Running title:

Trade-offs in virus-infected plants to drought

Virulence determines beneficial trade-offs in the response of virus-infected plants to drought via induction of salicylic acid

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Abstract

It has been hypothesized that plants can get beneficial trade-offs from viral infections when grown under drought conditions. However, experimental support for a positive correlation between virus-induced drought tolerance and increased host fitness is scarce. We investigated whether increased virulence exhibited by the synergistic interaction involving *Potato virus X* (PVX) and *Plum pox virus* (PPV) improves tolerance to drought and host fitness in *Nicotiana benthamiana* and *Arabidopsis thaliana*. Infection by the pair PPV/PVX and by PPV expressing the virulence protein P25 of PVX conferred an enhanced drought-tolerant phenotype compared to single infections with either PPV or PVX. Decreased transpiration rates in virus-infected plants were correlated with drought tolerance in *N. benthamiana* but not in Arabidopsis. Metabolite and hormonal profiles of Arabidopsis plants infected with the different viruses showed a range of changes that positively correlated with a greater impact on drought tolerance. Virus infection enhanced drought tolerance in both species by increasing salicylic acid accumulation in an abscisic acid-independent manner. Viable offspring derived from Arabidopsis plants infected with PPV increased relative to non-infected plants, when exposed to drought. By contrast, the detrimental effect caused by the more virulent viruses overcame potential benefits associated with increased drought tolerance on host fitness.

Summary statement

It has been hypothesized that tolerance of virus-infected plants to abiotic stresses is a conditional phenotype that could act as a pay-off that offsets the detrimental effect of virus infection on plant fitness. However, this might not be the case if fitness costs associated with virulence outweigh the beneficial effects conferred by stress tolerance on plant growth. We experimentally tested the hypothesis that virus infection would increase the reproductive fitness of Arabidopsis thaliana and Nicotiana benthamiana under variable drought conditions. Our results challenge the general validity of the hypothesis that viruses behave as conditionally beneficial to their hosts, and emphasize the need of considering the effect of virulence in the analysis of plant responses to combined abiotic and biotic stress.

Keywords: virulence; virus-induced drought tolerance; salicylic acid; *Potato virus X; Plum pox virus*; host fitness

Introduction

Under field conditions, plants are concurrently exposed to a number of abiotic and biotic stresses. Combined abiotic and biotic stresses result in plant responses which are different and sometimes contrasting to those seen under individual stresses (Ramegowda & Senthil-Kumar 2015). Occurrence of abiotic stresses such as drought, heat, cold, salinity, and nutrient stress dramatically alters the response of the plants to biotic stresses. Similarly, interactions of plants with pathogens affect their responses to abiotic stresses (Prasch & Sonnewald 2015). The outcomes of these interactions can either provide resistance or susceptibility toward any of the two stresses depending on the plant species, pathogen and stress intensity. The combination of drought and pathogen infection is of particular interest in the context of changes in environmental conditions associated with global warming. Current climate prediction models indicate a gradual increase in CO_2 levels and an enhancement of the frequency and amplitude of heat and drought episodes (IPCC 2007). Moreover, changing environmental conditions may affect the incidence and severity of plant diseases and influence the further co-evolution of plants and their pathogens (Eastburn *et al.* 2011; Aguilar *et al.* 2015a).

Plants have developed a range of strategies to reduce the negative effects of drought on their physiology (Verslues *et al.* 2006; Fang & Xiong 2015). At an early stage of water deficit, water content is maintained within relatively narrow limits by increasing water uptake through a well-developed root system, and by limiting water loss from transpiration by partially closing stomata. As stress conditions increase, some osmoprotectants such as proline, spermine, betaine, and soluble sugars accumulate in plant cells to maintain the cell turgor pressure. Traditionally, abscisic acid (ABA) has been considered as the primary regulator of drought stress response in plants (Fujita *et al.* 2011). By contrast, salicylic acid (SA) and jasmonic acid (JA) have been considered signals of biotic stress responses because they fulfill essential roles in plant defense. In particular, SA signaling is critical for defense responses against a wide range of pathogens, including viruses (Carr *et al.* 2010). However, recent findings on the molecular mechanisms underlying hormonal regulation in response to drought have uncovered a

complex and dynamic regulatory network in which JA, SA and ABA participate (Harb *et al.* 2010; Miura *et al.* 2013; Okuma *et al.* 2014).

Viruses are obligate intracellular parasites, relying on their hosts to provide the basic machinery to allow them to replicate, spread and survive. This extreme dependence from host resources may cause physiological, metabolic and developmental disturbances in their hosts leading to plant diseases (Pallas & García 2011). Some of these alterations do not necessarily provide an advantage to the virus but nevertheless may have adverse effects on host fitness. Because virulence, defined as the deleterious effects of parasites on their hosts, does not represent any clear advantage for obligate parasites, it is not obvious why viruses damage their hosts (Pagán *et al.* 2007). However, there may be environments in which it would be more advantageous for plants to be infected by viruses (Roossinck 2011). For instance, it has been shown that virus infection can enhance the resilience of host plants to cold stress (Xu *et al.* 2008; Fernández-Calvino *et al.* 2014). In addition, virus-infected plants can exhibit enhanced tolerance to drought as a result of pathogen-induced acclimation. Xu *et al*. (Xu *et al.* 2008) showed that several susceptible hosts infected with *Brome mosaic virus* (BMV), *Cucumber mosaic virus* (CMV), and *Tobacco mosaic virus* (TMV) had both a delayed appearance of leaf wilting and stem dehydration when exposed to drought compared to uninfected plants. In addition, infection by CMV induced an increase in the tolerance to drought of *Arabidopsis thaliana*, which was attributable to the virulence protein 2b of CMV (Westwood *et al.* 2013). Metabolic profiling analysis in BMV-infected rice (*Oryza sativa*) plants and CMV-infected beet (*Beta vulgaris*) plants revealed that the levels of several plant osmoprotectants were higher in virus-infected plants than in non-infected plants (Xu *et al.* 2008). Although some viral infections increase ABA concentration in plants (Alazem & Lin 2015), it is unclear if this is a general response to viral infection and whether this plays a role in virus-induced drought tolerance. Besides metabolic acclimation, pathogen infection can also bring physiological adaptations in plants resulting in enhanced tolerance to drought. For example, infection of *A. thaliana* by *Pseudomonas syringae* caused stomatal closure, which resulted in reduced water loss from the infected plant (Beattie 2011). Thus, in

the context of host-virus co-evolution, it has been hypothesized that tolerance of infected plants to abiotic stresses is a conditional phenotype that could act as a pay-off that offsets the detrimental effect of virus infection on plant fitness (Roossinck 2011). However, experimental data available so far assume that increased survival of infected plants under stress conditions conveys an increase in fecundity and, hence, in fitness, compared to non-infected plants. However, this scenario might not be the case if fitness costs associated with virulence outweigh the beneficial effect conferred by stress tolerance on plant growth (Xu *et al.* 2008; Westwood *et al.* 2013).

The synergistic interaction involving *Potato virus X* (PVX) and members of the *Potyvirus* genus constitutes an excellent system for investigating the molecular and physiological mechanisms that underlie virus-induced drought tolerance and its dependence on virulence (Vance *et al.* 1995). Compared to single infections, co-infection of *Nicotiana benthamiana* plants with PVX and *Plum pox virus* (PPV) resulted in increased virulence that led to necrotic symptoms in the newly emerging leaves. Furthermore, the expression of the P25 protein of PVX by a PPV vector was sufficient to induce an increase of PPV virulence that resembled that of the PPV/PVX synergistic interaction (Aguilar *et al.* 2015b a).

In this study, we investigated whether increased levels of virulence facilitates tolerance to drought induced by virus infection in two experimental hosts, *N. benthamiana* and *A. thaliana*. Estimates of virus effects on vegetative growth, transpiration rates, metabolite profiles, and water content in drought-stressed plants supported a positive association between virulence and tolerance to drought. However, studies on reproductive fitness showed that a higher tolerance to drought in virus-infected plants was not always accompanied by an increase in host fitness. Nevertheless, infections showing moderate virulence were able to increase fitness of *A. thaliana* plants grown under drought conditions. In addition, the role of hormonal regulation in the enhanced tolerance to drought stress in virus-infected plants was analyzed using mutant plants deficient in ABA, JA or SA signaling.

Materials and Methods

Plant materials

The following *A. thaliana* transgenic and mutant lines used in this study were derived from ecotype Columbia (Col-0): *aba2-1* (Rook *et al.* 2001), NahG (Lawton *et al.* 1995), *sid2-2* (Wildermuth *et al.* 2001), and *coi1-1* (Feys *et al.* 1994). The *coi1-1* mutant plants were selected from a heterozygote population using root sensitivity to 50 μM methyl jasmonate. The transgenic *N. benthamiana* plants expressing the salicylate hydroxylase gene have been described previously (Ying *et al.* 2010).

Binary vector constructs

The binary vector pGR107, which contains the infectious cDNA of PVX, was provided by D. C. Baulcombe (University of Cambridge). The infectious cDNA clone of PVX carrying PPV HCPro (pPVX-HC) sequences was described before (Aguilar *et al.* 2015b). pGWBinPPV-3xHA is a derivative of pBinPPV which contains the infectious cDNA of PPV (Alamillo *et al.* 2006). PPV expressing either PVX P25 (PPV-P25) or GFP (PPV-GFP) sequences were described before (Aguilar *et al.* 2015b). *A. tumefaciens* carrying pCAMBIA1305.1 containing a gene encoding GUS was used as negative control.

Agro-inoculation

N. benthamiana plants were grown in 10 cm diameter pots containing a mixture of soil and vermiculite (3:1 [v/v]). Four-week-old plants were agro-infiltrated with *A. tumefaciens* bearing the indicated binary vectors (Tenllado & Díaz-Ruíz 2001). Plants were grown at 25 °C using a 16 h light photoperiod.

A. thaliana plants were agroinoculated *in vitro* with the indicated binary vectors as described (Pasin *et al.* 2014). At different intervals after inoculation, plants were transferred to 5 X 5 cm individual pots

containing a mixture of soil and vermiculite (3:1 [v/v]), and covered with a transparent plastic bag to prevent desiccation. Plants were grown at 21 °C using a 16 h light photoperiod.

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RNA and protein gel blot analysis

Total RNA was extracted from upper, non-inoculated leaves as described previously (Pacheco *et al.* 2012). Northern blot hybridization was carried out overnight at 65°C using digoxigenin-labeled riboprobes corresponding to PVX CP sequences (González-Jara *et al.* 2005). Detection of *ARGININE DECARBOXYLASE1* (*ADC1*; At2g16500) mRNA was performed using a ^{32}P-labeled probe corresponding to a 199–bp fragment from *ADC1* (Fernández-Calvino *et al.* 2014).

qRT-PCR for virus detection was performed using primers that amplify a region from nucleotides 4668 to 4810 of the PPV sequence (Table S1). qRT-PCR for the analysis of *PR1* and *RD29B* gene expression was performed with gene-specific primers. The relative quantification of PCR products was calculated by the comparative cycle threshold (ΔΔCt) method as described (García-Marcos *et al.* 2013). Amplification of *β-TUBULIN5* (TUB5; At1g20010) and 18S rRNA were chosen for normalization in Arabidopsis and *N. benthamiana*, respectively.

Viral proteins were analyzed by western blot using virus-specific antisera as described (Tena Fernández *et al.* 2013). Hemagglutinin (HA)-tagged P25 or GFP were detected with a rat monoclonal antiserum to HA. Blotted proteins were detected using commercial secondary antibodies. Bound antibodies were visualized using either the ECL system (Amersham Biosciences) or with SigmaFast™ BCIP/NBT substrate tablets (Sigma-Aldrich).

Multicolor fluorescence imaging

Plants were excited under UV light (355 nm) and fluorescence images were acquired using an Open FluorCamFC 800-O (Photon Systems Instruments). Fluorescence images were analysed with

Fluorcam7 software. Measurements and image analysis were carried out on attached leaves according to Pérez-Bueno *et al.* (2015).

Chlorophyll fluorescence analysis

The maximum efficiency of photosystem II (F_V/F_M) was calculated as (F_M-F_0)/F_M being F_M and F_0 the maximum and minimum chlorophyll fluorescence in the dark-adapted state. Measurements and image analysis were carried out using a FluorCam 700MF and Fluorcam5 software (Photon Systems Instruments) on attached leaves as described by Pineda *et al.* (2008).

Thermal imaging

Infrared images of attached leaves were taken using a FLIR A305sc camera (FLIR Systems) as described (Pérez-Bueno *et al.* 2016). The thermal camera was vertically positioned approximately 0.3 m over the plants. Numerical data were obtained from digital video data by the Research & Development software (FLIR Systems).

Drought and water content measurements

Plants were bottom-watered for 3h to saturate the soil at 10 days after inoculation (dai) (*N. benthamiana*) or 16 dai (Arabidopsis), and then moved to dry trays where water was withheld. An equal number of plants were kept well-watered over the same period as a control. To minimize experimental variation, the position of the trays in the grown chamber was changed periodically. Experiments were repeated at least three times using approximately 40 plants per virus treatment.

Water content analysis in both drought-stressed and well-watered plants was performed according to Xu *et al.* (2008). The percentage water content of each plant was calculated by dividing the water weight with the fresh weight for each sample. At least 15 mock-inoculated and 15 virus-infected plants were analyzed in each of three separate experiments. Statistical analyses were performed using the statistical software IBM SPSS Statistics v.20 (IBM Corp).

The relative soil water content (RSWC) was calculated following the formula: (fresh weight-dry weight)/(initial weight-dry weight) X 100, as described before (Miura *et al.* 2013).

Water loss, stomatal conductance and root analyses

For Arabidopsis plants, 12 rosettes per virus treatment were excised from the soil and placed in Petri dishes at room temperature (22°C). For *N. benthamiana* plants, eight leaf discs from individual plants (4 replicates per treatment) were cut off and placed in Petri dishes with their abaxial face up. Two independent water loss experiments were conducted in each plant species according to Westwood *et al.* (2013).

Stomatal conductance was measured using a leaf porometer (SC-1 Decagon-T) at 25°C, 65% relative humidity. Attached, fully expanded leaves of plants were placed in the chamber and repeat measurements of conductance from 12 plants per treatment were taken.

Epidermal impressions of leaves were carried out as described by Delgado *et al.* (2012). Impressions were photographed with a Leica DM 2500 microscope equipped with a Leica DFC 320 camera. Stomatal density (stomatal number per area), epidermal cell density (epidermal cell number per area) and stomatal index (stomatal number divided by epidermal cell and stomatal number) were measured on abaxial side of the leaves.

For root measurements, Arabidopsis seeds were germinated in MS-containing vertical plates. Seedlings were agro-inoculated 10 days after germination with the different agrobacterium cultures. At 11 dai, measurements were made on 12 plants for each virus treatment by scanning with image-analysis software ImageJ (http://imagej.nih.gov/ij/index.htmls).

Fitness assays

For experiments in Arabidopsis we imposed two water stress regimes defined as moderate (standard) and extended drought. In moderate drought conditions, plants that had been transferred to

soil at 11 dai were bottom-watered for 3h after removing the plastic bag at 16 dai. In extended drought, plants were transferred to soil at 6 dai and after removing the plastic bag at 11 dai no more water was added. RSWC under the two water stress regimes was calculated as described above. For experiments in *N. benthamiana*, the standard drought regime as described above was followed.

The number of Arabidopsis and *N. benthamiana* plants producing seed and seed production per plant were determined at complete senescence. Seeds were weighted separately after threshing and recorded as seed weight per plant. Seed weight was estimated after determining the weight of 200 seeds derived from each of four plants per treatment. Seed viability was measured as the germination percentage of ca 100 seeds per plant. Germination efficiency was determined after 10 days of cultivation in MS-containing plates following 3 days of stratification.

Determination of metabolite and hormone levels

Leaf samples from *A. thaliana* corresponding to four independent biological replicates were collected according to a water regime scheme; each replicates consisting of a pool of 10-13 plants. Extraction was performed according to Osorio *et al.* (2013). The sample corresponding to PPV-P25 infection under SD treatment was lost during processing. Derivatization and gas chromatography coupled to time-of-flight mass spectrometry analyses were carried out as described (Lisec *et al.* 2006). Both chromatograms and mass spectra were evaluated using TAGFINDER (Luedemann *et al.* 2008). The metabolite profiling data generated was then subjected to statistical analysis by Dunnett´s test. Hierarchical clustering and principal component analysis for clustering metabolite data was performed using R package.

For SA, ABA and JA analysis, sample preparation, extraction and derivatization were performed according to Vallarino & Osorio (2016). A volume of 1 µl of each sample was injected into a GC/TOF-MS system. The samples were quantified using internal [$^2H_6$]-ABA, [$^2H_4$]-SA, and [$^2H_6$]-JA standards

(OlChemIm Ltd), and relative quantification was subjected to statistical analysis by Dunnett´s test (Table S2).

Results

Drought tolerance in virus-infected *N. benthamiana*

N. benthamiana plants were agroinoculated with PVX, PPV expressing the green fluorescent protein (PPV-GFP), PPV expressing the virulence protein P25 of PVX (PPV-P25), and the combination PPV-GFP + PVX. Virus accumulation was assayed by Western blot analyses of extracts derived from upper leaves of infected plants (Fig. S1A). Mock-inoculated and virus-infected plants were normally irrigated or deprived from irrigation at 10 dai resulting in water stress. The detrimental effect of viral infection on the above-ground biomass of well-watered plants was higher for both PPV-P25 and PPV-GFP + PVX than for PPV-GFP and PVX, at 17 dai (Fig. 1A).

After withholding water, drought symptoms in mock-inoculated plants first appeared as drooped, curled, or wilted leaves. The prolonged water deficit eventually led to plant collapse (Fig. 1B). In plants infected with PPV-P25 and PPV-GFP + PVX and, to a lesser extent, in infections with PVX and PPV-GFP, the appearance of drought symptoms was delayed by several days and they appeared clearly less wilted than mock-inoculated plants throughout the experiment.

Imaging techniques, such as chlorophyll fluorescence (Chl-F) and multicolor fluorescence (MCFI) imaging are valuable tools for providing information about alterations of primary and secondary metabolism associated with stress responses (Pérez-Bueno *et al.* 2016). Increased blue (F440) and green (F520) fluorescence emissions were observed in plants infected with PPV-P25 or the combination PPV-GFP + PVX under well-watered growth conditions or even more drastically at 3 days after the water was withdrawn (daww) (Fig. S1B). Concomitantly, maximum efficiency of PSII (F_V/F_M)

was decreased in PPV-P25 infected plants at 3 daww relative to the values in mock-inoculated plants and plants infected with the other viruses (Fig. S1C).

The water content of mock- and virus-infected plants growing under normal and non-watered conditions was compared at 17 dai (7 daww, Fig. 1C). Under non-watered growth conditions, average water content was higher in plants infected with PVX, PPV-P25 and PPV-GFP + PVX, compared to mock-inoculated plants, whereas infection by PPV-GFP had a marginal effect on water content. Furthermore, a significantly lower water content was observed in well-watered plants infected with PPV-P25 compared to plants infected with either PPV-GFP or PVX. When the ratio of water content in drought-stressed *vs.* well-watered plants was calculated, a significant difference was observed in plants infected with PPV-P25 or in the combination PPV-GFP + PVX when compared to the levels observed in mock-inoculated plants and in plants infected with single viruses (Fig. 1D). These results indicated that virus-induced drought tolerance in *N. benthamiana* correlated with virulence.

Transpiration decreases in infected *N. benthamiana* plants

Since decrease in transpiration is an important trait of plants under drought stress, weight loss due to water loss over time was calculated from leaf discs excised from well-watered, mock-inoculated and infected *N. benthamiana* plants. Discs from plants infected with PVX, PPV-GFP + PVX and PPV-P25, but not those from plants infected with PPV-GFP, dried out at significantly slower rates than discs from mock-inoculated plants (Fig. 2A). Leaf temperature can be used as an indicator to detect differences in transpiration, since transpiration causes leaf cooling (Merlot *et al.* 2002). Leaf temperature measured by thermal imaging in plants under well-watered growth conditions increased in virus-infected plants compared to mock-inoculated plants, particularly in the case of plants infected with PPV-P25 (Fig. 2B). These results suggest that stomata on leaves of virus-infected *N. benthamiana* plants have a decreased conductance compared to mock-inoculated plants. Indeed, measurements of stomatal conductance in plants under well-watered growth conditions showed that virus infection caused a

several-fold decrease in conductance, particularly in the case of plants infected with either PPV-P25 or the combination PPV-GFP + PVX (Fig. 2C). Thus, relative differences in transpiration were correlated with differences in drought tolerance in infected *N. benthamiana* plants.

After several days without watering, leaves of mock-inoculated plants and of plants infected with either PVX or PPV-GFP showed a gradual decrease in stomatal conductance compared to well-watered conditions, whereas leaves of plants infected with either PPV-P25 or PPV-GFP + PVX did not exhibit any significant change in conductance (Fig. 2C). Accordingly, leaf temperature did not vary in plants infected with either PPV-P25 or PPV-GFP + PVX when grown under drought or well-watered conditions (Fig. 2B). Furthermore, stomatal conductance and leaf temperature in plants infected with either PPV-P25 or the combination PPV-GFP + PVX grown under well-watered conditions were similar to those observed in mock-inoculated plants when grown under water deficit.

We next investigated whether virus-infected plants showed an alteration in the number of stomata. The leaf abaxial stomatal density increased in plants infected with the different viruses when compared to mock-inoculated plants (Fig. 2D, upper panel). Because the epidermal cells in virus-infected plants were smaller in size than those in mock-inoculated plants (Fig. 2D, middle panel), leaf stomatal index (stomata:epidermal cell ratio) in infected plants was found to be similar to control plants (Fig. 2D, lower panel). These findings suggested that, despite the increase in stomatal density, infection of *N. benthamiana* by these viruses regulated stomatal conductance in the same direction as non-infected plants responded to water deficit, i. e., closing stomata to prevent water loss.

Drought tolerance in infected Arabidopsis plants

In vitro grown Arabidopsis seedlings were agroinoculated with PVX, PPV-GFP, PPV-P25, and the combination PPV-GFP + PVX. Virus accumulation was assayed by Western and Northern blot analyses of extracts derived from upper, systemically-infected leaves at 11 dai (Figs. S2A and S2B). *A. thaliana* was not a systemic host for PVX. Nevertheless, it became susceptible to PVX if co-infected with PPV-

GFP. However, a recombinant PVX expressing the suppressor of RNA silencing helper-component proteinase (HCPro) of PPV (PVX-HC) did not accumulate in plants. These findings suggest that expansion of host range of PVX by a heterologous virus is not simply due to suppression of RNA silencing (Valli *et al.* 2017).

Eleven days after inoculation, plants were transferred to soil. Mock-inoculated and virus-infected plants were normally irrigated or subjected to water stress resulting from irrigation deprivation at 16 dai. Symptoms in plants infected with PPV-P25 were more severe than those of plants infected with PPV-GFP or the PPV-GFP + PVX combination, and included necrotic spots and marked chlorosis (Fig. S2D). The detrimental effect of viral infection on the above-ground biomass of well-watered plants was higher for PPV-P25 than for PPV-GFP and PPV-GFP + PVX at 30 dai (Fig. 3A).

After withholding water, drought symptoms in mock-inoculated plants first appeared as drooped and wilted leaves or dehydrated stems. The prolonged water stress eventually led to plant collapse. In virus-infected plants, the appearance of drought symptoms was delayed by several days and they were less wilted than mock-inoculated plants throughout the experiment (Fig. 3B). RSWC was similar in each treatment, indicating that evaporation was the major cause of soil water depletion (Fig. 3C). *ADC1* is involved in the biosynthesis of putrescine, a polyamine that modulates abiotic stress tolerance (Alet *et al.* 2011). We confirmed the increased expression of *ADC1* in virus-infected plants by Northern blot analysis (Fig. S2C).

The alteration of the primary and secondary metabolism in virus-infected plants was analyzed in leaves by Chl-F and MCFI. Under well-watered growth conditions, F_V/F_M was not significantly different between virus-infected and mock-inoculated plants, and only minor differences in F440 fluorescence emission were detected between treatments (Fig. S3). However, at a later stage of infection, 7 daww, virus-infected plants showed higher levels of F440 and F520 than mock-inoculated plants, indicating upregulation of the secondary metabolism, particularly in the case of plants infected with either PPV-

P25 or in the combination PPV-GFP + PVX. There was also a decrease in F_V/F_M in PPV-P25 infected plants, indicating loss of activity of PSII.

At 30 dai (14 daww), the water content of mock-inoculated and virus-infected plants was compared. The average water content was higher in virus-infected plants than in mock-inoculated plants under non-watered growth conditions, whereas under normal irrigation conditions infected plants had marginally lower, but statistically significant, water contents than mock-inoculated plants (Fig. 3D). Furthermore, statistically significant lower water content was observed in well-watered plants infected with PPV-P25 compared to plants infected with either PPV-GFP or the combination PPV-GFP + PVX. When the ratio of water content in drought-stressed *vs.* well-watered plants was calculated, a significant difference was observed between infection by PPV-P25 and PPV-GFP (Fig. 3E). These results indicate that, like in *N. benthamiana*, virus-induced drought tolerance in Arabidopsis correlated with virulence, and also that expression of the PVX P25 protein in a PPV infection affected water balance.

Transpiration increases in infected Arabidopsis plants

Water loss over time was calculated from rosettes excised from well-watered, mock-inoculated and infected Arabidopsis plants as a measurement of transpiration rate. Paradoxically, plants infected by PPV-P25 dried out at a significantly faster rate than mock-inoculated plants, whereas plants infected with either PPV-GFP or the combination PPV-GFP + PVX showed an intermediate phenotype (Fig. 4A). Leaf temperature measured by thermal imaging in plants under well-watered growth conditions decreased in virus-infected plants compared to mock-inoculated plants, particularly in the case of plants infected with either PPV-P25 or the combination PPV-GFP + PVX (Fig. 4B). After 7 days without watering, leaf temperature of mock-inoculated plants and plants infected with the different viruses increased when compared to well-watered plants. Nevertheless, leaf temperatures of plants infected with PPV-P25 or PPV-GFP + PVX were significantly lower, and hence their transpirations were higher,

than that of mock-inoculated plants. Thus, relative differences in transpiration rates prior to and during the establishment of water deficit did not correlate with drought tolerance in infected Arabidopsis plants.

Like in *N. benthamiana*, leaf stomatal density, but not stomatal index, increased in Arabidopsis plants infected with PPV-P25, and in the cases of plants infected with PPV-GFP or PPV-GFP + PVX, there was a trend towards increased stomatal numbers compared to mock-inoculated plants (Fig. 4C). These findings indicate that infection by these viruses increased stomatal densities in Arabidopsis plants, which could contribute to higher transpiration rates when compared to non-infected plants, even under drought conditions.

It has been shown that alteration of root system architecture may improve drought tolerance (Uga *et al.* 2013). The primary and lateral roots, as well as lateral root densities of mock-inoculated and virus-infected seedlings were measured at 11 dai. No significant differences in root architecture were observed between virus-infected and mock-inoculated seedlings (Fig. S4).

Reproductive fitness in infected plants

The effect of virus infection on the fitness of both, well-watered and drought-stressed Arabidopsis plants was estimated by determining the number of infected plants producing seed, and the weight of those seeds, and compared with those produced by mock-inoculated plants. Under normal irrigation, virus infections did not affect the number of plants producing seed (Table 1). However, plants infected with PPV-GFP or PPV-GFP + PVX showed a four-fold reduction in seed production compared to mock-inoculated plants, whereas infection by PPV-P25 imposed a more severe penalty to host fitness (Fig. 5A). Under standard moderate drought conditions, seed production in plants infected with either PPV-GFP or PPV-GFP + PVX equaled that of mock-inoculated plants, with negligible differences in the number of seed-producing plants (Fig. 5B, Table 1). In contrast, infection by PPV-P25 still caused a detrimental effect on seed production.

Next, we imposed a severe water stress regime (extended drought) by transferring *in vitro* grown plants to soil at 6 dai instead of at 11 dai, followed by irrigation deprivation at 11 dai. Statistical significant differences in the relative water content of the soil were observed under the two water stress regimes assayed (Fig. S5). The extended period of drought led to an enhanced fitness (two-fold) of plants infected with PPV-GFP and PPV-GFP + PVX compared to mock-inoculated plants, as shown by the number of plants producing seed (Table 1). No significant differences in seed production were observed between virus-infected and mock-inoculated plants under these conditions of extended drought (Fig. 5C). Virus infections did not affect seed weight or the viability of individual seeds when plants were grown at moderate or extended drought conditions (Fig. 6). Thus, infection by PPV-GFP or the PPV-GFP + PVX combination, but not by PPV-P25 infection, conferred beneficial trade-offs to Arabidopsis plants grown under severe water deficit.

In *N. benthamiana*, infection by PVX, PPV-P25 and PPV-GFP + PVX had a detrimental effect on seed production under well-watered conditions. Under standard drought conditions, however, seed production and the number of PVX-infected plants producing seed did not differ significantly from that of mock-inoculated plants, whereas infection by PPV-P25 and PPV-GFP + PVX still caused a detrimental effect on host fitness (Fig. 7).

Metabolic and hormonal analyses in infected Arabidopsis plants

In order to gain a deeper comprehension of the effects of virus infection and drought on plant responses, we next determined metabolite and hormone levels in samples taken from Arabidopsis leaves of infected and mock-inoculated plants at 16, 23 and 26 dai, i.e. at 0 (watered, W), 7 (drought , D) and 10 (severe drought, SD) daww, respectively, using an established gas chromatography/mass spectrometry (GC/TOF-MS)-based metabolite profiling method (Osorio *et al.* 2013); Table S2). In addition, samples from plants held without watering for 10 days were taken at 6 hours after reinstatement of watering (R) and subjected to metabolic and hormonal analyses. The identified

compounds were categorized as sugars and sugar alcohols (14), amino acids and amines (21) and organic acids (9), and their levels were estimated relative to their concentration in well-watered, mock-inoculated plants.

Hierarchical clustering was used to group the entire data sets from all the treatments by the similarity of their overall metabolite profiles (Fig. 8A). Grouping was strongly influenced by different water regimes (W, D, SD and R), especially W samples which formed an isolated branch. Within W branch, a separation of plants infected with PPV-P25 from the other virus-infected and mock-inoculated plants was evident, which suggests a differential metabolic status in PPV-25-infected plants prior to water deprivation. Upon D conditions, the metabolic profile of PPV-P25 infection was still separated from those corresponding to the other virus infections. Within the branches corresponding to water stressed samples, metabolic data from mock-inoculated plants could be clearly distinguished from plants infected with the different viruses, suggesting that infection produces significant metabolic changes in addition to those imposed by water deficit. Samples corresponding to R treatment were grouped apart from SD samples, indicating that within 6 hours after reinstatement of watering these drought-stressed plants have already started to respond at the metabolic level to water availability.

To validate overall differences in metabolic profiles, data were also examined by principal component analysis (PCA). Two principal components explained 80.8% of the overall variance of the metabolite profiles (62.53 and 18.23% for principal component 1 [PC1] and principal component 2 [PC2], respectively; Fig. S7A). Again, PCA score plot revealed a clear separation according to the water status of the samples as the main source of variance underlying PC1. The most important metabolites for the PC1 loading included Trp, galactinol, Fru, Pro, raffinose, 4-hydroxy-Pro, Ile, Phe as well as Glc (Table S3), whose levels increased in water stressed samples (D, SD and R) compared to W samples. In addition, PC2 defined a clear separation of R from SD samples in the PCA score plot. The metabolites that exhibited higher levels in R versus SD samples mostly belonged to the amino acids class, whereas those showing lower levels in R versus SD belonged to the sugars class.

Next, we identified metabolites whose levels were significantly altered in virus-infected plants compared to mock-inoculated plants in each of the water regimes examined (Table S2). In W samples, the accumulation levels of 18, 29 and 17 metabolites showed significant changes after infection with PPV-GFP, PPV-P25 and PPV-GFP + PVX, respectively (Table 2). Most of the differences between PPV-P25 and the other infections occurred in the number of metabolites with increased levels. Following the progression of infection, a higher number of up-regulated metabolites was also found in D samples corresponding to plants infected with PPV-P25 compared to PPV-GFP and PPV-GFP + PVX infections. However, the total number of metabolites altered by the different viruses converged under the D, SD and R regimes. We used a PCA analysis to evaluate the contribution of individual metabolites in W samples (Fig. S6B and Table S3). The main contributors for the PC1 loading, accounting for 58% of the variance, were Ser and Thr, whose levels were increased by all three virus infections compared to mock-inoculated plants. Other important metabolites for the PC1 loading included Lys, fucose and Glc, which were only up-regulated by the PPV-P25 infection. The main contributors for the PC2 loading (25% of the variance) were threonic acid, Fru and raffinose, whose levels were increased by all three virus infections. Moreover, significant changes in raffinose levels were detected in virus-infected plants across the different water regimes examined (Fig. 8B). Remarkably, elevated putrescine levels were found in W and D samples corresponding to plants infected with PPV-P25 but not in infections with PPV-GFP or PPV-GFP + PVX (Fig. 8C). Both raffinose and putrescine function as osmoprotectants during drought stress (Seki *et al.* 2007).

The dynamics of accumulation of SA, JA and ABA in Arabidopsis leaves across the different water regimes were monitored in parallel to the metabolic analysis by GC/TOF-MS (Table S2 and Fig. 9). ABA accumulated at similar levels in both virus-infected and mock-inoculated plants under normal irrigation (W) (Fig. 9A). Water deprivation (D and SD treatments) caused a progressive increase of ABA content in mock-inoculated plants, whereas plants infected with viruses did not exhibit any significant increase in ABA content upon drought conditions. Under W conditions, SA accumulated in plants

infected with all viruses tested whereas remained undetectable in mock-inoculated plants (Fig. 9B). Intriguingly, infection by PPV-P25 induced a higher increase in the production of SA compared to infections by PPV-GFP and PPV-GFP + PVX under all water regimes assayed. The level of ABA in R samples corresponding to plants infected by PPV-P25 and PPV-GFP + PVX was below detection limit, which could be explained by the high levels of SA detected in these samples (Figs. 9A and 9B). Antagonistic cross talk between ABA and SA has been observed upon viral infection (Sade *et al.* 2014). Indeed, we found a significant negative correlation in the accumulation of ABA and SA in our entire dataset (Pearson coefficient, $P < 0.01$), which was not observed in any other hormone combination (Fig. S7). PPV-P25 induced a strong accumulation of JA in W conditions, whereas the JA content in plants infected with the other viruses or the mock-inoculated plants was undetectable (Fig. 9C). After water deprivation, JA was detected to highly variable levels in mock-inoculated and virus-infected plants, suggesting that JA modulated plant responses to drought. Overall, these findings argue for a differential hormonal homeostasis in plants infected by PPV-P25 prior to water deprivation. This is more clearly revealed in the 3D representation of hormone profiles shown in Fig. 9D, where the W sample corresponding to PPV-P25 was located close to water stressed samples and apart from other W samples.

Virus-induced drought tolerance is altered in hormone-deficient plants

In order to determine whether elevated hormone levels contribute to virus-induced drought tolerance, Arabidopsis mutant lines deficient in ABA biosynthesis (*ABA deficient2* [*aba2-1*]) or JA perception (*coronatine insensitive1* [*coi1*]), along with a transgenic line expressing the SA-degrading enzyme salicylate hydroxylase (NahG) and Col-0 as the wild-type (WT) control line were assessed for their tolerance to water deprivation upon PPV-P25 infection. Mock-inoculated and virus-infected plants were normally irrigated or subjected to water withdrawal. At 30 dai (14 daww), the water content of mock-inoculated and virus-infected plants was compared (Figs. 10A and 10B). Under non-watered growth

conditions, the water content in mock-inoculated NahG plants was similar to WT plants. As expected, the average water content in mock-inoculated plants was lower in *aba2-1* than in WT, whereas it was higher in *coi1-1* than in WT plants (Harb *et al.* 2010). Interestingly, virus infection did not induce drought tolerance in the SA-deficient NahG line. Water content in virus-infected NahG plants reached the levels observed in mock-inoculated NahG controls, whereas statistically significant higher water content was observed in virus-infected *aba2-1* and *coi1-1* plants compared to their respective mock-inoculated controls. Furthermore, the mutant line *aba2-1* displayed a higher relative increase in water content compared to WT when infected by PPV-P25, i. e. 3.5- *vs*. 2-fold increase compared to their mock-inoculated controls, albeit responses were less intense than in WT. In a separate experiment, the average water content in the SA biosynthesis mutant *salicylic acid induction deficient2* (*sid2-2*) infected by PPV-P25 was lower than in WT plants under non-watered growth conditions (Fig. 10C). PPV-P25 accumulation was significantly lower in NahG and *sid2-2* plants than in the WT controls (11.1- and 1.8-fold, respectively), whereas virus accumulated at 3.3-fold higher levels in *aba2-1* compared to WT plants (Fig. 10D). Virus titers have been reported to be reduced in SA-deficient Arabidopsis plants at late stages of infection (Wang *et al.* 2011).

When we performed the same experiment on NahG-transgenic *N. benthamiana* plants, a significant reduction in water content was observed in PPV-P25-infected NahG plants compared to infected WT plants (Fig. S8). Like in Arabidopsis, water content in virus-infected, NahG *N. benthamiana* plants reached the levels observed in mock-inoculated NahG controls. The accumulation of PPV-P25 did not show significant differences between the SA-deficient plants and the WT controls, confirming previous report that the effect of SA-mediated defenses on PPV infection in *N. benthamiana*, if it exists, is minor (Ying *et al.* 2010).

To confirm the linkage between SA and virus-induced drought tolerance, we also assessed the expression of the SA- and drought-responsive genes *PATHOGENESIS-RELATED PROTEIN 1* (*PR1;* At2g14610) and *RESPONSIVE TO DESICCATION* 29B (*RD29B;* At5g52300) in hormone-deficient

Arabidopsis plants before water deprivation (15 dai) by real-time quantitative RT-PCR (qRT-PCR). *PR1* expression was higher in both mock-inoculated and PPV-P25-infected *aba2-1* plants compared with WT controls, suggesting that enhanced activation of SA signaling in the ABA-deficient line led to increased drought tolerance upon virus infection (Fig. 10E). No significant differences in *RD29B* expression was observed in PPV-P25-infected *aba2-1* plants compared to WT plants (Fig. 10F). Altogether these findings suggest that virus-induced drought tolerance was positively affected by SA signaling, which seems to be negatively controlled by ABA signaling. The contribution of JA signaling to virus-induced drought tolerance, if any, is negligible.

Discussion

Estimates of virus effects on vegetative growth, stress response, reproductive fitness and the ratio of water content in drought-stressed *vs.* well-watered plants was consistent with a positive association between virulence and tolerance to drought; that is, infection by PPV-P25 in both Arabidopsis and *N. benthamiana*, and by the combination PPV-GFP + PVX in *N. benthamiana*, conferred an enhanced drought-tolerant phenotype compared to single infections with PPV-GFP or PVX.

In a previous work, a transcriptome profiling analysis of *N. benthamiana* plants co-infected with PVX and *Potato virus Y* (another member of the *Potyvirus* genus) revealed a greater impact on the transcriptional up-regulation of genes associated with carbohydrate metabolism and stress compared with single infections (García-Marcos *et al.* 2009). Accordingly, PVX/potyvirus infection induced a severe oxidative stress in *N. benthamiana* leaves, as judged by the generation of reactive oxygen species (ROS), which correlated with increased virulence (García-Marcos *et al.* 2009). However, co-infection of PPV-GFP and PVX in Arabidopsis did not increase virulence caused by single infection with PPV-GFP (the present study), most likely because PVX accumulated at subliminal levels in this host (Andika *et al.* 2015). Recent findings suggest that the P25 protein of PVX is the major viral determinant involved in PVX/potyvirus-associated virulence (Aguilar *et al.* 2015b). Thus, the expression of P25 by

PPV led to an increase of PPV virulence in both Arabidopsis and *N. benthamiana*. Metabolic and hormonal data shown in this work further support an altered metabolic status in Arabidopsis plants infected with PPV-P25 of more amplitude than the one triggered by PPV-GFP infection. Recent works have linked trade-offs in compatible plant-virus interactions to a group of viral proteins commonly known as virulence determinants. For instance, the 2b protein of CMV has been demonstrated to participate in both drought tolerance in Arabidopsis (Westwood *et al.* 2013) and insect pollinator attraction in tomato (Groen *et al.* 2016). Similarly, we propose that the P25 protein of PVX would induce metabolic acclimation in plants to abiotic stresses as a result of its virulence properties.

Several reports have hinted at a possible connection between the establishment of plant responses to disease and drought tolerance. For instance, transgenic *N. benthamiana* plants expressing the *Phytophthora sojae* effector protein CRN161 exhibited increased resistance to two oomycete pathogens and showed enhanced tolerance to salinity and drought stresses (Rajput *et al.* 2015). The cause for this increased tolerance to abiotic stresses may reside in that plants use a network of interconnected signaling pathways to respond to various environmental stresses, and that there may be a crosstalk between biotic and abiotic stress signaling (Fujita *et al.* 2006). We conceive that virus infection that leads to an ample reprogramming of plant metabolism might offer a competitive advantage, i. e., metabolic acclimation, to the infected plant against environmental stresses. Such a metabolic shift would prove beneficial to the overall plant fitness, provided that virus-infected plants could subsequently produce offspring at higher rates than non-infected individuals under stress growth conditions.

Previous reports on virus-induced drought tolerance did not measure reproductive fitness of the plant host (Xu *et al.* 2008; Westwood *et al.* 2013). Here, we experimentally tested the hypothesis that virus infection would increase the reproductive fitness of Arabidopsis and *N. benthamiana* under variable drought conditions. Virus infection dramatically reduced seed production in well-watered Arabidopsis plants. However, Arabidopsis plants infected with PPV-GFP or the combination PPV-GFP

+ PVX produced similar amounts of seed per plant as non-infected plants when grown under moderate and extended drought conditions. More importantly, the number of plants infected by these viruses that produced seeds doubled in comparison to non-infected plants when plants were exposed to extended drought conditions. These findings substantiate the possibility argued by others (Roossinck 2011; Westwood *et al.* 2013) that under natural conditions, especially in areas where drought episodes are common, infection by single viruses or their combinations might provide strong fitness benefits to the host. By contrast, the detrimental effect caused by PPV-P25 infection on host fitness overcame the beneficial effects associated with virus-induced metabolic acclimation to drought. Similarly, virus-induced drought tolerance could compensate for decreased yield of progeny on *N. benthamiana* plants infected with PVX, but not in those infected with either PPV-P25 or PPV-GFP + PVX. Thus, the level of virulence determined the outcome of beneficial trade-offs in the response of virus-infected plants to drought, and possibly other abiotic stresses.

A recent work has shown that infection with TMV and *Turnip vein clearing virus* influenced stomatal development in *N. tabacum* and *A. thaliana* respectively, which was associated with a reduction in stomatal density and index (Murray *et al.* 2016). Accordingly, transpiration rate was significantly reduced in *N. tabacum* infected with TMV, since maximum stomatal conductance is controlled mainly by stomatal density and size (Franks *et al.* 2009). In this study, infection with other viruses caused an increase in stomatal density in leaves of Arabidopsis and *N. benthamiana* plants. However, whereas transpiration rate increased in infected Arabidopsis plants and this could be attributed to the increase in stomatal density, viral infections induced lower transpiration rates in *N. benthamiana*. The reductions in transpiration rates in infected *N. benthamiana* plants were roughly proportional to the intensities of the drought-tolerant phenotype associated with the different viral infections. These findings suggest that plant viruses may regulate tolerance to drought through distinct, but not mutually exclusive, host-dependent mechanisms. Stomatal closure, which reduces water loss through reduced transpiration rate, is an important plant survival strategy in response to drought stress (Acharya & Assmann 2009). Thus,

virus infection positively affects drought tolerance in *N. benthamiana* probably because of reduced stomatal aperture. Nevertheless, other mechanisms like osmotic adjustment (see below) might also play a role in virus-induced drought tolerance in *N. benthamiana*.

On the other hand, virus-induced drought tolerance in Arabidopsis plants did not result from a decreased rate of transpiration. These findings are in agreement with Westwood *et al.* (2013), where drought tolerant, CMV-infected Arabidopsis plants exhibited greater conductivity of the stomata in their leaves. Similarly, changes in root architecture to maximize water uptake cannot be argued to explain the increased drought tolerance in virus-infected Arabidopsis plants observed in this study. When water uptake and loss cannot be balanced, plant resists to water deficit by accumulating solutes (osmoprotectants) to tolerate reduced water content. Osmoprotectants protect plants from stress by an osmotic adjustment which helps in turgor maintenance, detoxification of ROS, and stabilization of the structure of membranes and proteins (Fang & Xiong 2015). In this study, a metabolite profiling analysis of Arabidopsis plants infected with different viruses under distinct water regimes showed a range of metabolic and hormonal changes that positively correlated with a greater impact on plant tolerance to drought. Hierarchical clustering and PCA analyses indicated that the water regime at which plants were grown had the greatest influence on metabolite profiles. An earlier study focusing on a combination of stresses also found responses to drought more dominant than virus infection (Prasch & Sonnewald 2013). Some of the metabolites with increased levels under water deficit included Trp, galactinol, Fru, Pro, raffinose, Ile, and Phe, which had already been described to increase under drought stress (Seki *et al.* 2007; Vasquez-Robinet *et al.* 2008; Bowne *et al.* 2012; Prasch & Sonnewald 2013). Comparing the metabolic response of plants within each water regime revealed that virus infections altered more than 40% of the primary metabolites analyzed. Differences between virus treatments were particularly evident in plants before being subjected to water deprivation, with the number of metabolites induced by PPV-P25 doubled compared to the other infections. The main metabolites contributing for differences between infected and non-infected plants under watered conditions were amino acids such

as Ser, Thr, Lys, and Glc; and sugar derivatives, threonic acid, fucose, Fru and raffinose, whose levels were increased by virus infections. High concentrations of individual and total amino acids have also been reported in different host species following infection with several viruses (Llave 2016). It is unclear, however, whether those increments in amino acids other that Pro might contribute to enhanced stress tolerance in infected plants. Fructans and raffinose have been proposed to act as osmoprotectants in several species, stabilizing membranes and proteins from oxidative damage caused by different types of stress conditions, included drought (Taji *et al.* 2002; Nishizawa *et al.* 2008; Van den Ende 2013). In addition, increased accumulation of putrescine was observed in plants infected with PPV-P25, which could have contributed to the better tolerance of these plants under drought stress. Putrescine acts as a mediator of osmotic adjustment, a stabilizer of subcellular structures and a scavenger of ROS (Seki *et al.* 2007). The enhanced biosynthesis of putrescine in the *Tobacco rattle virus*-Arabidopsis interaction has been reported to induce tolerance to freezing, another abiotic stress that affect plant water status (Fernández-Calvino *et al.* 2014). Moreover, osmoprotectants such as Pro and putrescine have also been described to increase in different plant-virus interactions leading to drought tolerance (Xu *et al.* 2008). Thus, despite their increased rate of transpiration, the greater accumulation of osmoprotectants in virus-infected Arabidopsis plants would confer a stage of metabolic acclimation that enables plants to cope with water deficit. The trade-off in osmotic adjustment is that increased accumulation of compatible solutes can be energy and resource intensive for the plant (Bohnert & Shen 1999), and may have a detrimental effect on reproductive fitness as observed in plants infected with PPV-P25.

Several biotic and abiotic stresses may cause an increase in the accumulation of SA (Janda *et al.* 2007). Besides the involvement of ABA in drought signaling (Fujita *et al.* 2011), recent evidence has suggested that SA might also provide tolerance to water stress in an ABA-independent manner. For instance, Arabidopsis SA-accumulating mutants *acd6* and *cpr5* showed more tolerance to drought stress than controls (Miura *et al.* 2013; Okuma *et al.* 2014). Furthermore, plants pre-treated with an appropriate concentration of SA showed increased tolerance to drought and salt stresses (Senaratna *et*

al. 2000). In the present study, Arabidopsis plants infected with PPV-P25 accumulated more SA than infections by PPV-GFP and PPV-GFP + PVX, suggesting a correlation between SA signaling and drought tolerance in virus-infected plants. Similarly, the activation of SA signaling has been reported in PVX/potyviruses-associated synergism in *N. benthamiana* (Pacheco *et al.* 2012). SA also accumulated to high concentrations in both BMV-infected rice and CMV-infected beet plants, although no clear association with improved drought tolerance could be established (Xu *et al.* 2008). In our study, a role for SA in the establishment of drought tolerance in virus-infected plants was substantiated from the analysis of SA-deficient transgenic lines. Preventing SA accumulation in Arabidopsis and *N. benthamiana* plants possessing the NahG transgene abolished tolerance to drought induced by PPV-P25 infection. Furthermore, drought tolerance was also substantially attenuated in the Arabidopsis SA biosynthesis mutant *sid2-2*.

A previous work reporting CMV-induced drought tolerance in Arabidopsis indicated that virus infection did not affect ABA accumulation, but did interfere with the expression of ABA-responsive genes (Westwood *et al.* 2013). It has been reported that activation of SA signaling, a defense response induced by CMV infection, suppressed the expression of ABA-responsive genes (Yasuda *et al.* 2008; Lewsey *et al.* 2010). Similarly, ABA levels were not increased in PPV-P25-infected plants, although the drought-responsive gene *RD29B* was induced by virus infection in an ABA-independent manner. Here, we also showed that the drought-sensitive phenotype of the Arabidopsis ABA-deficient mutant *aba2-1* was alleviated by infection with PPV-P25. This further substantiates that additional non-ABA-dependent mechanisms contribute to drought tolerance in virus-infected Arabidopsis plants. The SA-responsive gene *PR1* was induced at higher levels in the *aba2-1* mutant compared to WT plants before and after infection with PPV-P25. Several works have indicated that steps in the SA signaling pathway can be enhanced by ABA deficiency (Yasuda *et al.* 2008; Muñoz-Espinoza *et al.* 2015). As expected, the water content in non-infected *aba2-1* plants drop abruptly compared with WT plants under drought. Upon virus infection the relative increase in water content in *aba2-1* was ca. 75% higher than that in WT

plants, which was associated with a two-fold increase in *PR1* levels. However, overall tolerance to drought was reduced in *aba2-1* compared to WT plants, as the mutant background was impaired in ABA-mediated response to drought.

How can virus-induced SA confer a drought tolerance phenotype in plants? It has been reported that SA accumulation plays an important role in stomatal closure via production of ROS (Khokon *et al.* 2011; Miura *et al.* 2013). Different hormone signaling pathways can lead to stomatal closure, but SA does not seem to be an intermediate of the ABA-dependent pathway (Montillet *et al.* 2013). One possibility is that stomatal closure induced by ROS production may cause a reduction of transpiration, resulting in the storage of water in leaves for survival under drought conditions. This scenario is supported by the severe oxidative stress induced by PVX/potyviruses-associated synergism in *N. benthamiana* (García-Marcos *et al.* 2009). In addition, SA can regulate several plant metabolic processes and modulate the production of various osmoprotectants and secondary metabolites, and also maintain nutrient status to protect plants under abiotic stress conditions (Khan *et al.* 2015). For instance, it has been documented that SA is involved in increasing Pro metabolism under abiotic stresses (Misra & Saxena 2009). Moreover, exogenous application of SA caused an increase in putrescine content in maize (Németh *et al.* 2002). SA can also significantly modulate the uptake and metabolism of mineral elements and thereby improve growth and development in abiotic stressed plants (Gunes *et al.* 2007). Similarly, Arabidopsis plants infected with PPV-P25 showed an increased production of SA, and accumulated metabolites with known functions as osmoprotectants involved in stress tolerance.

In summary, virus-induced tolerance to drought was not always accompanied by an increase in the ability to produce seed. Although virus infection generally renders plants more tolerant to drought, crossing of a virulence threshold leads to detrimental effects on host fitness, even under drought conditions. Nevertheless, infections showing moderate virulence were able to increase host fitness under drought conditions, although this conditional phenotype varied in a host-dependent manner. Thus, viruses would modulate plant tolerance toward abiotic stresses by mechanisms which include trade-offs

between biotic and abiotic stress responses, and would lead to alterations in the response of plants to changing environmental conditions. In this sense, recent findings suggest that the genetic variability of certain nucleotide binding leucine-rich repeat genes in *A. thaliana* populations is not only shaped by coevolution between plants and pathogens but also the need to balance responsiveness to biotic and abiotic stresses in the environment (Ariga *et al.* 2017). Moreover, our results challenge the general validity of the hypothesis that viruses behave as conditionally beneficial to their hosts, and emphasize the need of considering the effect of virulence in the analysis of plant responses to combined abiotic and biotic stresses.

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Table 1: Effects of virus infection on the number of Arabidopsis plants producing seed when grown under well-watered conditions, moderate and extended drought.

Treatment	Number of plants	% plants producing seed
Well-watered		
Mock	12	100a
PPV-GFP	19	100a
PPV-P25	27	100a
PPV + PVX	21	100a
Moderate drought		
Mock	35	45.7a
PPV-GFP	37	40.5a
PPV-P25	38	31.6a
PPV + PVX	38	39.5a
Extended drought		
Mock	63	20.6a
PPV-GFP	77	44.1b
PPV-P25	79	10.1a
PPV + PVX	72	43b

Statistically significant differences between means were determined among treatments within each watering condition by employing Fisher's exact test with a Bonferroni correction for multiple comparisons of α to α= 0.008. Different letters indicate significant differences.

Table 2: Number of metabolites showing significant change in virus-infected Arabidopsis plants compared with mock-inoculated plants in each of the water regimes examined.

Treatment	Increased levels	Decreased levels	Total
Watered			
PPV-GFP	12	6	18
PPV-P25	23	6	29
PPV + PVX	10	7	17
Drought			
PPV-GFP	7	15	22
PPV-P25	14	13	27
PPV + PVX	2	25	27
Severe Drought			
PPV-GFP	10	15	25
PPV + PVX	11	18	24
Rewatered			
PPV-GFP	6	18	24
PPV-P25	15	12	27
PPV + PVX	13	12	27

Changes were determined significant by employing Dunnett test ($P < 0.05$).

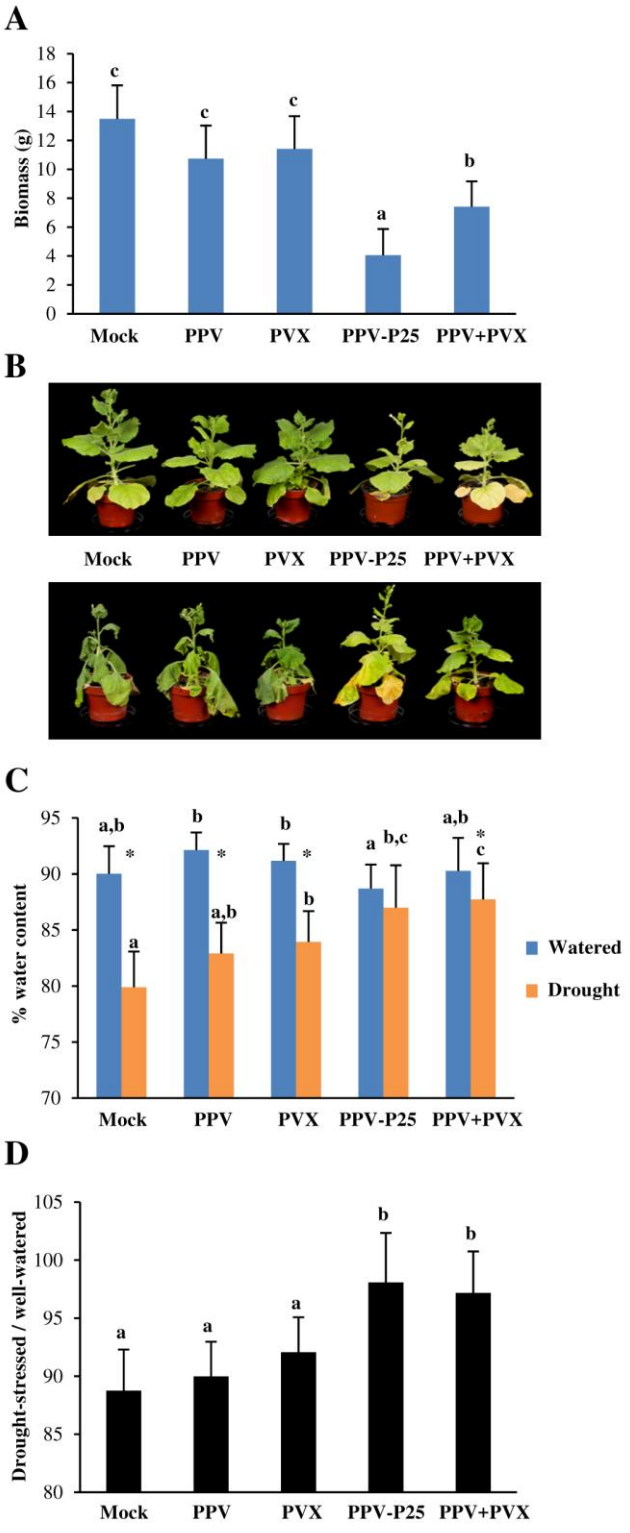

Figure 1: Comparison of the drought tolerance in mock-inoculated and virus-infected *N. benthamiana* plants. (A) Above-ground biomass of well-watered, virus-infected plants at 17 days after inoculation. Data represent the means ± standard errors of at least 14 plants that received the same treatment. Statistical comparisons between means were determined by employing Scheffé's multiple range test (*P*

< 0.05). (B) Seven days after the water was withdrawn (daww) (bottom panel), representative plants were photographed next to their watered counterparts (upper panel). (C) Water content percentage in mock-inoculated and virus-infected plants at 7 daww. (D) Ratio of water content in drought-stressed *vs.* well-watered plants that received the same treatment. Data represent the means ± standard errors of 14 plants that received the same treatment. Statistical comparisons between means were made among treatments within each watering condition by employing Mann-Whitney U test with a Bonferroni correction for multiple comparisons of α to α= 0.005. For pairwise comparisons, asterisks indicate the statistical significance of drought-stressed plants compared to well-watered plants (Mann-Whitney U test, $P < 0.05$).

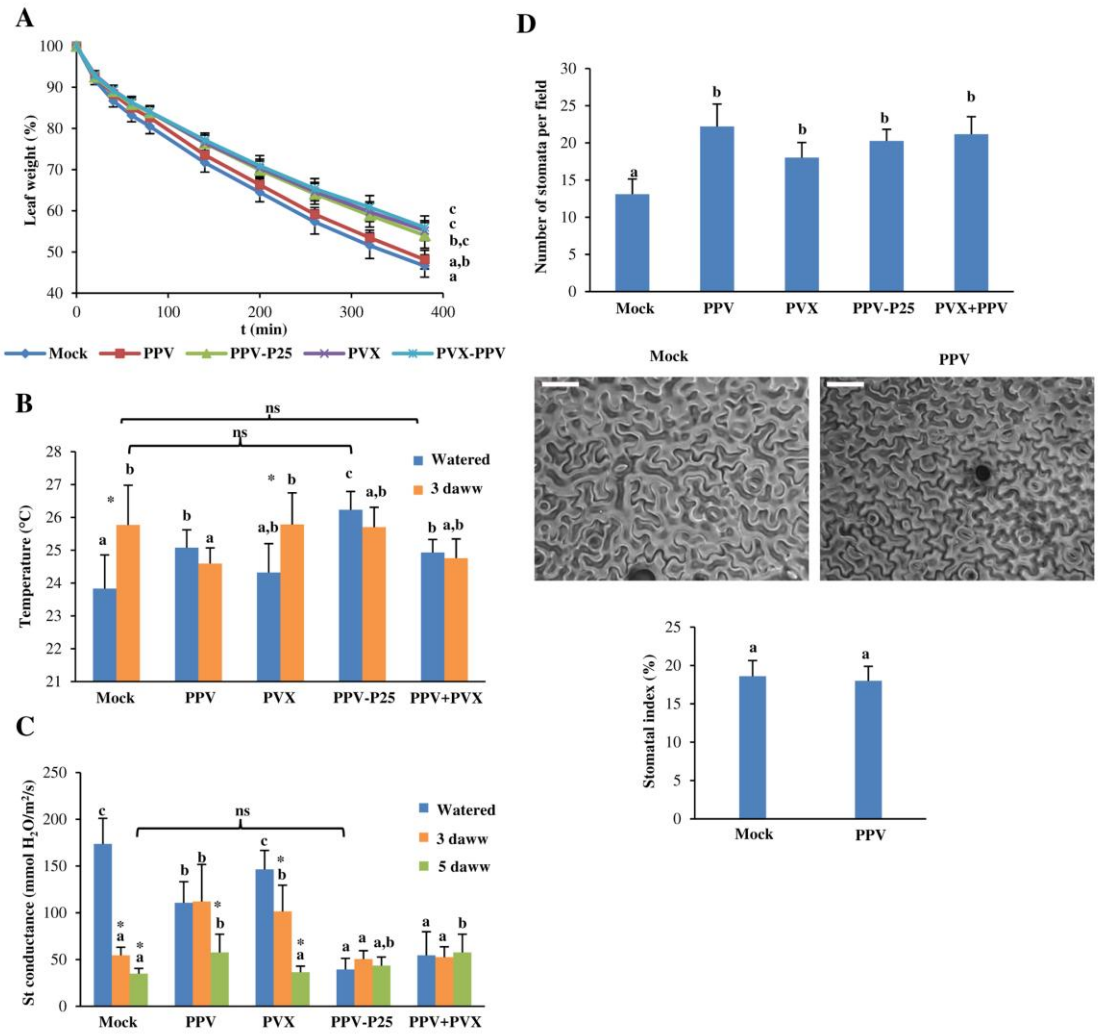

Figure 2: Comparison of water loss in mock-inoculated and virus-infected *N. benthamiana* plants. (A) Eight leaf discs from individual plants per treatment were cut off at 3 days after inoculation and placed in Petri dishes with their abaxial face up. Weight was measured at various intervals over a period of 380 min, and related to the first weight measured. Statistically significant differences between means were determined at t=380 by employing Scheffé's multiple range test. Different letters indicate significant differences at $P < 0.05$. (B) Averaged temperature values in leaves of mock-inoculated and virus-infected plants under well-watered growth conditions or 3 days after the water was withdrawn (daww). Data represent the means ± standard errors of 10 replicates (plants) that received the same treatment. A minimum of 4 leaves from each plant were positioned for simultaneous imaging. Statistically significant differences between means were determined by employing Scheffé's multiple range test.

Different letters indicate significant differences at $P < 0.05$. For pairwise comparisons, asterisks indicate significant differences between treatments (Student's t-test, $P < 0.05$); ns: not significative. (C) Effect of drought on stomatal conductance in mock-inoculated and virus-infected plants grown under well-watered conditions or at 3 and 5 daww. Statistical comparisons between means were made by employing Mann-Whitney U test for between-groups comparisons with a Bonferroni correction for multiple comparisons of α to α= 0.005. Different letters indicate significant differences at $P < 0.005$. For pairwise comparisons, asterisks indicate the statistical significance of drought-stressed plants compared to well-watered plants (Mann-Whitney U test, $P < 0.05$); ns: not significative. (D) Agarose impressions taken from mock-inoculated and virus-infected plants grown under well-watered conditions were examined under a light microscope. Images were taken at 20X magnification and the numbers of stomata (upper panel) and epidermal cells were counted (n= 40 fields). The photographs (middle panels) are representative images from mock-inoculated and PPV-GFP-infected plants. Scale bars represent 40 μm. The stomatal index (lower panel) is the number of stomata divided by the total number of epidermal cells (including stomata). Statistically significant differences between means were determined by employing Scheffé's multiple range test. Different letters indicate significant differences at $P < 0.05$.

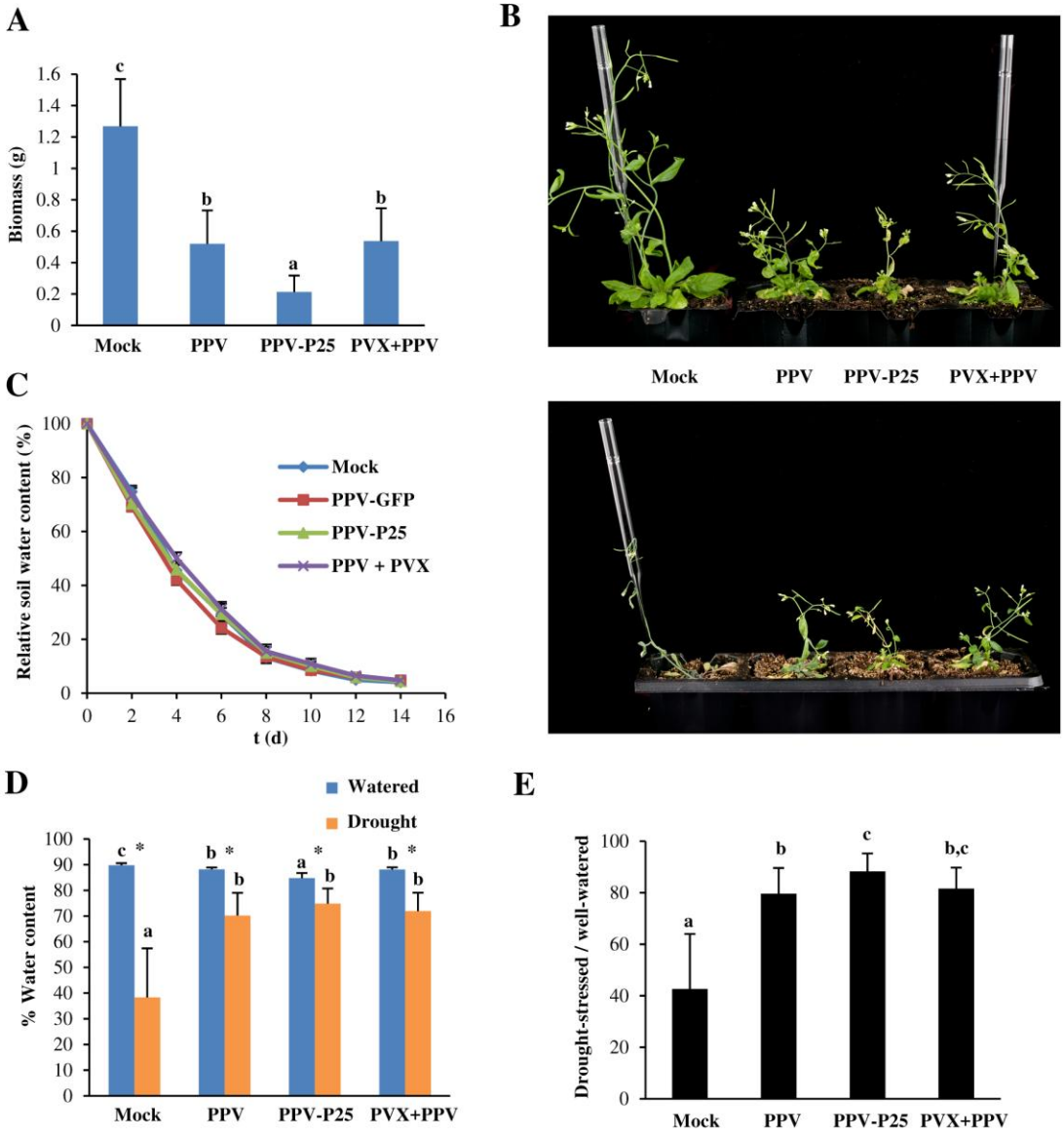

Figure 3: Comparison of the drought tolerance in mock-inoculated and virus-infected Arabidopsis plants. (A) Above-ground biomass of well-watered, virus-infected plants at 30 days after inoculation. (B) Twelve days after the water was withdrawn (daww) (bottom panel), representative plants were photographed next to their watered counterparts (upper panel). (C) The relative soil water content was measured in each pot after withholding water. (D) Water content percentage in mock-inoculated and virus-infected plants at 14 daww. (E) Ratio of water content in drought-stressed *vs.* well-watered plants that received the same treatment. Data represent the means ± standard errors of at least 15 plants that received the same treatment. Statistical comparisons between means were made among treatments

within each watering condition by employing Scheffé's multiple range test. Different letters indicate significant differences at $P < 0.05$. For pairwise comparisons, asterisks indicate the statistical significance of drought-stressed plants compared to well-watered plants (Student's t-test, $P < 0.05$).

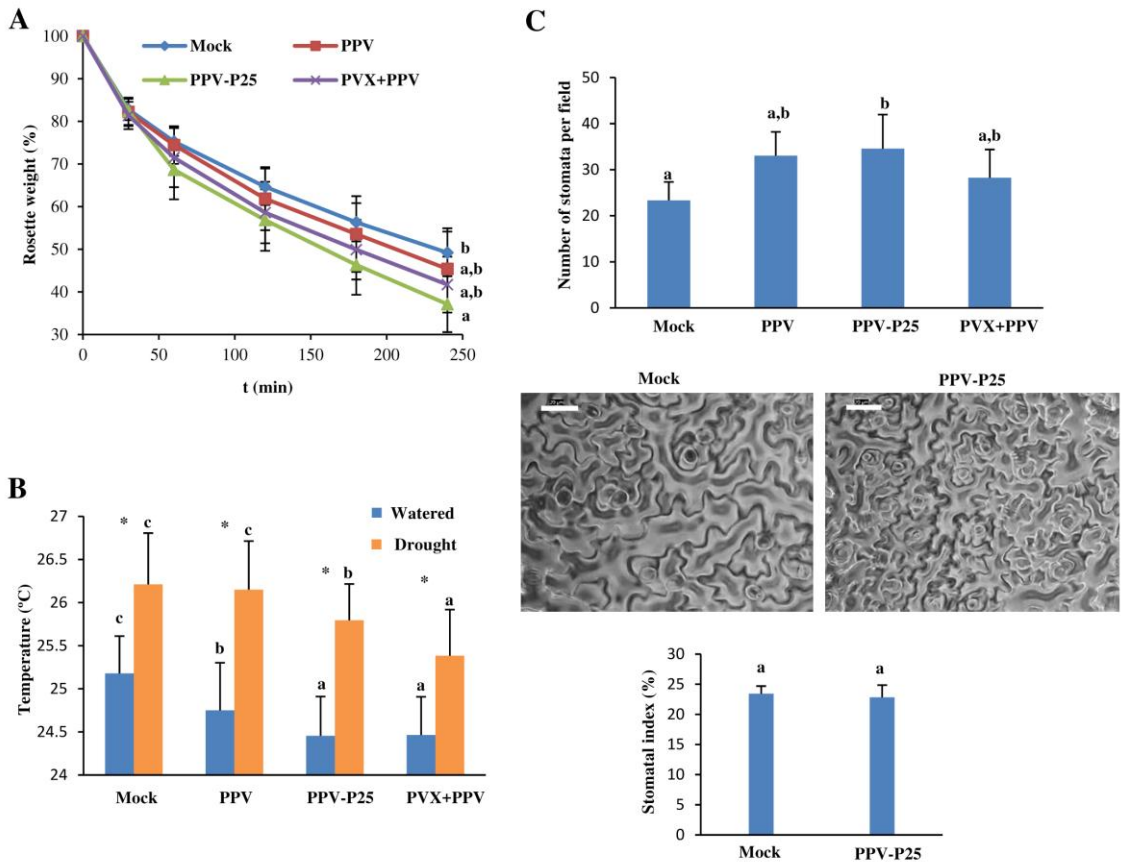

Figure 4: Comparison of water loss in mock-inoculated and virus-infected Arabidopsis plants. (A) Well-watered rosettes were detached from soil-grown plants at 23 days after inoculation and placed in plates with their abaxial face up. Weight was measured at various intervals over a period of 4h, and related to the first weight measured. Statistically significant differences between means were determined by employing Scheffé's multiple range test. Different letters indicate significant differences at $P < 0.05$. (B) Average temperature values in rosette leaves of mock-inoculated and virus-infected plants under well-watered growth conditions or 7 days after the water was withdrawn. Data represent the means ± standard errors of 5 replicates, each consisting of 4 plants that received the same treatment. A minimum of six leaves from each plant were positioned for simultaneous imaging. Statistical comparisons between means were made by employing Mann-Whitney U test for between-groups comparisons with a Bonferroni correction for multiple comparisons of α to α= 0.008. Different letters indicate significant differences at $P < 0.008$. For pairwise comparisons, asterisks indicate the statistical significance of drought-stressed plants compared to well-watered plants (Mann-Whitney U

test, $P < 0.05$). (C) Agarose impressions taken from mock-inoculated and virus-infected plants grown under well-watered conditions were examined under a light microscope. Images were taken at 20X magnification and the numbers of stomata (upper panel) and epidermal cells were counted (n = 40 fields). The photographs (middle panels) are representative images from mock-inoculated and PPV-P25 infected plants. Scale bars represent 40 μm. The stomatal index (lower panel) is the number of stomata divided by the total number of epidermal cells (including stomata). Statistically significant differences between means were determined by employing Scheffé's multiple range test. Different letters indicate significant differences at $P < 0.05$.

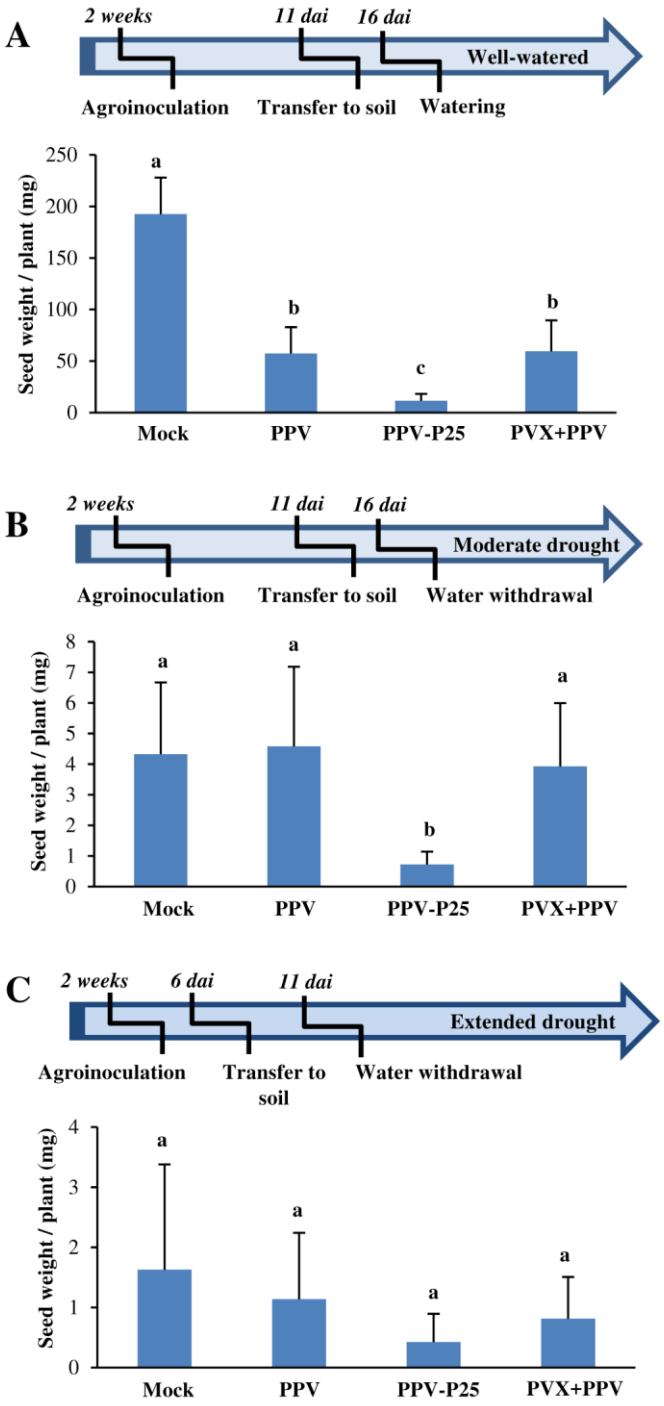

Figure 5: Effect of virus infection on seed production in Arabidopsis plants grown under different watering conditions. The schemes illustrate the experimental design for exposing plants to (A) well-watered conditions, (B) moderate and (C) extended drought. Seeds were weighted separately after threshing and recorded as seed weight per plant. The number of individuals analyzed were 12 (Mock), 27 (PPV-GFP), 19 (PPV-P25) and 21 (PPV-GFP + PVX) for well-watered condition; 30 (Mock), 26

(PPV-GFP), 24 (PPV-P25) and 23 (PPV-GFP + PVX) for moderate drought; and 11 (Mock), 23 (PPV-GFP), 8 (PPV-P25) and 26 (PPV-GFP + PVX) for extended drought. Statistically significant differences between means were determined by employing Scheffé's multiple range test (A) and Dunnett´s T3 test (B and C). Different letters indicate significant differences at $P < 0.05$.

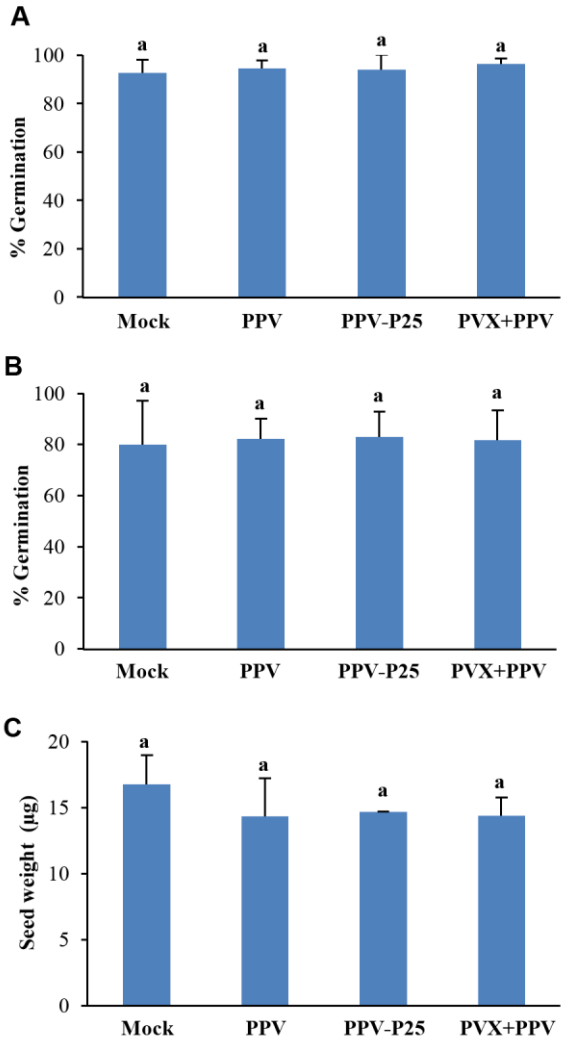

Figure 6: Effect of virus infection on seed viability and on the weight of seeds in Arabidopsis plants grown under moderate and extended drought conditions. Effect of virus infection on seed viability (A and B) and on the weight of seeds (C) of Arabidopsis plants grown under moderate or extended drought conditions. Seed viability was measured as the germination percentage of ca 100 seeds per plant, using 11 and 4 individuals per treatment in moderate and extended drought, respectively. Seed weight was estimated after determining the weight of 200 seeds derived from each of four plants per treatment. Statistically significant differences between means were determined by employing Dunnett´s T3 test (A) and Scheffé's multiple range test (B and C). Different letters indicate significant differences at $P < 0.05$.

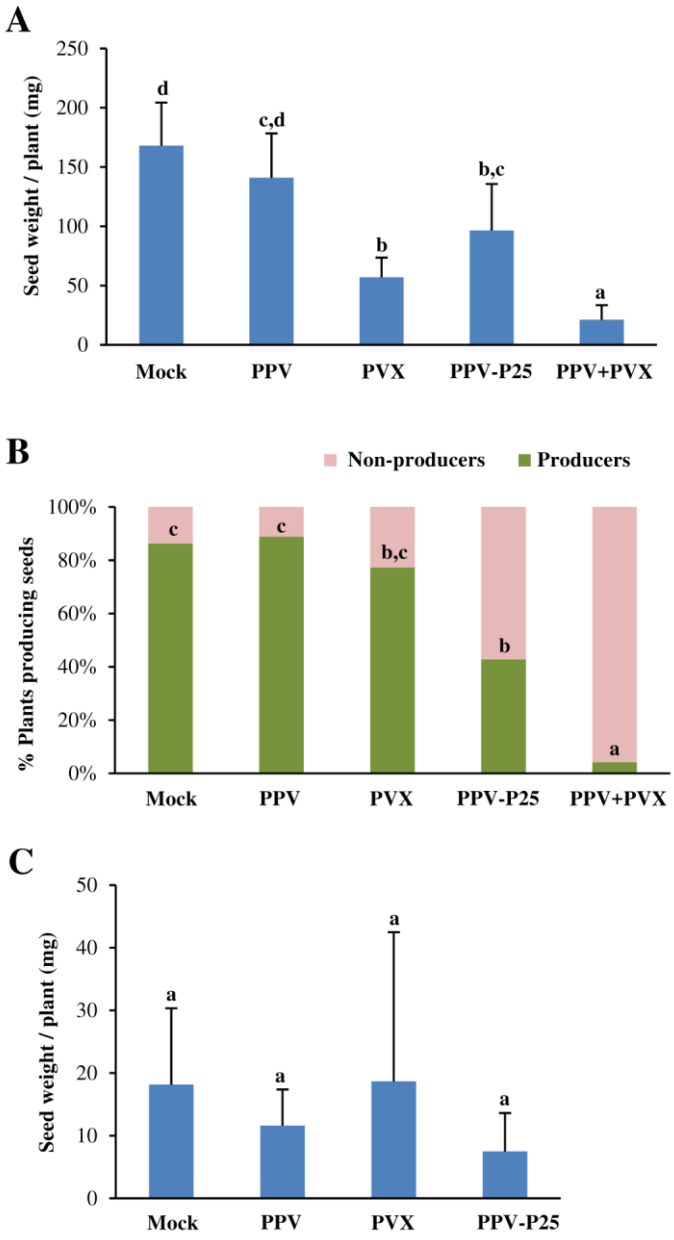

Figure 7: Effect of virus infection on the number of *N. benthamiana* plants producing seed and on seed production. Plants were grown under (A) well-watered and (B and C) drought conditions. All the mock-inoculated and virus-infected plants (seven plants per treatment) grown under well-watered conditions produced seeds. Seeds were weighted separately after threshing and recorded as seed weight per plant. The number of individuals analyzed in seed production were 7 (Mock), 7 (PPV-GFP), 7 (PVX), 7 (PPV-P25) and 6 (PPV-GFP + PVX) for well-watered condition; and 7 (Mock), 6 (PPV-GFP), 4 (PVX) and 4 (PPV-P25) for drought. Only one plant infected with PPV-GFP + PVX produced seeds under drought conditions. Statistically significant differences between means were determined by employing

Scheffé's multiple range test (A and C) and Fisher's exact test with a Bonferroni correction for multiple comparisons of α to α= 0.005 (B). Different letters indicate significant differences.

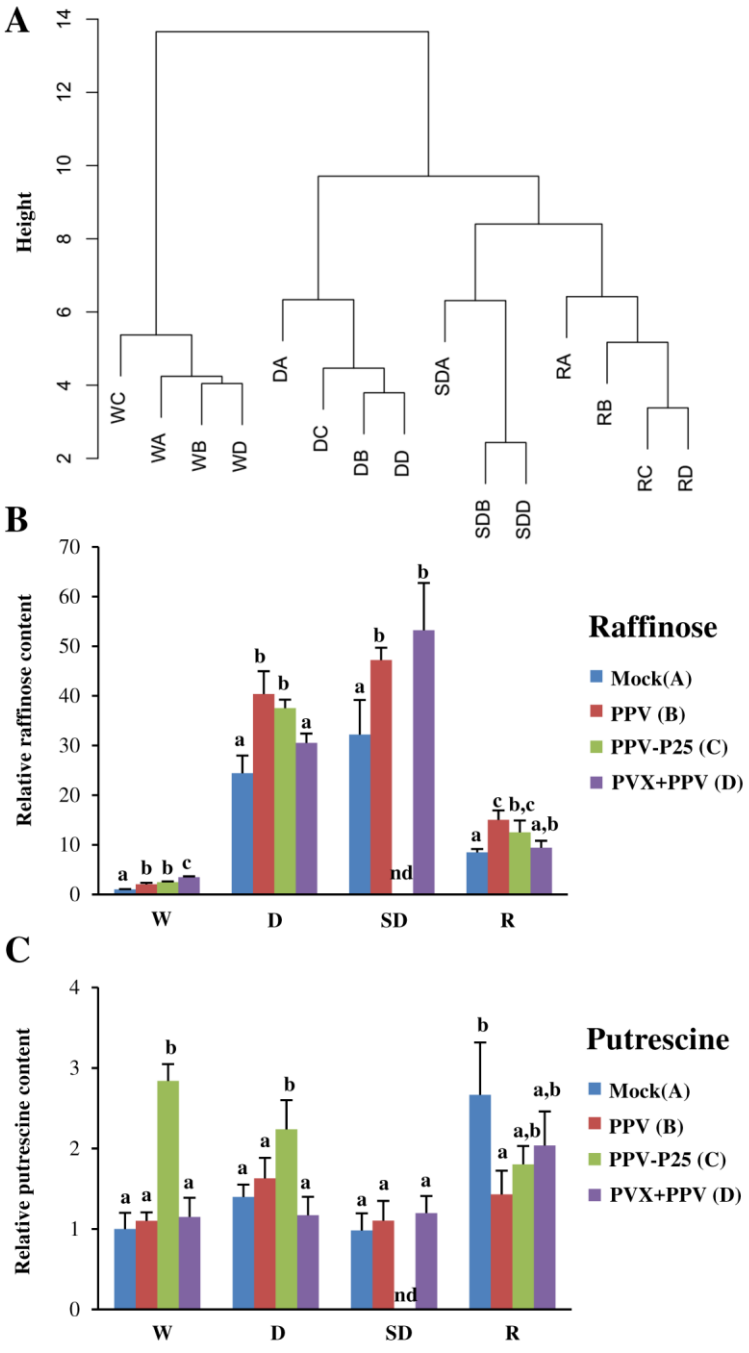

Figure 8: Metabolic profiles of mock-inoculated and virus-infected Arabidopsis plants grown under different water regimes. (A) Hierarchical clustering of metabolic data separating treatments of each water regime. Data are given as mean values of 44 metabolites with n = 4. Water regimes are as follows: watered (W), drought (D), severe drought (SD), and reinstatement of watering (R). Treatments are as follows: mock (A), PPV-GFP (B), PPV-P25 (C), PVX + PPV-GFP (D). Hierarchical clustering was performed using R package. (B) Relative raffinose and (C) putrescine contents in mock-inoculated and

virus-infected Arabidopsis leaves. The value of WA sample was set at 1 and other data calculated relative to this value. Statistically significant differences between means were determined by employing Scheffé's multiple range test. Different letters indicate significant differences at $P < 0.05$. nd, not determined.

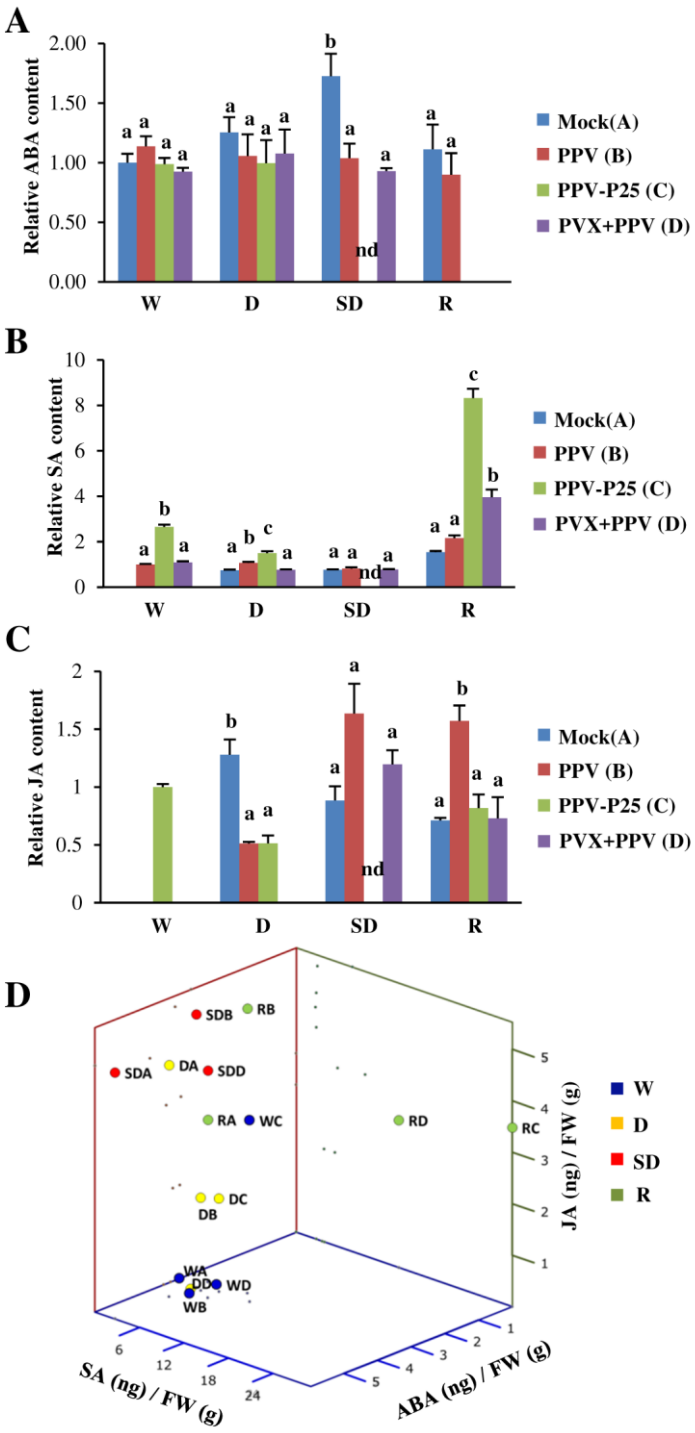

Figure 9: Hormonal profiles of mock-inoculated and virus-infected Arabidopsis plants grown under different water regimes. Rossete leaves were collected from plants at different time points after inoculation and relative levels of (A) abscisic acid (ABA), (B) salicylic acid (SA) and (C) jasmonic acid (JA) were determined by GC/TOF-MS. Statistically significant differences between means were determined for between-groups comparisons within each watering condition by employing Mann-

Whitney U test with a Bonferroni correction for multiple comparisons of α to α= 0.008 (A), and Scheffé's multiple range test ($P < 0.05$) (B and C). (D) Tridimensional representation of the hormonal profiling data. Water regimes were as follows: watered (W), drought (D), severe drought (SD), and reinstatement of watering (R). Treatments were as follows: mock (A), PPV-GFP (B), PPV-P25 (C), PVX + PPV-GFP (D). The value of WA sample in (A), WB in (B), and WC in (C) was set at 1 and other data calculated relative to this value. nd, not determined.

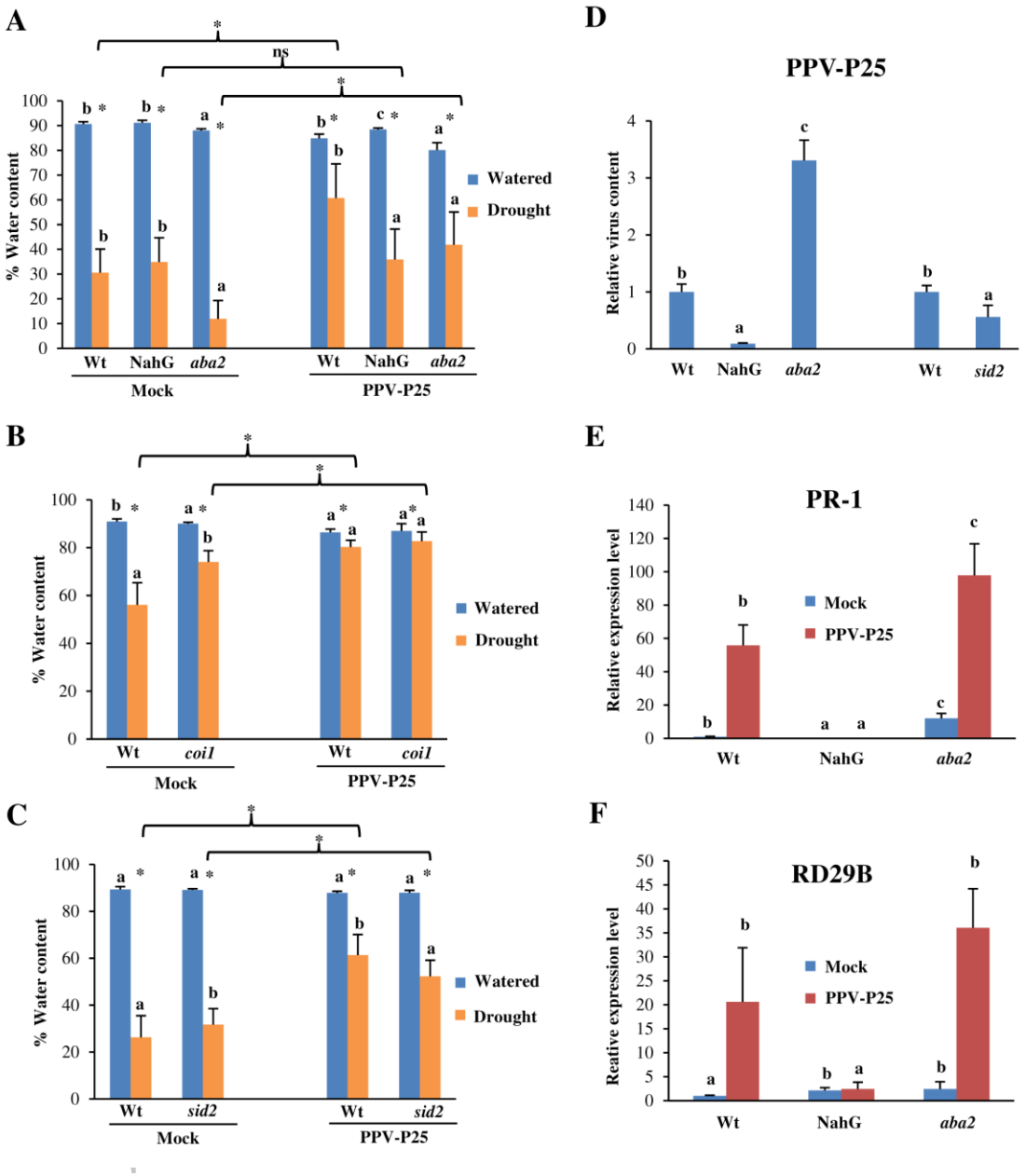

Figure 10: Virus-induced drought tolerance in hormone-deficient Arabidopsis plants. (A) Water content percentage in mock-inoculated and PPV-P25 infected WT, NahG, and *aba2-1* plants at 14 days after the water was withdrawn (daww). Statistical comparisons between means were made among treatments within each watering condition by employing Scheffé's multiple range test. Different letters indicate significant differences at $P < 0.05$. For pairwise comparisons, asterisks indicate significant differences between treatments (Student's t-test, $P < 0.05$); ns: not significative. (B) Water content percentage in mock-inoculated and PPV-P25 infected WT and *coi1-1* plants at 14 daww. Statistical

comparisons between means were made among treatments within each watering condition by employing Student's t-test ($P < 0.05$) (C) Water content percentage in mock-inoculated and PPV-P25 infected WT and *sid2-2* plants at 14 daww. Statistical comparisons between means were made among treatments within each watering condition by employing Student's t-test ($P < 0.05$). (D) Comparative analysis of viral load estimated by qRT-PCR in WT and hormone-deficient plants at 15 days after inoculation (dai). In each of two separate experiments the value of the WT sample was set at 1 after normalization to the *TUB5* internal control, and other data calculated relative to this value. (E) Comparative analysis of PR1 expression, estimated by qRT-PCR in WT and hormone-deficient plants at 15 dai. (F) Comparative analysis of RD29B expression, estimated by qRT-PCR in WT and hormone-deficient plants at 15 dai. Statistical comparisons between means were made by employing Mann-Whitney U test for between-groups comparisons with a Bonferroni correction for multiple comparisons of α to α= 0.0167. Different letters indicate significant differences at $P < 0.0167$. Data represent the means ± standard errors of 13 to 20 plants that received the same treatment.