JOURNAL OF GENERAL VIROLOGY

RESEARCH ARTICLE

Aguilar *et al.*, *Journal of General Virology* 2020;101:122–135

DOI 10.1099/jgv.0.001353

MICROBIOLOGY SOCIETY

Virus infection induces resistance to *Pseudomonas syringae* and to drought in both compatible and incompatible bacteria–host interactions, which are compromised under conditions of elevated temperature and CO_2 levels

Emmanuel Aguilar[1], Francisco J. del Toro[1], David Figueira-Galán[1], Weina Hou[2], Tomás Canto[1] and Francisco Tenllado[1,*]

Abstract

Plants are simultaneously exposed to a variety of biotic and abiotic stresses, such as infections by viruses and bacteria, or drought. This study aimed to improve our understanding of interactions between viral and bacterial pathogens and the environment in the incompatible host *Nicotiana benthamiana* and the susceptible host *Arabidopsis thaliana*, and the contribution of viral virulence proteins to these responses. Infection by the *Potato virus X* (PVX)/*Plum pox virus* (PPV) pathosystem induced resistance to *Pseudomonas syringae* (Pst) and to drought in both compatible and incompatible bacteria–host interactions, once a threshold level of defence responses was triggered by the virulence proteins P25 of PVX and the helper component proteinase of PPV. Virus-induced resistance to Pst was compromised in salicylic acid and jasmonic acid signalling-deficient *Arabidopsis* but not in *N. benthamiana* lines. Elevated temperature and CO_2 levels, parameters associated with climate change, negatively affected resistance to Pst and to drought induced by virus infection, and this correlated with diminished H_2O_2 production, decreased expression of defence genes and a drop in virus titres. Thus, diminished virulence should be considered as a potential factor limiting the outcome of beneficial trade-offs in the response of virus-infected plants to drought or bacterial pathogens under a climate change scenario.

INTRODUCTION

Plants are constantly exposed to a variety of combined biotic and abiotic stresses throughout their lifespan, including fungal, bacterial and viral infections, as well as situations of drought and heat waves [1]. Each of these stresses impacts negatively on plant growth and development, and causes considerable losses in crop yields worldwide. Emerging evidence suggests that combined abiotic and biotic stresses result in plant responses that are different from, and sometimes in contrast to, those observed for each individual stress [2]. In addition, plants exhibit shared responses that are common to individual stresses and to stress combinations. For instance, it has been shown that in some cases virus-infected plants exhibit improved tolerance to drought and other abiotic stresses [3–6]. Similarly, plant responses to viruses involve metabolic, physiological and gene expression changes that overlap, at least partially, with the defence responses elicited by other microbial pathogens [7–9].

There is a large body of literature studying the effects of both abiotic and biotic stresses on plant performance, including interactions between these stresses [1, 10]. However, virus–bacteria interactions have been studied less frequently than other microbial inter-kingdom interactions [11]. A number of interactions between plant viruses and bacteria have been investigated where bacteria provide protection against viral pathogens. Specifically, it has been reported that different rhizobacteria protect against multiple plant pathogens, including viruses, through the induction of

Received 01 July 2019; Accepted 21 October 2019; Published 15 November 2019

Author affiliations: [1]Departamento de Biotecnología Microbiana y de Plantas, Centro de Investigaciones Biológicas, CSIC, Madrid 28040, Spain; [2]Centre for the Research and Technology of Agro-Environmental and Biological Sciences, Department of Biology, University of Minho, 4710-057, Braga, Portugal.
***Correspondence:** Francisco Tenllado, tenllado@cib.csic.es

Keywords: viral virulence proteins; *Potato virus X*; *Plum pox virus*; climate change; incompatible interaction.

Abbreviations: CaMV, *Cauliflower mosaic virus*; CCC, climate change-associated conditions; Col-0, ecotype Columbia; CP, coat protein; dai, days after agro-infiltration; GUS, β-glucuronidase; GUS, β-glucuronidase; HC-Pro, helper component-proteinase; HR, hypersensitive response; NahG, salicylate hydroxylase gene; ppm, parts-per-million; PPV, *Plum pox virus*; Pst, *P. syringae* pv. *tomato*; PVX, *Potato virus X*; ROS, reactive oxygen species; SA, salicylic acid; Std, standard; ZYMV, *Zucchini yellow mosaic virus*.

Three supplementary figures are available with the online version of this article.

systemic resistance [12]. Similarly, the interactions of plants with viruses affect their responses to bacterial pathogens. For instance, *Arabidopsis thaliana* infected with *Cauliflower mosaic virus* (CaMV) exhibited increased susceptibility to *Pseudomonas syringa*e [13]. In a study by Tollenaere *et al.* [14], *Xanthomonas oryzae* load was significantly increased in rice plants when co-infected with *Rice yellow mottle virus*, whereas the titres of the virus were reduced in the presence of *X. oryzae*. On the other hand, it has been reported that gourd plants (*Cucurbita pepo* ssp. *texana*) infected with *Zucchini yellow mosaic virus* (ZYMV) contract a bacterial wilt infection caused by *Erwinia tracheiphila* at significantly lower rates than healthy plants [15]. Thus, the outcomes of these interactions can provide increased resistance or susceptibility toward any of the two pathogens depending on the plant species, pathogen and environmental conditions.

Plant diseases are the result of interactions between a susceptible host plant, a virulent pathogen (or pathogens) and the environment. Changes in environmental conditions are strongly associated with differences in the level of losses in crops caused by diseases. The build-up in the atmosphere of greenhouse gases deriving from anthropogenic emissions is very likely going to increase global average temperatures and the CO_2 levels of the troposphere [16]. The changes associated with global warming (i.e. heat waves, drought, etc.) may thus affect the incidence and severity of plant diseases and influence the further co-evolution of plants and their pathogens [17]. Moreover, changing environmental conditions may weaken or enhance plant defence responses, depending on the timing, nature and severity of each particular stress [18, 19]. Most studies that address climate change scenarios involve single parameters, such as temperature and CO_2, and single pathogens, but none have been conducted so far that examine the effects on mixed infections caused by viruses and bacteria [20].

The synergistic interaction involving *Potato virus X* (PVX) and members of the genus *Potyvirus* constitutes an excellent system for investigating interactions between plant viruses and bacteria and their dependence on virulence [21]. Compared to single infections, co-infection of PVX and *Plum pox virus* (PPV) resulted in increased virulence in *Nicotiana benthamiana* plants, which correlated with severe oxidative stress and up-regulated expression of defence-related genes [22]. Recent findings indicate that the P25 protein of PVX is the major virulence determinant involved in PVX–potyvirus-associated synergisms, with the helper component proteinase (HC-Pro) protein from potyviruses having an auxiliary role as a suppressor of the host defence mechanism based on RNA silencing [23]. Indeed, the expression of the PVX P25 protein was sufficient to increase the virulence of PPV and, conversely, the expression of HC-Pro from PPV was sufficient to increase the virulence of PVX [24, 25]. Thus, the expression of the PVX P25 and the PPV HC-Pro proteins by either a PPV or a PVX vector induced an increase of virulence comparable to that observed in plants doubly infected with PVX and PPV.

P. syringae pv. *tomato* (Pst) DC3000, one of the most widely studied strains of Pst, is pathogenic in the model plant *A. thaliana* and induces an incompatible interaction associated with a hypersensitive response (HR)-like response in *N. benthamiana*, thus providing a model pathosystem for studying both compatible and incompatible host–pathogen interactions [26]. Pst elicits the expression of defence responses, including callose deposition, phytoalexins and reactive oxygen species (ROS), as well as transcriptional activation of defence-related genes. Plant hormones also play a role in modulating resistance to Pst, with the defence hormone salicylic acid (SA) having a more prominent effect [27, 28].

The present study was aimed at understanding the interactions that occur between viral and bacterial pathogens and two model plants, *N. benthamiana* and *A. thaliana*, under different environment conditions, and the contribution of viral virulence proteins to these responses. In particular, we assessed beneficial trade-offs that the host may obtain from infection by the PVX/PPV pathosystem when confronted by either a bacterial pathogen or drought under standard or altered ambient conditions of elevated temperature and CO_2 levels. Although there is already substantial information available on the metabolic, physiological or molecular changes that take place in plants in response to individual stresses, basic and experimental studies on the effects of combined stresses, i.e. virus–bacteria and virus–drought, on plants and their relationship with virus virulence under a climate change scenario are entirely lacking.

METHODS

Plant materials

The transgenic *N. benthamiana* plants expressing the salicylate hydroxylase gene (*NbNahG*) and the *NbCOI1* IR line, in which *NbCOI1* was silenced by an RNAi hairpin construct, have been described previously [29, 30]. The following *A. thaliana* transgenic and mutant lines used in this study were derived from ecotype Columbia (Col-0): *AtNahG* [31] and *Atcoi1-1* [32]. The *Atcoi1-1* mutant plants were selected from a heterozygote population using root sensitivity to 50 µM methyl jasmonate.

N. benthamiana and *A. thaliana* plants were kept in environment-controlled growth chambers with a 16/8 h day/night photoperiod and daylight intensity of ~2500 lux. Plants were kept in either one of two sets of environmental conditions with regard to temperature and CO_2 levels: standard (Std) conditions of 23 °C and current atmospheric CO_2 partial pressures [~405 parts per million (p.p.m.)] and conditions of simultaneously elevated temperature (30 °C) and CO_2 partial pressures of ~970 p.p.m. [climate change-associated conditions (CCC)].

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 HC-Pro (PVX-HC) sequences under the control of the PVX duplicated coat protein (CP) promoter has been described previously [24, 33]. PPV expressing either PVX P25 (PPV-P25) or GFP (PPV-GFP) sequences in the viral polyprotein have been described previously [24]. *A. tumefaciens* carrying pCAMBIA1305.1 containing a gene encoding *β-glucuronidase* (GUS) was used as a negative control.

Agro-infiltration

Four-week-old *N. benthamiana* plants were agro-infiltrated with *A. tumefaciens* bearing the indicated binary vectors [34]. Two-week-old *A. thaliana* seedlings were agro-infiltrated *in vitro* with the indicated binary vectors as described previously [35]. In those cases where expression was to take place at higher temperatures (CCC), plants were maintained during the first 24 h after infiltration at 23 °C to allow for the *Agrobacterium*-mediated T-DNA transfer into plant tissues to take place, as described previously [36]. For drought experiments, *A. thaliana* seedlings were transferred to 5×5 cm individual pots containing a mixture of soil and vermiculite [3:1 (v/v)] at 8 days after agro-infiltration (a.i.) with viruses.

RNA and protein gel blot analysis

Total RNA was extracted from upper, non-inoculated leaves as described previously [37]. Real-time quantitative RT-PCR (qRT-PCR) for virus detection was performed using primers that amplify a region from nucleotides 4668 to 4810 of the PPV sequence (Table S1, available in the online version of this article). qRT-PCR for the analysis of defence gene expression was performed with gene-specific primers [3, 38]. Gene expression analyses were performed using three biological replications. The relative quantification of PCR products was calculated by the comparative cycle threshold ($\Delta\Delta C_t$) method as described previously [30]. Amplification of *β-TUBULIN5* (*TUB5*) and 18S rRNA was chosen for normalization in *Arabidopsis* and *N. benthamiana*, respectively, because of their similar levels of expression across all treatments.

PPV CP was detected with a rabbit polyclonal antiserum (1:2000 dilution) (Instituto Valenciano de Investigaciones Agrarias, Valencia, Spain) using an appropriate secondary antibody conjugated with HRP. Detection was performed using the ECL system (Amersham Biosciences).

For 3,3′-diaminobenzidine (DAB) staining to detect H_2O_2 production, leaves were vacuum infiltrated for 10 min with DAB solution at 1 mg ml^{-1}.

Analyses of bacterial growth

The bacterial strains used in this study were *P. syringae* pv. *tomato* (Pst) DC3000, which is a non-pathogenic bacteria in *N. benthamiana* despite proliferating 10^5 times for 3 days, and Pst DC3000 *avrRpm1*, which is avirulent in *A. thaliana* [39]. Bacterial inoculations were performed by infiltration and spray inoculation. Prior to inoculation, bacteria were grown overnight at 28 °C in Petri plates with King's B medium. For infiltration assays, three non-agroinfiltrated, systemic upper leaves of at least six plants were infiltrated with a bacterial suspension at a concentration of 10^5 colony-forming units (c.f.u.) ml^{-1}. For spray inoculation, 20 plants were sprayed with a bacterial suspension at 10^8 c.f.u. ml^{-1} with 0.03% Silwet L-77 (OSI Specialties, Inc., Danbury, CT, USA). For analyses of systemic acquired resistance, three leaves of each independent plant were first infiltrated with a bacterial suspension at 10^7 c.f.u. ml^{-1} and 48 h later the upper leaves of pretreated plants were subjected to a second bacterial infiltration at 10^5 c.f.u. ml^{-1}. Discs from infected leaves were excised at the times of interest, pooled in triplicate, homogenized and used for counting bacterial growth in Petri plates. In all cases, the reported results are the means and standard errors of the values obtained in two or three independent experiments.

An *Arabidopsis* seedling flood-inoculation method was followed according to the method described by Ishiga *et al.* [40]. Briefly, 50 ml of bacterial suspension in sterile distilled H_2O containing 0.025% Silwet L-77 was dispensed into the plate containing *Arabidopsis* seedlings at 10 days a.i. with viruses, and the plates were incubated for 2–3 min at room temperature. After the bacterial suspension was removed by decantation, plates containing inoculated plants were sealed with 3 M Micropore tape (3 M, St Paul, MN, USA) and incubated with a light intensity of 150–200 μE m^{-2} s^{-1} and a 16 h light/8 h dark photoperiod. Internal bacterial populations were evaluated from six biological replicates and each replicate represented a pooled sample of four independent seedlings from a single experiment grown in a single Petri dish. Inoculated seedlings were collected by cutting the hypocotyls to separate the above agar parts (whole rosettes) from the plate, and the total weight of inoculated seedlings was measured. The seedlings were surface-sterilized with 5% H_2O_2 for 3 min. After being washed three times with sterile distilled water, a pooled sample of four seedlings was homogenized in 5 ml sterile distilled water using a mortar and pestle, and diluted samples were plated onto King's B medium containing rifampicin (50 mg l^{-1}). Two days after the plating of diluted samples, the bacterial c.f.u. were counted and normalized as c.f.u. mg^{-1} using the total weights of the inoculated plants. The bacterial populations were evaluated in three independent experiments.

Drought and water content measurements

Plants were bottom-watered for 3 h to saturate the soil at 10 days a.i. for *N. benthamiana* plants or 18 days a.i. for *Arabidopsis* plants, 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.

Water content analysis was performed in both drought-stressed and well-watered plants according to Westwood *et al.* [5]. Whole aerial tissue of both drought-stressed and well-watered plants was harvested and fresh weights were recorded. Samples were then dried over a period of 7 days at 65 °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 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 analysed in each of the three separate experiments.

The relative soil water content (RSWC) was calculated following the formula: (fresh weight−dry weight)/(initial weight−dry weight)×100, as described previously [41]. Water loss experiments were conducted according to Westwood *et al.* [5].

Stomatal conductance was measured using a leaf porometer (SC-1 Decagon-T, Pullman, USA) at 25 °C, 65% relative humidity. Attached, fully expanded leaves of *N. benthamiana* plants were placed in the chamber and repeat measurements of conductance from 12 plants per treatment were taken. Stomatal conductance was not performed in *Arabidopsis* because the leaves did not adequately cover the aperture of the sensor head in our assay conditions.

Statistical analysis

All statistical analyses were performed using the statistical software IBM SPSS Statistics v.20 (IBM Corp). For each experiment, samples were assessed for normality via the Shapiro–Wilk test, and for equality of variances using Levene's test. For experiments with approximately normally distributed samples of equal variance, one-way analysis of variance (ANOVA) followed by Scheffé's post hoc test was performed. Otherwise, a nonparametric Mann–Whitney U test was employed, with the Bonferroni correction for multiple comparisons between samples applied. For comparisons between pairs of means (pairwise comparisons), Student's *t*-tests or Mann–Whitney U tests were employed, depending on the normality of the data.

RESULTS

Viral infection enhances incompatible host response against Pst

To test if viral infection affects incompatible interaction against bacterial pathogens, we examined the growth of *P. syringae* pv. *tomato* DC3000 (Pst) in *N. benthamiana* plants infected with PVX, PPV expressing green fluorescent protein (PPV-GFP) or PPV expressing the virulence protein P25 of PVX (PPV-P25). Control plants were infiltrated with *Agrobacterium* harbouring a binary construct expressing GUS. After he appearance of viral symptoms at 10 days a.i., the plants were inoculated with the avirulent strain Pst at 10^5 c.f.u. ml^{-1}. The bacterial population was estimated by plating serial dilutions of leaf extracts at 3 days post-inoculation (p.i.). Pst exhibited decreased growth on PVX- and PPV-P25-infected plants compared with control virus-free plants and plants infected with PPV-GFP (Fig. 1a). Accordingly, besides vein clearing symptoms induced by virus infections, the Pst-induced HR-like response was diminished in *N. benthamiana* plants infected with PVX and PPV-P25 compared to controls at 4 days after inoculation with Pst (Fig. 1b).

Expression of P25 by PPV-P25 conferred enhanced virulence in *N. benthamiana* as compared to PPV expressing GFP [24]. We examined defence-associated markers in plants infected with either PPV-GFP or PPV-P25 before Pst inoculation. PPV-P25 infection led to a greater increase of H_2O_2 production, a contributor to oxidative stress, compared to plants infected with PPV-GFP or the GUS control (Fig. 1c). Comparative analysis of virus accumulation revealed that the level of PPV CP in PPV-P25-infected plants was similar to that in plants infected with PPV-GFP, ruling out the possibility that the PPV-P25-enhanced incompatible host response against Pst was due to increased accumulation of PPV-P25 (Fig. 1d). We also assessed the expression of the SA-responsive gene *pathogenesis-related protein 1* (*PR1*), as well as transcript levels of the defence-related genes *Hin1* and *Hsr203j* in mock-inoculated and virus-infected plants. *PR1*, *Hin1* and *Hsr203j* transcript levels were increased at much greater levels in PPV-P25-infected plants compared with plants infected with PPV-GFP and control plants (Fig. 1e–g). Thus, virus-enhanced incompatible host response against Pst correlated with the level of defence responses induced by virus infection.

It has been reported that the expression of the HC-Pro protein of PPV from a PVX vector (PVX-HC) was sufficient to induce an increase of PVX virulence [25]. To determine if virulence affects virus-enhanced incompatible host response against Pst as a general phenomenon, *N. benthamiana* plants were infected with either PVX-HC or the parental PVX vector. Bacterial titres were significantly lower in PVX- and PVX-HC-infected plants than in virus-free plants, particularly for the virulent recombinant virus, indicating that the increased incompatible host response against Pst in virus-infected plants correlated with the level of virulence (Fig. 1g).

Virus-enhanced incompatible host response against Pst is not altered in hormone signalling-deficient plants

In order to determine whether hormone signalling contributed to the virus-enhanced incompatible host response against Pst in *N. benthamiana* plants, transgenic lines deficient in jasmonic acid (JA) perception (*NbCOI1* IR line) or SA accumulation (*NbNahG* line), as well as the wild-type (WT) control, were assessed for bacterial accumulation upon infection by either PPV-P25 or PPV-GFP at 10 days a.i. Bacterial titres were determined in virus-infected, transgenic and control plants at 0 and 3 days p.i. Statistically significant differences in bacterial growth were observed in PPV-P25-infected WT, *NbCOI1* IR and *NbNahG* plants compared to their respective mock-inoculated controls at 3 days p.i (Fig. 2a). However, there was no significant difference in Pst titres in PPV-GFP-infected plants of the three genotypes compared with control virus-free plants. Next, we monitored the amount of PPV-P25 RNA in plants just prior to bacterial inoculation (10 days a.i.) by qRT-PCR (Fig. 2b). PPV-P25 accumulation was significantly lower in *NbCOI1* IR plants than in the WT controls, whereas virus accumulated at similar levels in *NbNahG* compared to WT plants, confirming

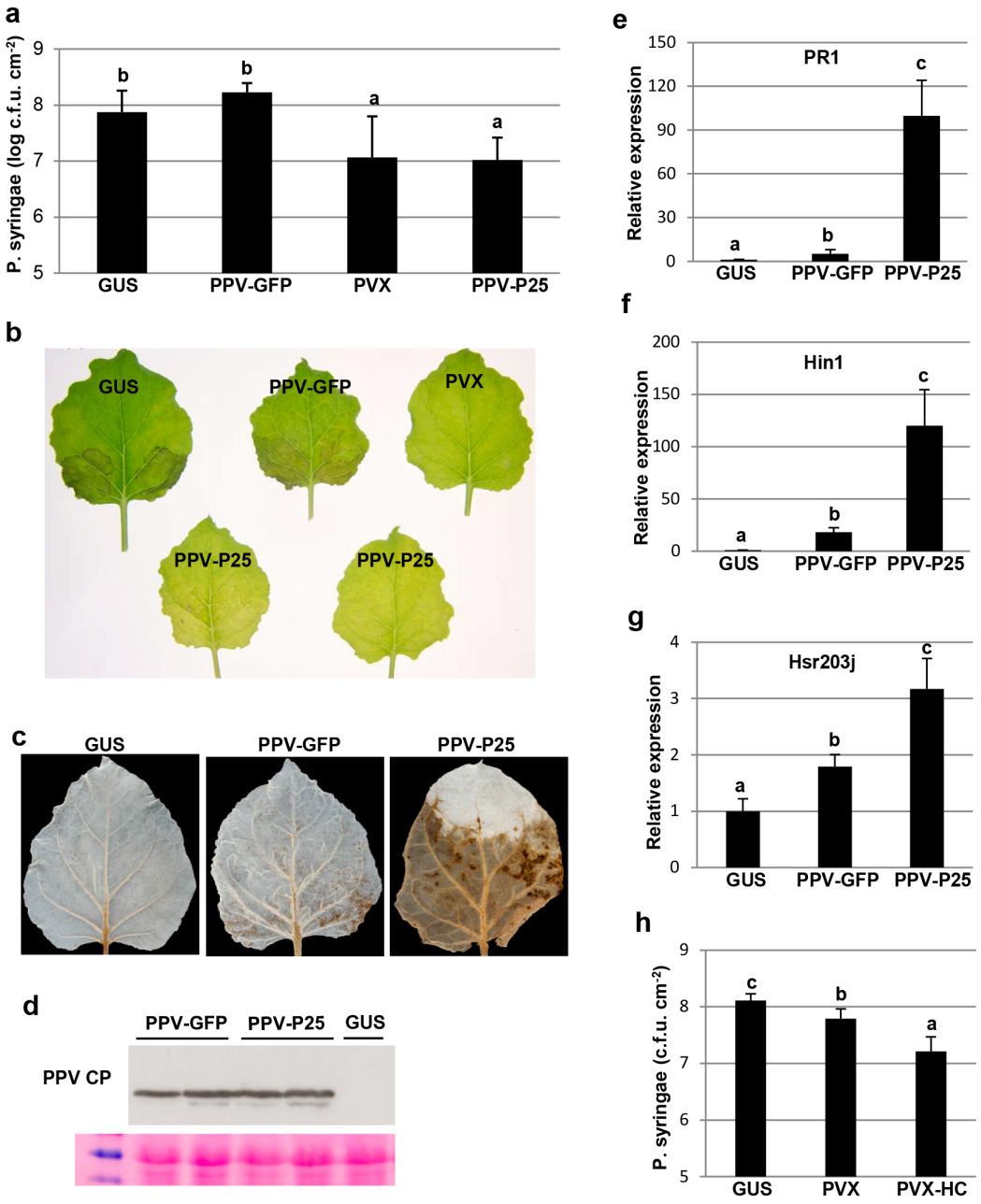

Fig. 1. Effects of viral infections on growth of *Pseudomonas syringae* pv. *tomato* DC3000 (Pst) in *Nicotiana benthamiana*. (a) Plants infected with *Potato virus X* (PVX), *Plum pox virus* expressing green fluorescent protein (PPV-GFP), PPV expressing the P25 protein of PVX (PPV-P25) or GUS as a control, were inoculated with Pst (10^5 c.f.u. ml^{-1}) at 10 days after agro-infiltration (a.i. Bacterial populations were determined at 3 days post-inoculation (p.i.) Data represent the means±standard errors of 18 replicates in 3 independent experiments, each consisting of 6 plants that received the same treatment. Statistical comparisons between means were made by employing the Mann–Whitney U test with a Bonferroni correction for multiple comparisons of alpha=0.008. Different letters indicate significant differences at $P<0.008$. (b) Pst-induced HR-like response in *N. benthamiana* plants agroinfiltrated with the different viruses or GUS as a control at 4 days p.i. (c) Leaves infected with PPV-GFP, PPV-P25 and control leaf were stained with 3,3′-diaminobenzidine (DAB) solution before Pst inoculation. DAB formed a polymerization product upon reaction with H_2O_2. (d) Western blot analysis of extracts from plants infected with PPV-GFP, PPV-P25 or GUS as a control before Pst inoculation, using antibodies against PPV CP. Two independent pooled samples were analysed for each inoculum. The lower panel shows the Ponceau S-stained membrane after blotting, as a control for loading. The relative expression of *PR1* (e), *Hin1* (f) and *Hsr203j* (g) in PPV-GFP, PPV-P25 and control plants was estimated before bacterial inoculation by qRT-PCR. Expression of the 18S rRNA gene served as a control. Statistical comparisons between means were made by employing the Mann–Whitney U test with a Bonferroni correction for multiple comparisons of alpha=0.016 in (d) and (e) ($P<0.016$), and by Scheffé's multiple range test in (f) ($P<0.05$). Different letters indicate significant differences. (h) Bacterial populations in plants infected with PVX, PVX expressing the HC-Pro protein of PPV (PVX-HC), or GUS as a control were determined at 3 days p.i. Statistical comparisons between means were made by employing Scheffé's multiple range test ($P<0.05$).

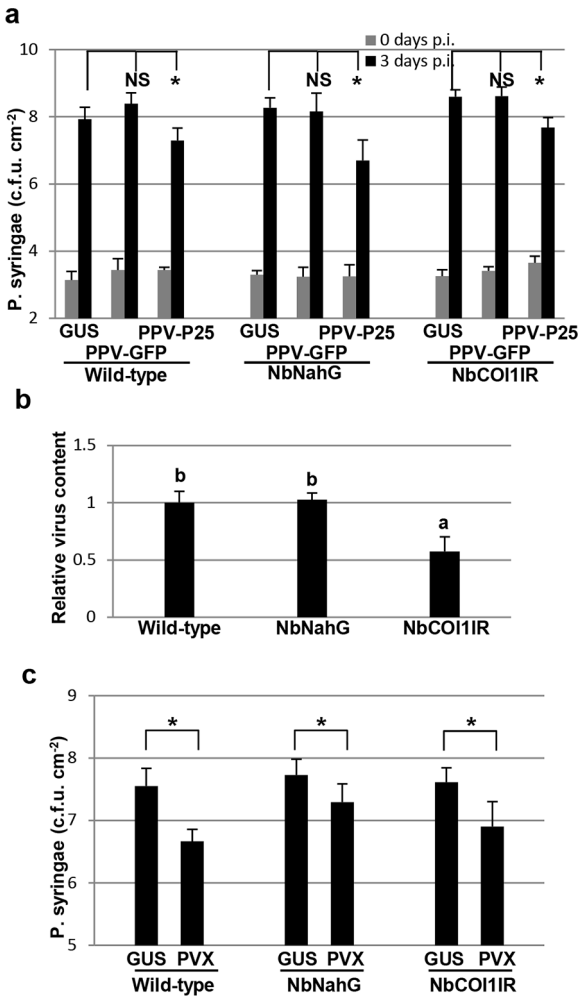

Fig. 2. Virus-enhanced incompatible host response against Pst in hormone-deficient *Nicotiana benthamiana* plants. (a) Transgenic lines deficient in JA perception (*NbCOI1 IR*) or SA accumulation (*NbNahG*), as well as the wild-type (WT) plants infected with PPV expressing the green fluorescent protein (PPV-GFP), PPV expressing P25 of PVX (PPV-P25), or GUS as a control, were inoculated with Pst (10^5 c.f.u. ml^{-1}) at 10 days after agro-infiltration (a.i.). Bacterial populations were determined at 0 and 3 days post-inoculation (p.i.). (b) qRT-PCR was used to analyse the accumulation of viral RNA in WT and transgenic plants infected with PPV-P25 just prior to bacterial inoculation. Multiple comparisons of means were made among genotypes by employing Scheffé's multiple range test ($P<0.05$). (c) Transgenic *NbCOI1 IR*, *NbNahG* and WT plants infected with PVX or GUS as a control, were inoculated with Pst at 10^5 c.f.u. ml^{-1}. Bacterial populations were determined at 3 days p.i. Data represent the means±standard errors of 12 replicates in 2 independent experiments, each consisting of 6 plants that received the same treatment. For pairwise comparisons, asterisks indicate significant differences between treatments (Student's *t*-test, $P<0.05$).

a previous report that the effect of SA-mediated defences on PPV accumulation in *N. benthamiana* is negligible [29].

We next inoculated Pst on WT, *NbCOI1* IR and *NbNahG* plants infected with PVX at 10 days a.i. As observed in plants infected with PPV-P25, a significant reduction in Pst titres was monitored in PVX-infected plants of the three genotypes compared to their respective mock-inoculated controls at 3 days p.i. (Fig. 2c). Overall, these findings suggest that defence responses beyond the SA- and JA-dependent signalling pathways may be involved in the enhanced incompatible host response of virus-infected *N. benthamiana* plants to Pst.

Virus-induced resistance to Pst also occurs in *Arabidopsis*, but is compromised in hormone signalling-deficient plants

To test if viral infection can induce resistance to Pst in a susceptible host, we first inoculated Pst on *A. thaliana* by infiltration with a bacterial suspension at 10^6 c.f.u. ml^{-1} into the abaxial side of leaves from plants infected with PPV, PPV-P25 or control virus-free plants. We omitted PVX from these experiments because *A. thaliana* is not a systemic host for PVX. There was no significant difference in Pst titres or disease expression in virus-infected plants compared with control virus-free plants, indicating that virus infection did not induce resistance to Pst in *Arabidopsis* when the bacteria was inoculated by infiltration (Fig. S1a,b). As a control, Pst induced systemic acquired resistance in virus-free *A. thaliana* plants when lower leaves were first inoculated with Pst and, 48 h later, distal leaves located above the primarily inoculated tissues were challenged with Pst (Fig. S1c).

P. syringae generally enters host tissues through natural openings such as stomata or wounds, and multiplies in the apoplast to cause disease [42]. As pressure infiltration of bacteria into the apoplast bypasses the first steps of invasion through stomata, we used spray inoculation to mimic the natural infection process of *P. syringae* in *A. thaliana*. Statistically significant differences in bacterial growth were observed in PPV-P25-infected *Arabidopsis* plants compared to mock-inoculated controls at 3 and 4 days p.i. (Fig. S1d), suggesting that virus-induced resistance acts in the early steps of bacterial infection and probably affects the control of stomata opening. Accordingly, the symptoms induced by Pst were diminished in plants infected with PPV-P25 compared to controls at 4 days after inoculation with Pst (Fig. S1e).

Wild-type and hormone signalling-deficient *Arabidopsis* seedlings infected with PPV-P25 and control virus-free seedlings were inoculated with a Pst suspension (5×10^6 c.f.u. ml^{-1}) at 10 days a.i. using a rapid seedling flood-inoculation method [40]. Bacterial growth was determined by measuring bacterial titres in whole seedlings at 0 and 3 days p.i. Pst exhibited decreased growth on PPV-P25-infected WT seedlings compared with control virus-free seedlings (Fig. 3a, b). Interestingly, there was no significant difference in Pst titres in virus-infected lines deficient in JA perception (*Atcoi1-1* line) and SA accumulation (*AtNahG* line) compared to their respective mock-inoculated controls at 3 days p.i. To examine whether virus-induced resistance to Pst was associated with accumulation levels of PPV-P25, we monitored the amount of viral RNA in seedlings just prior to bacterial inoculation (10 days a.i.) by qRT-PCR. PPV-P25 accumulation was halved in *Atcoi1-1* and *AtNahG* seedlings compared to wild-type seedlings (Fig. 3c). PPV-P25 titres

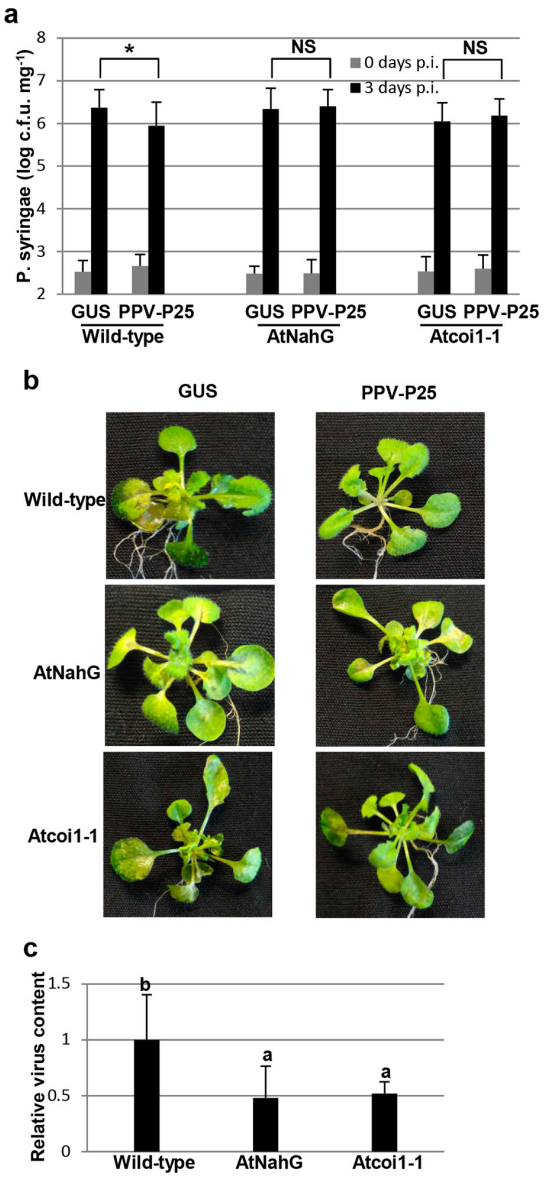

Fig. 3. Virus-induced resistance to Pst in hormone-deficient *Arabidopsis thaliana* seedlings. (a) Transgenic lines deficient in JA perception (*Atcoi1-1*) or SA accumulation (*AtNahG*), as well as the wild-type (WT) seedlings infected with PPV expressing P25 of PVX (PPV-P25) or GUS as a control, were flood-inoculated with Pst (5×10^6 c.f.u. ml^{-1}) at 10 days after agro-infiltration (a.i.). Bacterial populations were determined at 0 and 3 days p.i. Data represent the means±standard errors of 16 replicates in 3 independent experiments, each consisting of 6 seedlings that received the same treatment. Asterisks indicate significant differences between treatments (Student's *t*-test, $P<0.05$). NS, not significant. (b) Disease phenotype of *Arabidopsis* seedlings inoculated with Pst at 3 days p.i. (c) qRT-PCR was used to analyse the accumulation of viral RNA in wild-type and transgenic seedlings infected with PPV-P25 just prior to bacterial inoculation. Expression of the *β-TUBULIN5* gene served as a control. Multiple comparisons of means were made among genotypes by employing Scheffé's multiple range test ($P<0.05$).

have been reported to be reduced in SA-deficient *Arabidopsis* plants [3].

Since SA and JA dependence on virus-induced resistance to Pst was found in the *Arabidopsis*–Pst compatible interaction but not in the *N. benthamiana*–Pst incompatible interaction, we speculated that these divergences could be attributed to differences between a compatible and an incompatible interaction. To examine this possibility, we measured the growth of Pst *avrRpm1* in WT, *AtNahG* and *Atcoi1-1* seedlings by flood inoculation (5×10^7 c.f.u. ml^{-1}). Pst *avrRpm1* is an avirulent strain that triggers a gene-for-gene response in Col-0. Pst *avrRpm1* exhibited decreased growth on PPV-P25-infected WT seedlings compared to mock-inoculated controls at 3 days p.i. (Fig. S2). Interestingly, there was no significant difference in Pst *avrRpm1* titres in virus-infected *AtNahG* and *Atcoi1-1* seedlings compared to their respective mock-inoculated controls, suggesting that virus-induced defences against Pst are species-specific.

Elevated temperature and CO_2 levels affect resistance to Pst induced by virus infection

We next addressed how changes in two of the main parameters related to global warming, ambient CO_2 levels and temperature, may affect virus-induced resistance to Pst. To this end, *N. benthamiana* and *Arabidopsis* plants were mock-inoculated or infected with PPV-P25, and were kept under CCC of 30 °C, and~970 p.p.m. of CO_2 or under Std conditions of 23 °C and ~405 p.p.m. of CO_2 for the time span of these experiments. In plants kept under CCC, viral symptoms were very attenuated compared to those shown by plants under Std conditions. At 10 days a.i., plants were inoculated (*N. benthamiana*) or flood-inoculated (*Arabidopsis*) with Pst, and bacterial titres were determined in virus-infected and control plants at 0 and 3 days p.i. In *N. benthamiana*, Pst exhibited decreased growth on PPV-P25-infected plants compared with control virus-free plants under both CCC and Std conditions at 3 days p.i. However, Pst titres were significantly lower in virus-infected plants kept under Std conditions than in virus-infected plants kept under CCC ($P=0.001$ and $P=0.024$, respectively) when compared with the controls (Fig. 4a, b). In *A. thaliana*, a significant reduction in Pst titres induced by virus infection was observed in plants kept under Std conditions at 3 days p.i., whereas there was no significant difference in Pst titres in virus-infected plants compared with control plants under CCC.

To determine if elevated CO_2 and temperature affect defence responses induced by virus infection, we examined defence-associated markers in virus-infected plants kept under CCC or Std conditions before Pst inoculation. In *N. benthamiana*, PPV-P25 infection led to an increase of H_2O_2 production compared to control virus-free plants grown under Std conditions (Fig. 4c). No such increase was observed in plants infected by PPV-P25 under CCC. Similarly, PPV-P25-infected *Arabidopsis* plants grown under CCC exhibited a reduction in H_2O_2 production compared to virus-infected plants grown under Std conditions (Fig. 4d).

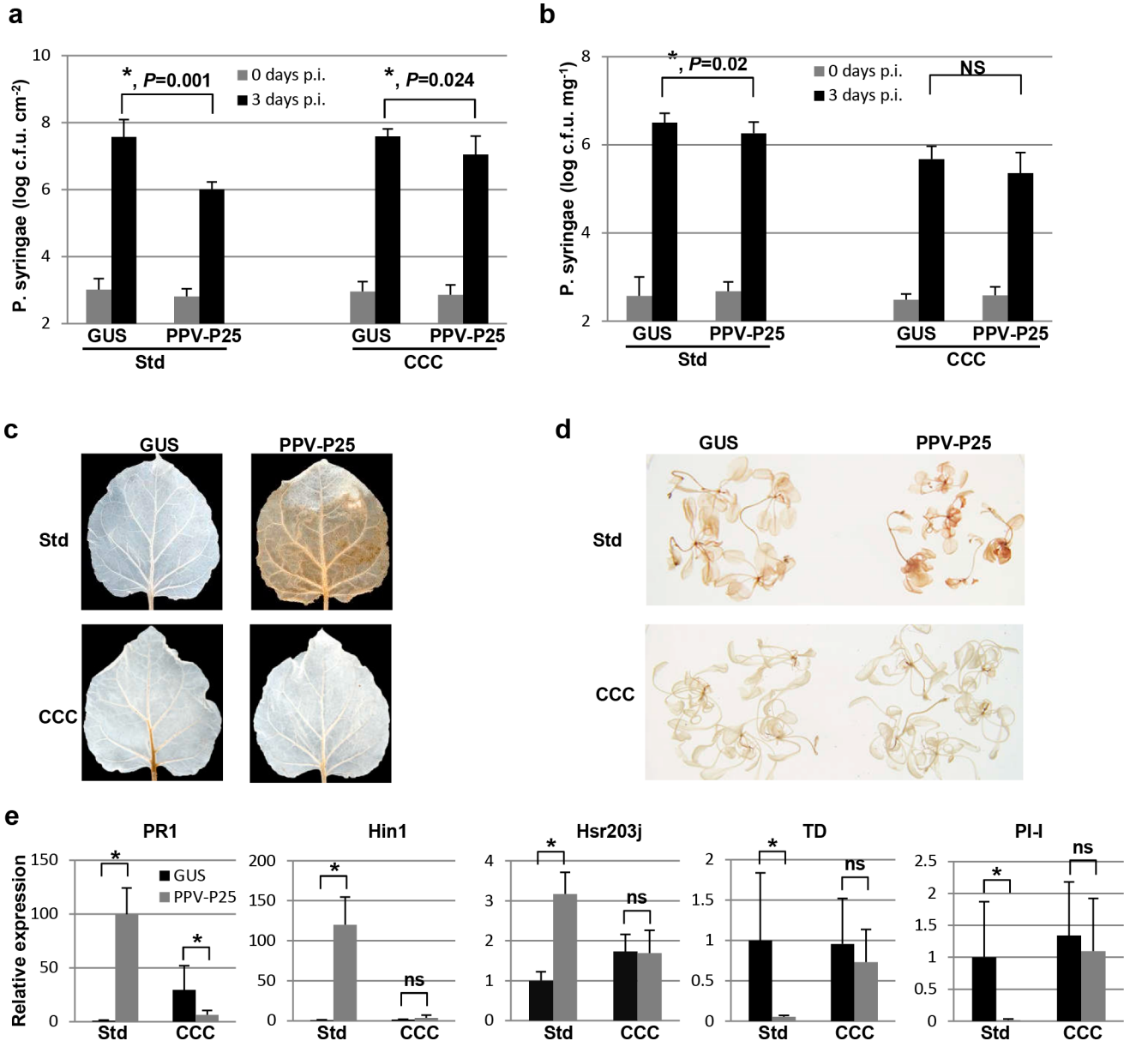

Fig. 4. Effects of virus-induced responses on grown of Pst in plants kept under standard (Std) conditions or under climate change-related conditions (CCC). (a) *Nicotiana benthamiana* and (b) *Arabidopsis thaliana* plants infected with PPV expressing the virulence protein P25 of PVX (PPV-P25) or GUS as a control were inoculated (*N. benthamiana*) or flood-inoculated (*A. thaliana*) with Pst at 10 days after agro-infiltration (a.i.). Bacterial populations were determined at 0 and 3 post-inoculation (p.i.). Data represent the means±standard errors of 12 replicates in 2 independent experiments, each consisting of 6 plants that received the same treatment. Asterisks indicate significant differences between treatments (Student's *t*-test, $P<0.05$). NS, not significant. (c) *N. benthamiana* leaves infected with PPV-P25 and control leaves were stained with 3,3′-diaminobenzidine (DAB) solution before Pst inoculation. (d) *A. thaliana* plants infected with PPV-P25 and control plants were stained with DAB solution before Pst inoculation. (e) The relative expression of *PR1*, *Hin1*, *Hsr203j*, *TD* and *PI-I* in PPV-P25 and control *N. benthamiana* plants was estimated before bacterial inoculation by qRT-PCR. Expression of the 18S rRNA gene served as a control. Asterisks indicate significant differences between treatments (Student's *t*-test, $P<0.05$).

To confirm the link between defence responses induced by virus infection and resistance to Pst, we also assessed the expression of the SA-responsive gene *PR1* and the JA-responsive genes, *threonine deaminase* (*TD*) and *proteinase inhibitor I* (*PI-I*), as well as transcript levels of *Hin1* and *Hsr203j*, in mock-inoculated and PPV-P25-infected *N. benthamiana* plants kept under CCC or Std conditions by qRT-PCR. Levels of *PR1*, *Hin1* and *Hsr203j* mRNAs were increased in virus-infected plants when compared to controls plants grown under Std conditions, whereas no such increases in gene expression were observed in plants infected with PPV-P25 kept under CCC (Fig. 4e). The

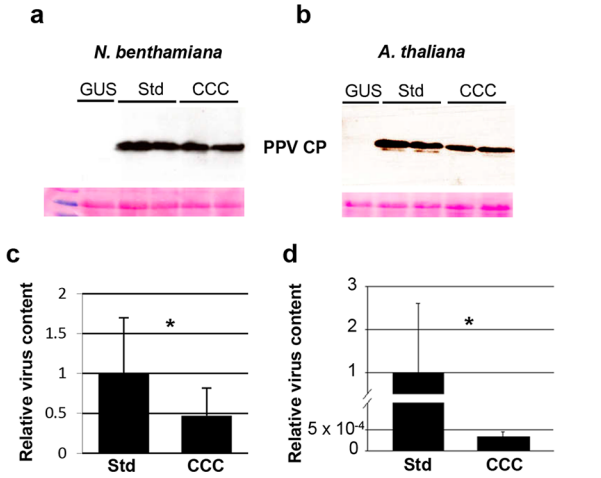

Fig. 5. Comparison of relative PPV-P25 levels in plants kept under standard (Std) conditions or under climate change-related conditions (CCC). The relative accumulation of PPV-P25 CP was estimated by Western blot in *Nicotiana benthamiana* (a) and *Arabidopsis thaliana* (b) plants grown under Std conditions or CCC using antibodies against PPV CP. Two independent pooled samples were analysed for each inoculum. The lower panel shows the Ponceau S-stained membrane after blotting, as a control for loading. The relative accumulation of PPV-P25 RNA was estimated by qRT-PCR in *N. benthamiana* (c) and *A. thaliana* (d) plants grown under Std conditions or under CCC. Expression of *β-TUBULIN5* and 18S rRNA was chosen for normalization in *Arabidopsis* and *N. benthamiana*, respectively. Statistical comparisons between means were made by employing Student's *t*-test (c) and the Mann–Whitney U test (d). Asterisks indicate significant differences between treatments ($P<0.05$).

JA-responsive genes *TD* and *PI-I* were down-regulated by PPV-P25 infection in plants grown under Std conditions, but not under CCC, most likely due to the antagonistic relationship between the JA and SA signalling pathways [27].

To examine whether environment-conditioned resistance to Pst was associated with the accumulation levels of PPV-P25, we monitored virus levels in plants at different times after agro-infiltration with viruses. Comparative analysis in the agro-infiltrated leaves by Western blot revealed that the level of PPV-P25 CP in virus-infected plants grown under CCC was similar to that in plants grown under Std conditions at 4 days a.i. (Fig. 5). However, at later stages of infection, just before bacterial challenge (10 days a.i.), the accumulation of PPV-P25 RNA was halved in *N. benthamiana* plants maintained under CCC compared to plants kept under Std conditions, whereas virus accumulated at 4000-fold lower levels in *Arabidopsis* plants grown under CCC compared to Std conditions.

Elevated temperature and CO_2 levels compromise the tolerance to drought induced by virus infection

It has been shown that virus-infected *N. benthamiana* and *Arabidopsis* plants exhibit enhanced tolerance to drought as a result of pathogen-induced physiological and metabolic adaptations [3, 6]. We studied whether virus infection still confers tolerance to drought in plants maintained under CCC. Mock-inoculated and PPV-P25-infected *N. benthamiana* plants kept under CCC or Std conditions were normally irrigated or deprived of irrigation at 10 days a.i. After withholding water, the appearance of drought symptoms in PPV-P25-infected plants grown under Std conditions was delayed by several days, and they appeared clearly less wilted than mock-inoculated plants throughout the experiment. By contrast, there were no systematic differences in leaf turgor between virus-infected and control plants grown under CCC. At the end of the experiment (22 days a.i.), a detrimental effect of viral infection on the above-ground biomass of well-watered plants was observed in Std conditions but not in CCC (Fig. S3a,b). Remarkably, the biomass of plants grown under drought treatment was greater in virus-infected plants than in control plants grown under Std conditions but not under CCC.

The water content of mock- and virus-infected plants grown under watered and drought treatments in both CCC and Std conditions was compared at 12 days after the water was withdrawn (a.w.w.), i.e. 22 days a.i. (Fig. 6a, b). Under drought growth conditions, the average water content was higher in plants infected with PPV-P25 compared to mock-inoculated plants when grown under Std conditions, whereas there was no significant difference in water content in virus-infected plants compared with controls under CCC. A small but statistically significant higher level of RSWC was observed in virus-infected plants compared with control plants under Std conditions, but not under CCC (Fig. 6c, d). Moreover, measurements of stomatal conductance, an indicator of transpiration rate, in plants before the water was withdrawn showed that virus infection caused a 2.5-fold decrease in conductance under Std conditions but not under CCC (Fig. 6e). Thus, relative differences in transpiration were correlated with differences in soil moisture and drought tolerance in virus-infected *N. benthamiana* plants.

Mock-inoculated and PPV-P25-infected *Arabidopsis* plants kept under CCC or Std conditions were normally irrigated or subjected to water stress at 18 days a.i. At the end of the experiment (33 days a.i.), a much greater detrimental effect of viral infection on the above-ground biomass of well-watered plants was observed in Std conditions than in CCC. The biomass of plants grown under drought treatment was lower in virus-infected plants than in control plants under both Std conditions and CCC (Fig. S3c,d). The water content of mock- and virus-infected plants growing under watered and drought treatments in both CCC and Std conditions conditions was compared at 15 days a.w.w. (33 days a.i.) (Fig. 7a, b). Under drought growth conditions, average water content was higher in plants infected with PPV-P25 compared to mock-inoculated plants grown under Std conditions. By contrast, the water content in virus-infected plants was lower compared with control plants when grown under CCC. A statistically significantly higher level of RSWC was monitored at the end of the experiment in virus-infected plants compared with control plants under Std conditions, whereas the opposite was the case under CCC (Fig. 7c, d). In agreement with previous

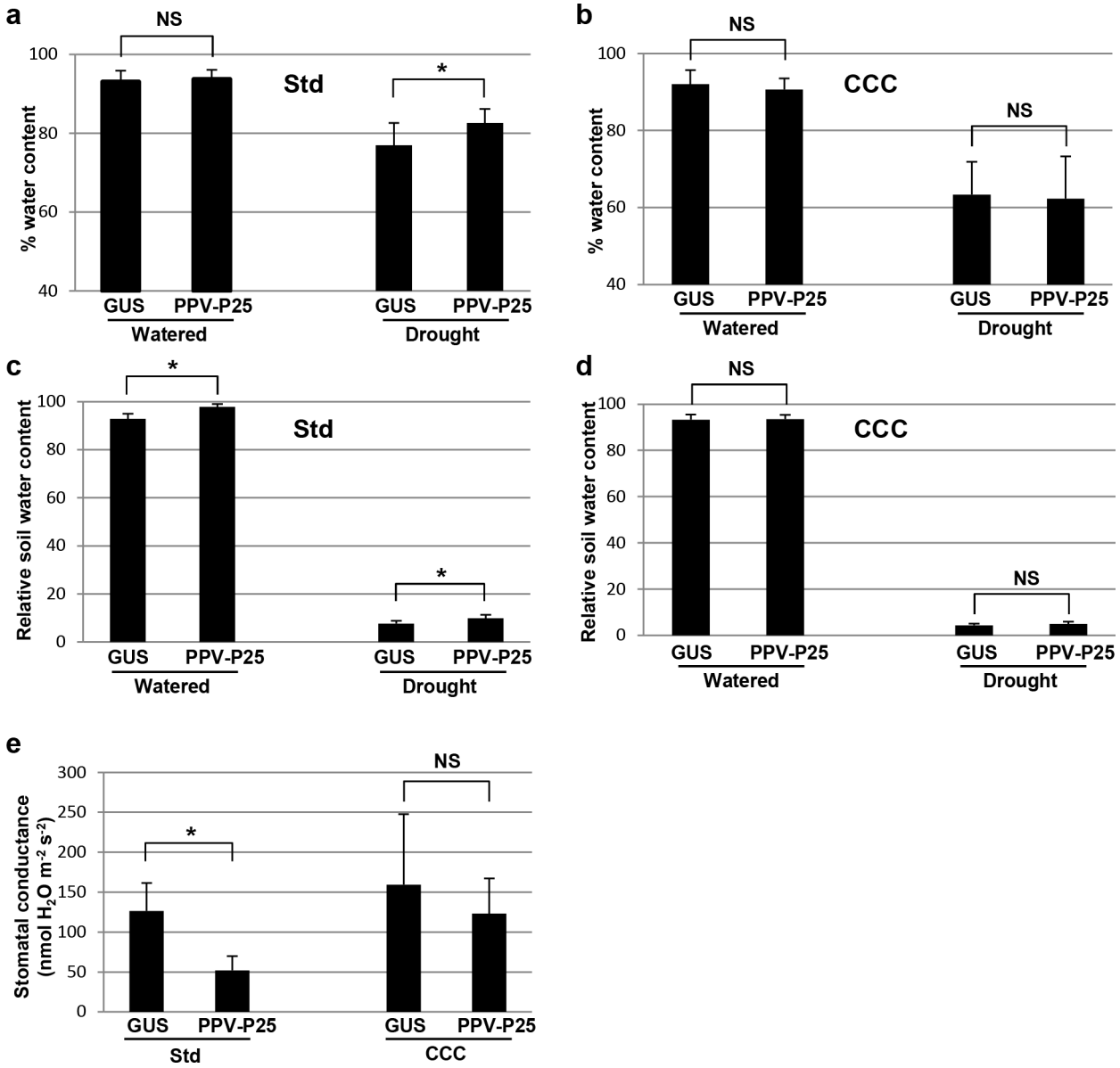

Fig. 6. Virus-induced drought tolerance in *Nicotiana benthamiana* plants grown under standard (Std) conditions or under climate change-related conditions (CCC). (a) Water content percentage in mock-inoculated and PPV–P25-infected plants grown under Std conditions at 12 days after the water was withdrawn (a.w.w.). (b) Water content percentage in mock-inoculated and PPV–P25-infected plants grown under CCC at 12 days a.w.w. Data represent the means±standard errors of 12 replicates that were normally irrigated or deprived of irrigation. (c) The relative soil water content was measured in each pot of plants grown under Std conditions at 12 days a.w.w. (d) The relative soil water content was measured in each pot of plants grown under CCC at 12 days a.w.w. (e) Stomatal conductance in mock-inoculated and virus-infected plants grown under Std conditions or under CCC before the water was withdrawn. Data represent the means±standard errors of six replicates that received the same treatment. Statistical comparisons between means were made among treatments within each watering condition by employing Student's *t*-test. Asterisks indicate significant differences between treatments ($P<0.05$). NS, not significant.

data [3, 5], *Arabidopsis* plants infected with PPV-P25 dried out faster than mock-inoculated plants when grown under Std conditions (Fig. 7e, f). Remarkably, there was no significant difference in weight loss due to water loss in virus-infected plants compared with controls under CCC, which correlated with the lack of virus-induced tolerance to drought under these conditions.

DISCUSSION

Our estimates of virus effects on oxidative stress, defence response, bacterial growth and HR-like response were consistent with a positive association between viral virulence and enhanced incompatible host response against *P. syringae* pv. *tomato* in *N. benthamiana*; that is, infection by PPV-P25

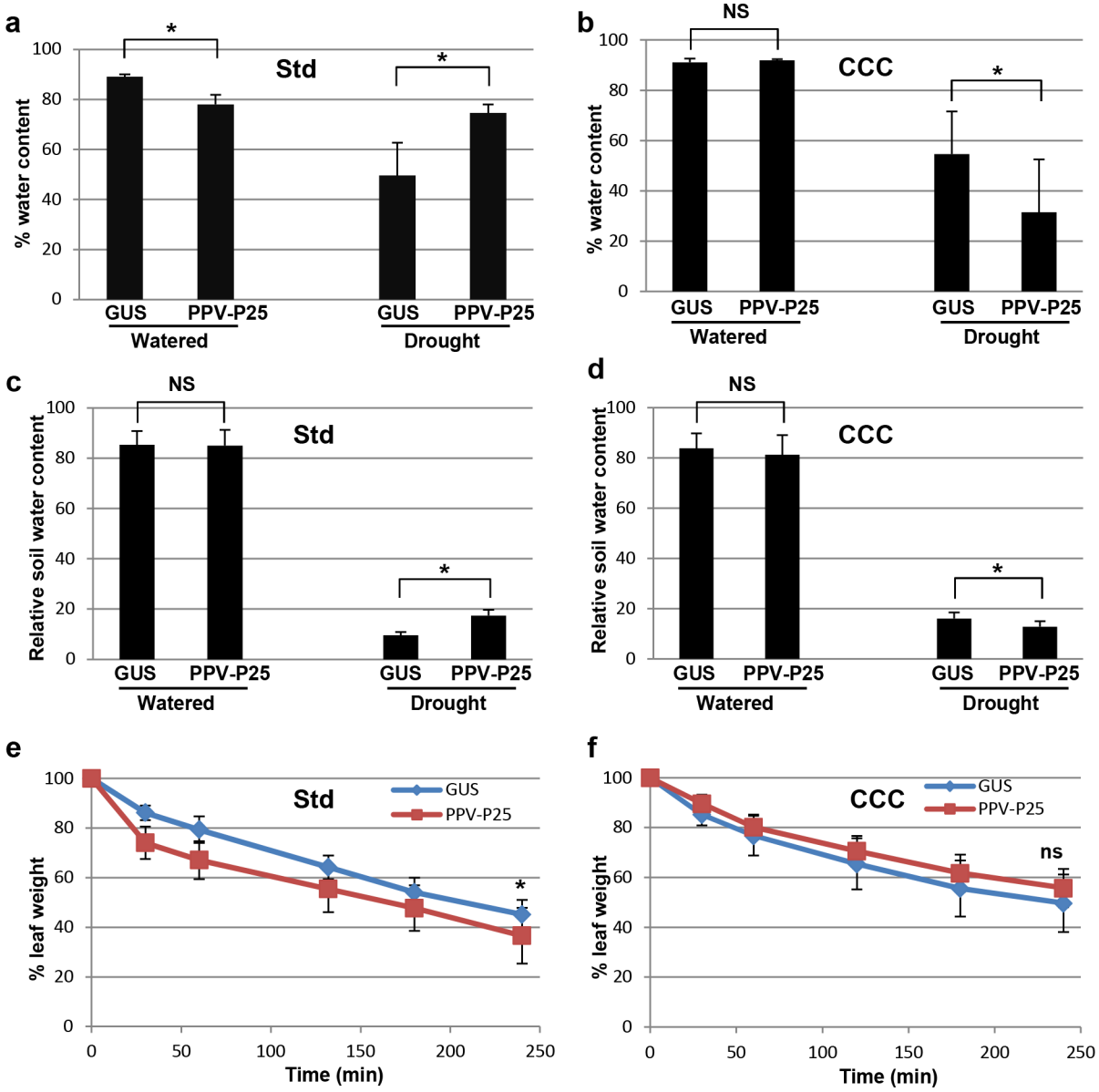

Fig. 7. Virus-induced drought tolerance in *Arabidopsis thaliana* plants grown under standard (Std) conditions or under climate change-related conditions (CCC). (a) Water content percentage in mock-inoculated and PPV–P25-infected plants grown under Std conditions at 15 days after the water was withdrawn (a.w.w.). (b) Water content percentage in mock-inoculated and PPV–P25-infected plants grown under CCC at 15 days a.w.w. Data represent the means±standard errors of 20 replicates that were normally irrigated or deprived of irrigation. (c) The relative soil water content was measured in each pot of plants grown under Std conditions at 15 days a.w.w. (d) The relative soil water content was measured in each pot of plants grown under CCC at 15 days a.w.w. (e) Comparison of water loss in mock-inoculated and virus-infected plants grown under Std conditions. (f) Comparison of water loss in mock-inoculated and virus-infected plants grown under CCC. Well-watered rosettes were detached from soil-grown plants at 18 days after inoculation and placed in plates with their abaxial face up. Weight was measured at various intervals over a period of 4 h, and related to the first weight measured. Data represent the means±standard errors of 12 replicates that received the same treatment. Statistical comparisons between means were made among treatments within each watering condition by employing Student's *t*-test. Asterisks indicate significant differences between treatments ($P<0.05$). NS, not significant.

and PVX-HC conferred an increased incompatible host response against Pst compared to infections with PPV-GFP or PVX, respectively. Similarly, PPV-P25 induced resistance to Pst in the susceptible host *A. thaliana* when plants were sprayed or flood-inoculated with bacterial suspensions.

Several reports have argued for a possible association between the establishment of plant responses to disease and subsequent resistance to biotic and abiotic stresses [1, 2, 43]. In particular, virus infection can improve plant resistance to biotic stress, including other viruses, unrelated pathogens, or herbivorous

insects [11, 44, 45]. Furthermore, recent studies have linked beneficial trade-offs in compatible plant–virus interactions to a group of viral proteins known as virulence determinants. For instance, the 2b protein of *Cucumber mosaic virus* has been demonstrated to participate in both drought tolerance in *Arabidopsis* [5] and insect pollinator attraction in tomato [46]. Moreover, transgenic tobacco plants expressing the HC-Pro protein from *Tobacco etch virus* were more resistant to *Tobacco mosaic virus* and to the oomycete *Peronospora tabacina* [47]. In our previous work, infection by PPV expressing the P25 protein of PVX conferred an enhanced drought-resistant phenotype to *N. benthamiana* and *Arabidopsis* plants compared to infections with either PPV-GFP or PVX [3]. Indeed, metabolic and hormonal studies supported an altered metabolic status in *Arabidopsis* plants infected with PPV-P25 with greater amplitude than the one triggered by PPV-GFP infection. Further, transcriptomic and hormonal analyses of *N. benthamiana* plants infected with PVX-HC revealed a greater impact on the transcriptional up-regulation of defence-related genes and hormone-responsive genes, as well as on the relative accumulation of several hormones, compared with infection by PVX [37]. Thus, both P25 and HC-Pro are major viral determinants involved in PVX/potyvirus-associated virulence that contribute to metabolic acclimation in plants to subsequent biotic and abiotic stresses [3, 21]. We propose that virus infections that trigger defence responses beyond a threshold level would induce a protective effect in plants against bacterial and possibly other microbial pathogens. However, this scenario might not be the case if viral proteins acting as virulence determinants interfere with innate immunity-based host defences against microbial pathogens. For instance, the growth rate of *P. syringae* was increased in *Arabidopsis* plants infected with either *Turnip mosaic virus* or CaMV [13, 48]. In this regard, the P6 protein from CaMV suppressed oxidative burst and accumulation of the defence hormone SA, facilitating the multiplication of the bacteria.

The cause of the increased resistance to bacterial growth in plants infected with viruses may be related to the fact that plants use a network of interconnected signalling pathways to respond to various environmental stresses, and that several of these responses are common in defence against viral and bacterial pathogens [7–9]. The defence responses induced by virus infection include the generation of ROS, hormonal signalling and the expression of defence-related genes that act to prevent the multiplication of bacterial pathogens [8]. This scenario is supported by the severe oxidative stress induced by PVX/potyvirus-associated synergism in *N. benthamiana* and *Arabidopsis* leaves, which correlated with increased virulence of PPV-P25 and PVX-HC when compared to infections with either PPV-GFP or PVX (this study; [33]). It has been reported that oxidative stress plays a key role in reducing the viability of *P. syringae* pv. *tomato* DC3000 [28]. In addition, Pst is sensitive to SA-dependent defence responses [27]. *N. benthamiana* and *Arabidopsis* plants infected with PVX-HC and PPV-P25, respectively, showed increased production of SA and enhanced expression of SA-responsive genes (this study; [3, 37]). Nevertheless, SA- and JA-independent defence responses were also elicited by virus infection in *N. benthamiana*, as virus-enhanced incompatible host response against Pst was not compromised in SA and JA signalling-deficient mutant lines, even though viral titre was halved in *NbCOI1* IR compared to WT plants. Likely, the reduced accumulation of PPV-P25 in *NbCOI1* IR was still enough to trigger defence responses beyond a threshold level to induce a protective effect in *N. benthamiana* against Pst.

Viral infection may regulate resistance to bacterial pathogens through distinct, species-specific mechanisms. Although *Arabidopsis* plants infected with PPV-P25 were as susceptible as virus-free plants when bacteria were infiltrated into leaves, they became more resistant to Pst when plants were sprayed or flood-inoculated with a bacterial suspension. The infiltration of bacteria with a syringe bypasses the first steps of the natural infection process, in particular the steps of invasion through wounds or natural openings such as stomata. Several studies have demonstrated that stomata play an active role in restricting bacterial invasion as part of the plant innate immune system [42]. Thus, host responses induced by PPV-P25 probably restrict bacterial invasion at an early step of the infection process, which contributes to disease resistance in *A. thaliana*. Recent works have shown that virus infection influenced stomatal development in *N. tabacum* and *Arabidopsis*, which was associated with a reduction in stomatal density [49]. Moreover, it has been reported that SA accumulation plays an important role in stomatal closure via production of ROS [41]. One possibility is that stomatal closure induced by ROS production during PPV-P25 infection may cause a reduction of bacterial entry into the apoplast, resulting in a resistant phenotype to Pst. In support of this hypothesis, virus-induced resistance to both virulent and avirulent strains of Pst was compromised in the SA signalling-deficient *Arabidopsis* mutant *NahG*. Virus-induced resistance to Pst was also impaired in the JA signalling-deficient mutant *coi1-1*. JA has been shown to participate in the establishment of systemic acquired resistance after infection by biotrophic bacteria [50], and *Arabidopsis* plants infected with PPV-P25 accumulated high levels of JA [3]. Moreover, it has been reported that the *coi1-1* mutation impaired JA-induced stomatal closing in *A. thaliana* [51]. Therefore, stomatal closure might also play a role in virus-induced resistance to bacterial pathogens through different signalling pathways. Nevertheless, we cannot rule out the possibility that reduced accumulation of PPV-P25 in *Atcoi1-1* and *AtNahG* plants was responsible for the lack of virus-induced resistance to Pst in SA and JA signalling-deficient *A. thaliana* mutant lines.

In contrast to *N. benthamiana*, virus-induced drought tolerance in *Arabidopsis* did not result from a decreased rate of transpiration [3, 5]. In our previous work, it was reported that infection with PPV-P25 conferred water stress resistance to *A. thaliana* by increased accumulation of amino acids and sugar derivatives acting as osmolytes, and by enhanced expression of hormones and drought-related genes [3]. Accumulation of osmolytes in plants is an adaptive response to water deficit, which helps in turgor maintenance, detoxification of ROS and stabilization of the structure of

membranes and proteins [52]. Thus, despite their increased rate of transpiration, the greater accumulation of osmolytes in PPV-P25-infected *Arabidopsis* plants would confer a stage of metabolic acclimation that enables plants to cope with water deficit.

Virus-induced resistance to Pst and drought in compatible and incompatible host interactions was abrogated or ameliorated, respectively, when plants were kept under CCC of elevated temperature and CO_2 levels. Ambient conditions of relative elevated temperatures have been reported to decrease viral titres in many compatible infections by positive-sense RNA viruses [33, 53, 54]. Moreover, it has been reported that elevated CO_2 levels increased SA-mediated antiviral defences in several pathosystems [55, 56]. In our study, elevated temperature and CO_2 levels caused the titres of PPV-P25 in *A. thaliana* and *N. benthamiana* plants to drop relative to Std conditions. This correlated with diminished H_2O_2 production and decreased expression of defence-related genes (*PR1*, *Hin1* and *Hsr203j*) in virus-infected plants grown under CCC compared to virus-infected plants grown under Std conditions. Thus, virus-induced tolerance to drought and bacterial infection was compromised under environmental conditions where virus infection did not trigger defence responses beyond a threshold level that would induce physiological and metabolic adaptations to subsequent biotic and abiotic stresses.

It has been hypothesized that there may be environments in which it would be more advantageous for plants to be infected by viruses [57]. For instance, it has been shown that virus infections may contribute to protecting host plants against herbivory and enhancing their resilience to abiotic stresses, as a result of pathogen-induced acclimation [3–6, 44]. However, in the context of host–virus co-evolution, this scenario might not be the case if the reproductive fitness costs associated with virulence, i.e. lowered fecundity, outweigh the beneficial effects conferred by stress resistance on plant growth. Indeed, it was reported that the detrimental effect of PPV-P25 infection on host fitness overcame the beneficial effects associated with virus-induced metabolic acclimation to drought [3]. Thus, virulence should be considered as a potential factor limiting the outcome of beneficial trade-offs in the response of virus-infected plants to drought, bacterial pathogens and possibly other biotic and abiotic stresses under the current and a future climate change scenario.

Funding information
This work was supported by the Ministry of Economy and Competitiveness of Spain [grant BIO2016-75619-R (AEI/FEDER, UE) to T. C. and F. T.].

Acknowledgements
We thank Dr J. Cubero (Instituto Nacional de Investigaciones Agrarias, Spain) and Dr C. Castresana (Centro Nacional de Biotecnología, Spain) for providing Pst DC3000 and Pst DC3000 *avrRpm1*, respectively.

Author contributions
E.A. performed most of the experiments and the statistical analysis of all the data. F.J.T., D.F. and W.H. performed some experiments related to resistance to bacteria in virus-infected plants. T.C. and F.T. designed the experiment, performed the analyses and wrote the manuscript. All authors read and approved the final manuscript.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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