the ability of CMV to induce drought tolerance may enhance the survival of its host and therefore of the virus itself during periods of environmental stress. Indeed, under drought conditions, plants resistant to CMV may be at a disadvantage compared with infected susceptible hosts that will benefit from increased drought tolerance. If viruses provide a conditional benefit to their hosts, stabilizing selection can apparently lead to antagonistic organisms becoming 'co-evolved for co-existence' (Alexander, 1981; Anderson and May, 1985). In the case of CMV, engendering drought tolerance could result in the long-term stability of the relationship between the plant and virus, and adds weight to the hypothesis that, under certain conditions, even pathogenic viruses can be mutualistic partners with their hosts (Roossinck, 2011).

Table 1 Rate of water loss from *Arabidopsis* rosettes.

EXPERIMENTAL PROCEDURES

Plants and viruses

The *2b*-transgenic lines [background: *Arabidopsis thaliana* (L.) Heynh. ecotype Col-0] used in this study, 2.30F and 3.13F, which constitutively express a *2b* transgene derived from *Fny*-CMV, and 4.31B, transformed with a construct containing the *2b* protein derived from *LS*-CMV, under the control of the cauliflower mosaic virus 35S promoter, have been described in Lewsey *et al.* (2007) and Lewsey (2006). A line constitutively expressing an untranslatable *2b* transcript (UNT) was used as a transgenic control (Lewsey *et al.*, 2007). Seeds for *ago1-25* (Morel *et al.*, 2002), *dcl1-9* (Jacobsen *et al.*, 1999) and *dcl2-5/3-1/4-2* were from pools previously authenticated for mutant alleles (Harvey *et al.*, 2011; Lewsey and Carr, 2009).

Virions of CMV (strain *Fny*: Roossinck and Palukaitis, 1990) and a *2b* gene deletion mutant of *Fny*-CMV, CMVΔ2b (Ryabov *et al.* 2001), were propagated in *Nicotiana glauca* and purified by the method of Ng and Perry (1999). Viruses were rub inoculated onto carborundum-dusted leaves of *Arabidopsis* plants at the four to six true-leaf stage with a suspension of purified virions at a concentration of 100 µg/mL in sterile water. Mock inoculation used water only. Successful infection was confirmed by symptom observation for wild-type CMV or by double-sandwich enzyme-linked immunosorbent assay (ELISA) for *Fny*-CMVΔ2b coat protein accumulation (Ziebell and Carr, 2009).

Drought and water loss experiments

Water content and water loss analyses were performed according to the methods of Xu *et al.* (2008). At least 10 4-week-old plants were drenched in water for 30 min to achieve 100% soil saturation. For test plants, this was the last water they received during the period of the study. An equal number of plants were kept well watered over the same period as a control. The position of each plant was randomized and plants were observed daily for up to 8 days without water. At this point, whole aerial tissue of both drought-stressed and well-watered plants was harvested,

Fig. 7 Lateral root growth is increased in *Fny2b*-transgenics and decreased in *ago1-25* mutants. Root growth was measured when seedlings were 12 days old, and results are expressed as the number of lateral roots per centimetre of main root (A) and mean lateral root length (B). Error bars represent standard error of the mean. Different letters are assigned to significantly different results. Tukey's tests, $P < 0.0001$. WT, wild-type.

and fresh weights were recorded. Samples were then dried over a period of 5 days at 50 °C. Dry weight was recorded and the weight loss for each plant, which is equal to the water weight, was calculated. The percentage water content of each rosette was calculated by dividing the fresh weight by the water weight for each sample. Each experiment was repeated at least three times with the same results.

For short-term water loss experiments, at least 12 plants per line were drenched in water as above, and rosettes were excised from the soil and placed in a weighing boat. The fresh weight of rosettes was recorded immediately following excision and weights were recorded at 30 min and at 1-, 2- and 4-h time points. Rosette weight at each time point was expressed as a percentage of the weight at the start of the experiment for

each line. To determine the rate of drying for each phase of water loss, the slope of each line between the start of the experiment and 30 min, as well as between 1 h and 4 h, was calculated for each line. The experiment was repeated three times with the same results.

Germination assays

Age-matched seeds were surface sterilized by washing with 70% ethanol for 2 min, followed by a solution containing 0.1% sodium dodecylsulphate (SDS) and 2.5% sodium hypochlorite for 15 min, and then rinsed three times with sterile, deionized water. Fifty seeds of each line were plated onto 9-cm-diameter Petri dishes containing $0.5 \times$ Murashige and Skoog (MS) medium (Melford Ltd., Ipswich, Suffolk, UK) at pH 5.7, supplemented with ABA dissolved in 0.05% ethanol at the appropriate concentration, or with an equivalent amount of only 0.05% ethanol, which acted as a control. Plates were stratified at 4 °C for 48 h before being transferred to a growth room with a 16-h : 8-h light : dark cycle at 21 °C with a light intensity of $200 \mu\text{E}/\text{m}^2/\text{s}$. The presence of cotyledons was scored at the fifth day after stratification (stage C as described by Boyes *et al.*, 2001) and the percentage germination was expressed for each plant line as a proportion of the total number of germinating seeds in the absence of ABA.

Stomatal counts and stomatal conductance

Epidermal peels were made from fully expanded 4-week-old nontransgenic and *2b*-transgenic plants using clear nail varnish. Three plants of each line were examined and at least 10 peels per plant were analysed using a Leica DFC425 C camera mounted on a Leica HC microscope (Leica Microsystems, Milton Keynes, Buckinghamshire, UK) at $20\times$ magnification to determine the number of stomata per field.

Leaf gas exchange of nontransgenic and *2b*-transgenic plants was measured by IRGA using a portable photosynthesis measurement system (LI-6400, LI-COR, Inc., Lincoln, NE, USA) equipped with a 6-cm^2 chamber maintained at 22 °C with an artificial light source providing a uniform quantum flux of $500 \mu\text{E}/\text{m}^2/\text{s}$. The molar CO_2 air flow rate inside the chamber was fixed at $125 \mu\text{mol}/\text{mol}/\text{s}$. All measurements were taken at a reference CO_2 concentration of $400 \mu\text{mol}$. Attached leaves of nontransgenic or *2b*-transgenic plants were placed in the chamber and repeat measurements of conductance from five plants per line were taken.

ABA content measurement

ABA, extracted from individual plants, was analysed by reverse-phase liquid chromatography-electrospray ionization/tandem mass spectroscopy using an Agilent high-performance liquid chromatograph (HPLC) (Agilent Technologies, Wokingham, Berkshire, UK) coupled to an Applied Biosystems Q-TRAP 2000 (Applied Biosystems, Foster City, CA, USA) by the method of Forcat *et al.* (2008). Samples were the entire aerial tissue from individual plants replicated five to seven times. The experiment was repeated twice with similar results. Extracts were spiked with 1 ng deuterated ABA as an internal standard to calculate the efficiency of recovery. Tissue destined for ABA analysis was also examined by double-sandwich ELISA for CMV coat protein accumulation (Bioreba AG, Reinach, Switzerland) to confirm CMV or CMV $\Delta 2b$ systemic infection.

Analysis of gene expression

Seedlings were grown for 12 days on $0.5 \times$ MS medium; 10 seedlings per treatment were removed from the medium and placed on filter paper soaked with the appropriate concentration of ABA. After 3 h, seedlings were flash frozen in liquid nitrogen. For the analysis of the response to water loss, drought experiments were performed as described above. Aerial tissue from three plants per treatment was pooled and flash frozen in liquid nitrogen. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, followed by precipitation with lithium chloride and an additional phenol–chloroform extraction and ethanol precipitation (Sambrook *et al.*, 1989). Total RNA preparations were then treated with DNase using the TURBO DNA-free kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Three independent biological replicates were performed. First-strand synthesis was performed using SuperScript III (Invitrogen) and random hexamer primers (Promega, Madison, WI, USA), according to the manufacturers' instructions. Real-time PCR was then conducted using SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich, St Louis, MO, USA), according to the manufacturer's instructions. A reference gene, *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), was identified as stable under the conditions tested. Primers were designed against the transcripts of interest (Table S1, see Supporting Information), and were verified as amplifying a single, specific product by agarose gel electrophoresis and sequencing products, as well as by melting curve analysis. Reactions were conducted in triplicate. Data were analysed using LinReg-PCR software to calculate the threshold cycle number and amplicon amplification efficiency (Ramakers *et al.*, 2003; Ruijter *et al.*, 2009). Fold changes in transcript abundance were calculated using $\Delta\Delta C_t$ methodology, adjusted for amplicon amplification efficiency, taking into account the reference transcript to control for variations in loading (Yuan *et al.*, 2008). Fold changes were expressed relative to transcript abundance in untreated Arabidopsis plants.

Lateral root measurements

Seedling were grown on vertical plates containing 1% Phytoagar and $0.5 \times$ MS medium for 12 days in 16-h days at 22 °C. The primary root and lateral roots were measured using ImageJ, and the lateral root density was expressed for each seedling as the total number of lateral roots per centimetre of main root (Lewsey *et al.*, 2007).

Statistics

All datasets were tested for statistical significance using R (R Development Core Team, 2011). One-way analysis of variance (ANOVA) tests were performed at the $P = 0.05$ significance level with *post-hoc* pairwise comparison of means using Tukey's test. Student's *t*-tests were used, when appropriate, to make simple pairwise comparisons, with *P* values corrected for multiple comparisons using the Bonferroni method when appropriate.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1 Primers used in quantitative reverse transcription polymerase chain reaction (Q-RT-PCR).