Effects of Elevated CO₂ and Temperature on Pathogenicity Determinants and Virulence of *Potato virus X*/Potyvirus-Associated Synergism

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Submitted 9 August 2015. Accepted 20 September 2015.

Infections of plants by multiple viruses are common in nature and may result in synergisms in pathologies. Several environmental factors influence plant-virus interactions and act on virulence and host defense responses. Mixed viral infections may be more frequent under environmental conditions associated with global warming. Here, we address how changes in the two main parameters behind global warming, carbon dioxide concentrations ([CO₂]) and temperature, may affect virulence of *Potato virus X* (PVX)/potyvirus-associated synergism compared with single infections in *Nicotiana benthamiana*. Elevated [CO₂] resulted in attenuated virulence of single infection by PVX, which correlated with a lower accumulation of virus. In contrast, virulence of PVX/potyvirus-associated synergism was maintained at elevated [CO₂]. On the other hand, elevated temperature decreased markedly both virulence and virus titers in the synergistic infection. We also show that the HR-like response elicited by transient coexpression of PVX P25 together with the potyviral helper component-proteinase protein was significantly enhanced by elevated temperature, whereas it was reduced by elevated [CO₂]. Both proteins are main pathogenicity determinants in PVX-associated synergisms. These findings indicate that, under environmental conditions associated with global warming, virulence of PVX/potyvirus-associated synergisms is expected to vary relative to single infections and, thus, may have pathological consequences in the future.

A plant disease is the result of interaction between a susceptible host plant, a one or more virulent pathogens, and the environment. Changes in environmental conditions are strongly associated with differences in the level of losses in crops caused by a disease. Moreover, changing environmental conditions may weaken or enhance plant defense responses, the outcome depending on the timing, nature, and severity of each stress (Atkinson and Urwin 2012). Due to the build-up in the atmosphere of greenhouse gases deriving from human activities (Shine and Sturges 2007), a realistic assumption of the doubling

of carbon dioxide concentrations ([CO₂]) within this century would likely imply a global temperature increase of over 3°C (IPCC 2007). The changes associated with global warming (i.e., increased temperatures, ozone levels, drought, and more), thus, may affect the incidence and severity of plant diseases and influence the further coevolution of plants and their pathogens (Eastburn et al. 2011; Garrett et al. 2006).

Temperature is known to influence disease resistance to bacteria, fungi, virus, and insects; and different host-pathogen interactions respond differently to different temperature ranges (Garrett et al. 2006). High temperatures very often suppress plant immunity based on protein-protein recognition (Zhu et al. 2010), whereas low temperature impairs RNA silencing, a potent plant defense against viral pathogens (Qu et al. 2005; Szittyá et al. 2003). For instance, temperature-dependent suppression of host resistance has been reported for *Tobacco mosaic virus* and *Tomato spotted wilt virus* in tobacco and pepper plants, respectively (Király et al. 2008; Moury et al. 1998). Moreover, heat stress increased the susceptibility of *Arabidopsis* plants to *Turnip mosaic virus* (Prasch and Sonnewald 2013).

Although the growth and physiological responses of numerous plant species to elevated [CO₂] are well documented (Ainsworth and Rogers 2007), few studies have assessed how plant interactions with viruses will change under future climatic conditions (Canto et al. 2009). Elevated [CO₂] led to increased resistance against infection by *Cucumber mosaic virus* (Fu et al. 2010) and *Potato virus Y* (PVY) (Matros et al. 2006; Ye et al. 2010). Furthermore, lower incidence and severity of *Tomato yellow leaf curl virus* (TYLCV) in elevated [CO₂] was accompanied by modulated plant-induced defense in tomato (Huang et al. 2012).

Infection of plants by multiple viruses is a common phenomenon in nature, with unpredictable pathological consequences (Hameed et al. 2014). In many cases, a mixed infection elicits disease symptoms that are more severe than those induced in single infections by either of the viruses, a phenomenon known as synergism in pathology (Hull 2002). Most plant viruses are transmitted by vectors and the majority by insects. Global warming can greatly influence insect populations by changing their dynamics, persistence, dispersal, and migration events, thereby affecting the spread of plant viruses (Harrington et al. 2007). Thus, with the increased incidence of diverse virus species associated with global warming, opportunities for mixed infection could become more frequent. There would then be greater chances of synergism between viruses, increasing

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*The e-Xtra logo stands for “electronic extra” and indicates that three supplementary figures are published online.

symptom severity, and weakening host resistance as well as the opportunities for genetic recombination and component exchange with possible increases in aggressiveness, virulence, and transmissibility (Jones 2009). Whether the altered environmental conditions associated with climate change will potentially modify disease expression in synergistic viral infections is largely unknown.

The best-studied synergistic pair is the interaction between *Potato virus X* (PVX) and members of the *Potyvirus* genus in *Nicotiana* spp. (González-Jara et al. 2005; Vance 1991). In comparison with single infections, coinfection of *N. benthamiana* with PVX and *Plum pox virus* (PPV) resulted in increased systemic symptoms that led to systemic necrosis in the newly emerging leaves and to plant death. Accordingly, it induced a severe oxidative stress in plant leaves, as judged by the generation of reactive oxygen species (García-Marcos et al. 2009). Further, the expression of the PPV helper component proteinase (HC) protein by a PVX vector (PVX-HC) was sufficient to induce an increase of PVX virulence comparable to that observed in plants doubly infected with PVX and PPV (González-Jara et al. 2005). Many viral pathogenicity determinants are suppressors of RNA silencing (VSR), and PVX/potyvirus-associated synergistic diseases have been suggested to result from suppression of this host defense mechanism by the potyviral VSR HC protein (Pruss et al. 1997). Recent findings, however, indicate that PVX P25, the VSR of PVX, is the major viral determinant involved in PVX-associated synergisms. Transient coexpression of P25 in the presence of HC or any of the other three VSR tested elicited a hypersensitive response (HR)-like response in *Nicotiana* spp. (Aguilar et al. 2015). These findings suggest that the increase in virulence caused by PVX/potyvirus synergism is a delayed immune response triggered by P25, once it reaches a threshold level by the RNA silencing suppression activity of potyviral HC.

In this work, we address how changes in the two main parameters behind global warming, $[\text{CO}_2]$ and, separately, temperature, may affect virulence of PVX/potyvirus-associated synergism in *N. benthamiana*. In addition, we use transient expression of PVX P25 in combination with VSRs by *Agrobacterium tumefaciens* to assess how elicitation of the HR-like response is influenced by these environmental conditions.

RESULTS

Effect of $[\text{CO}_2]$ on virulence of PVX-associated synergism.

To determine if elevated $[\text{CO}_2]$ affects the increase of PVX virulence mediated by the expression of PPV HC protein, *N. benthamiana* plants were infected with *Agrobacterium* cultures containing binary constructs expressing either PVX or PVX-HC, under ambient or elevated $[\text{CO}_2]$ (about 401 versus about 970 parts per million [ppm]). As a control, plants were inoculated with *Agrobacterium* cultures containing a binary construct expressing β -glucuronidase (GUS). Evaluation of electrolyte leakage was performed in upper, systemically infected leaves at 10 days postagroinoculation (dpa), to measure the extent of cell membrane injury as a quantitative indicator of virulence (De León et al. 2002). Plants infected with PVX exhibited significantly lower levels of electrolyte leakage under elevated $[\text{CO}_2]$ than under ambient $[\text{CO}_2]$. Indeed, electrolyte leakage level of PVX-infected plants returned to control level under elevated $[\text{CO}_2]$. However, elevated $[\text{CO}_2]$ was not able to alter the virulence of PVX-HC compared with ambient $[\text{CO}_2]$ (Fig. 1A). Moreover, PVX-HC infection led to an increase in H_2O_2 production, an indicator of stress-induced damage, compared with plants infected with PVX or the GUS control grown under both $[\text{CO}_2]$ conditions (Fig. 1B).

When *N. benthamiana* plants were infected with *Agrobacterium* cultures containing binary constructs expressing either PVX or PPV or with a mixture of both cultures, virulence of single infections was attenuated under elevated $[\text{CO}_2]$ compared with that under ambient $[\text{CO}_2]$, whereas virulence of dual infection was not altered (Supplementary Fig. S1). Thus, virulence of the synergistic pair PVX-PPV relative to single infections resembles that of the PVX recombinant expressing the HC protein from PPV under both $[\text{CO}_2]$ conditions.

To examine whether virulence was associated with accumulation levels of PVX and PVX-HC, we monitored the amount of viral genomic RNA (gRNA) in infected plants at 10 dpa by real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) (Fig. 2A). PVX gRNA accumulated at lower levels (twofold reduction) in plants grown under elevated $[\text{CO}_2]$ than under ambient $[\text{CO}_2]$, coincident with the lower levels of electrolyte leakage in these plants. However, there was a slight but significant increase in the level of PVX-HC gRNA in plants grown under elevated $[\text{CO}_2]$ compared with plants grown under ambient $[\text{CO}_2]$. Nevertheless, accumulation of gRNA in plants infected with PVX-HC was several fold less than in plants infected with PVX, irrespective of $[\text{CO}_2]$, arguing against an overall greater accumulation of gRNA as the cause

Fig. 1. Virus pathogenicity measured on *Nicotiana benthamiana* plants grown under ambient or elevated (970 ppm) carbon dioxide concentrations ($[\text{CO}_2]$). **A**, Electrolyte leakage from leaf discs of plants infected with *Potato virus X* (PVX), PVX-HC (helper component-proteinase), or β -glucuronidase (GUS) as a control, at 10 days postagroinoculation (dpa). Data represent the means $\pm$ standard errors of seven replicates, each consisting of four plants that received the same treatment. Statistical comparisons between means were made among treatments by employing Scheffé's multiple range test. Different letters indicate significant differences at $P < 0.05$. For each pair of bars, asterisks indicate significant differences between $[\text{CO}_2]$ levels (Student's t test, $P < 0.0005$). **B**, Leaves were stained with 3,3'-diaminobenzidine (DAB) solution at 10 dpa. DAB formed a polymerization product upon reaction with H_2O_2 .

Fig. 2. Comparison of relative viral levels in *Nicotiana benthamiana* plants grown under ambient or elevated (970 ppm) carbon dioxide concentrations ([CO₂]). **A**, Quantitative reverse transcription-polymerase chain reaction was used to analyze the accumulation of viral genomic (g) RNA levels in plants infected with *Potato virus X* (PVX) or PVX-HC (helper component-proteinase) at 10 days postagroinoculation (dpa). Data represent the means $\pm$ standard errors of two replicates, each consisting of four plants that received the same treatment. Statistical comparisons between means were made by employing the Mann-Whitney *U* test for between-groups comparisons with a Bonferroni correction for multiple comparisons of $\alpha = 0.008$. Different letters indicate significant differences at $P < 0.008$. Paired treatment bars within infected plants are significantly different at $P < 0.05$ (*) or $P < 0.0001$ (**). **B**, Diagrams show genome organization and approximate sizes of viral RNAs for PVX and PVX recombinant virus carrying *Plum pox virus* HC sequences (PVX-HC). **C**, Northern blot hybridization showing PVX RNA accumulation using PVX coat protein (CP) sequences as a probe. Expected sizes of genomic and subgenomic (sg) PVX RNAs are indicated in the margins. Ethidium bromide-stained RNA is shown as loading control. Two independent pooled samples were analyzed for each combination. In Elev X2, two times more RNA was loaded for comparative purposes.

of the increase in virulence of PVX mediated by HC protein. It is known that insertion of foreign sequences into the PVX vector has a debilitating effect on virus multiplication compared with the PVX empty vector (Chapman et al. 1992).

The triple gene block (TGB) subgenomic RNA (sgRNA)-encoded P25 protein was reported to be the main pathogenicity determinant responsible for symptom exacerbation in PVX-associated synergisms (Aguilar et al. 2015). To examine whether altered virulence at different [CO₂] was associated with changes in accumulation of TGB sgRNA, we measured the amount of total viral RNA by Northern blot, using the same RNA preparations analyzed above (Fig. 2C). Accumulation of TGB sgRNA but not that of coat protein (CP) sgRNA, in plants infected with PVX-HC was greater than in plants infected with PVX, irrespective of [CO₂]. Remarkably, the attenuated virulence of PVX under elevated [CO₂] correlated with a lower accumulation of TGB sgRNA in these plants.

Effect of [CO₂] on the HR-like response induced by PVX P25.

Previous studies have shown that transient expression by *A. tumefaciens* of PVX P25 in combination with other VSRs recapitulates, at local tissues, the effects of the increase in virulence induced by PVX recombinant viruses expressing those VSR proteins in *N. benthamiana* (Aguilar et al. 2015). To determine if elevated [CO₂] affects the HR-like response induced by P25, *N. benthamiana* leaf patches were infiltrated with P25, either alone or in combination with PPV HC, PPV HCLH (a variant of PPV HC containing a single point mutation [L₁₃₄H]) or *Tomato bushy stunt virus* (TBSV) P19 under ambient and elevated [CO₂]. HCLH is unable to increase virulence when expressed from a PVX vector (González-Jara et al. 2005). The expression of P25 or combinations of P25 together with HC and P19 resulted in lower levels of electrolyte leakage in plants grown under elevated [CO₂] compared with those grown under ambient [CO₂] at 7 dpa (Fig. 3A). We confirmed that P25 accumulated in plants under elevated [CO₂] at levels comparable to control conditions, as assayed by Western blot analysis (Fig. 3B). The higher levels of electrolyte leakage observed in combinations of P25 with VSRs compared with P25 alone or the combination of P25 with the nonfunctional HCLH has been reported to correlate with a greater accumulation of P25 in these patches (Aguilar et al. 2015).

Next, we assayed the performance of the silencing suppressor activity of P25 at elevated [CO₂] compared with that at ambient conditions. A green fluorescent protein (GFP) reporter construct was agroinfiltrated into *N. benthamiana* leaf patches together with constructs expressing either GUS or P25. After 3 dpa, GFP-derived fluorescence in the patches in which P25 was present was more intense than in the patches in which GFP was being expressed together with GUS (Fig. 3C). Moreover, both the visual intensity of fluorescence and the amounts of GFP detected by Western blot in the presence of P25 at 3 and 6 dpa were similar in both [CO₂] conditions (Fig. 3D, upper panel), indicating that P25 displayed comparable suppressor activity at ambient versus elevated [CO₂]. P25 was also detected serologically by Western blot in the corresponding patches (Fig. 3D, middle panel).

Effect of temperature on virulence of PVX-associated synergism.

Initial attempts to determine if elevated temperature affects the virulence of the PVX recombinant expressing the PPV HC protein were unsuccessful because of the rapid removal of the HC sequences at 30°C (data not shown). Thus, *N. benthamiana* plants were infected with *Agrobacterium* cultures containing binary constructs expressing either PVX or PPV or with a

mixture of both cultures under standard and elevated temperatures (25 versus 30°C). At 30°C, plants challenged with PVX plus PPV showed attenuated symptoms compared with infected plants kept at 25°C (Fig. 4A). Electrolyte leakage measurement revealed that virulence of dual infection was attenuated at the elevated temperature compared with that at the standard temperature at 13 dpa (Fig. 4B). Consistent with the above results, PVX-PPV infected plants grown under elevated temperature exhibited a reduction in H₂O₂ production compared with plants grown under the standard temperature (Fig. 4C). Plants singly infected with PPV exhibited a slight reduction in electrolyte leakage at elevated versus standard temperature. However, high temperature did not alter virulence of PVX.

To examine whether changes in virulence at elevated temperature were associated with changes in virus titers, we monitored the amount of virus accumulation in infected plants at 13 dpa. Comparative analysis of PVX accumulation by qRT-PCR revealed that the levels of PVX gRNA was halved in single- and doubly infected plants grown under an elevated temperature compared with the standard temperature (Fig. 5A). Remarkably, the attenuated virulence of PVX-PPV under the elevated temperature correlated with a lower accumulation of TGB sgRNA in these plants, as assayed by Northern blot analysis (Fig. 5B). Similarly, the levels of PPV HC (Fig. 5C) and CP (not shown) proteins were drastically reduced in both singly and doubly infected plants grown under an elevated temperature compared with the standard temperature.

Effect of temperature on the HR-like response induced by PVX P25.

To determine if elevated temperatures affect the HR-like response induced by P25, a time-course analysis of the production of H₂O₂ was performed with leaf patches infiltrated with P25, either alone or in combination with PPV HC, PPV HCLH, or TBSV P19, under standard and elevated temperatures. Subsequent to some preliminary experiments, we chose a temperature of 32°C instead of 30°C because, at 30°C, there were only minor differences compared with standard temperature (25°C) whereas, at 32°C, differences were clearer. The production of H₂O₂ was quicker and stronger in patches expressing combinations of P25 together with HC and P19 grown under the elevated temperature than under the standard temperature (Fig. 6A). The enhancement of P25-induced HR by functional VSRs was corroborated by electrolyte leakage measurements at 7 dpa (Fig. 6B). We confirmed that P25 accumulated in plants under the elevated temperature at levels comparable to standard conditions, as assayed by Western blot analysis (Fig. 6C). Conversely, the expression of P25 or the

Fig. 3. Transient expression of *Potato virus X* (PVX) P25 together with suppressors of RNA silencing in *Nicotiana benthamiana* plants grown under ambient or elevated (970 ppm) carbon dioxide concentrations ([CO₂]) by agroinoculation. **A**, Electrolyte leakage from leaf disks of *N. benthamiana* infiltrated with combinations of *Agrobacterium* cultures containing binary constructs expressing PVX P25 plus either β -glucuronidase (GUS), *Plum pox virus* (PPV) helper component-proteinase (HC), PPV HCLH, or *Tomato bushy stunt virus* (TBSV) P19, and GUS plus either PPV HC, PPV HCLH, or TBSV P19 at 7 days postagroinoculation (dpa), as indicated. Data represent the means $\pm$ standard errors of six replicates, each consisting of four plants that received the same treatment. Statistical comparisons between means were made among treatments within each carbon dioxide concentration ([CO₂]) condition by employing Scheffé's multiple range test. Different letters indicate significant differences at $P < 0.05$. For each pair of bars, asterisks indicate significant differences between [CO₂] treatments (Student's t test, $P < 0.05$). **B**, Western blot analysis of extracts derived from leaf patches infiltrated with combinations of *Agrobacterium* cultures containing P25 plus either GUS, PPV HC, PPV HCLH, or TBSV P19 at 7 dpa, using antibodies against the T7 epitope. The lower panel shows the Ponceau S-stained membrane after blotting, as control of loading. **C**, RNA silencing suppression activity of P25 in plants grown under ambient or elevated [CO₂]. Leaves were infiltrated with combinations of *Agrobacterium* cultures containing binary constructs expressing a free green fluorescent protein (GFP) reporter gene plus either PVX P25 or GUS, as indicated. Photographs were taken with long-wavelength UV light at 3 dpa. **D**, Western blot analysis of extracts derived from leaf patches at 3 and 6 dpa, using antibodies against GFP (upper panel) or the T7 epitope (middle panel). Below the selected lanes are densitometric analyses of protein bands. Protein levels in plants infiltrated with constructs GFP plus GUS kept at ambient [CO₂] are given the value of 1 and other data calculated relative to this value. It also shows the level of GFP accumulation induced by P25 compared with GUS controls. The lower panel shows the Ponceau S-stained membrane after blotting, as control of loading.

combination of P25 together with HC resulted in a delayed HR and a statistically significant reduction in electrolyte leakage in plants grown under a low temperature (16°C) compared with the standard temperature (Supplementary Fig. S2).

Fig. 4. Virus pathogenicity measured on *Nicotiana benthamiana* plants grown under standard (25°C) or elevated (30°C) temperatures. **A**, Plants infected with *Potato virus X* (PVX), *Plum pox virus* (PPV), or PVX plus PPV, at 13 days postagroinoculation (dpa). **B**, Electrolyte leakage from leaf discs of plants infected with PVX, PPV, or PVX plus PPV, at 13 dpa. Data represent the means $\pm$ standard errors of six replicates, each consisting of four plants that received the same treatment. Statistical comparisons between means were made among treatments by employing Scheffé's multiple range test. Different letters indicate significant differences at $P < 0.05$. Paired treatment bars within infected plants are significantly different at $P < 0.05$ (*) or $P < 0.005$ (**) (Student's *t* test). **C**, Leaves from plants grown at 25°C (upper row) or at 30°C (lower row) were stained with 3,3'-diaminobenzidine (DAB) solution at 13 dpa. DAB formed a polymerization product upon reaction with H_2O_2 .

Next, we assayed the performance of the silencing suppressor activity of P25 at the elevated temperature compared with that at standard conditions. Both the visual intensity of fluorescence (Fig. 7A) and the amounts of GFP detected by Western blot (Fig. 7B, upper panel) in the presence of P25 were similar in both temperature conditions, indicating that P25 displayed comparable suppressor activity at standard versus elevated temperatures. P25 was also detected serologically by Western blot in the corresponding patches (Fig. 7B, middle panel). However, the RNA silencing suppressor activity of P25 seemed to be necessary for the enhancement of HR-like response at the elevated temperature, as expression of HC together with P25 mutants unable to suppress silencing did not increase the levels of electrolyte leakage by temperature (Supplementary Fig. S3) (Aguilar et al. 2015).

DISCUSSION

Plant disease expression is partly determined by the impact of the environment on pathogenesis (McElrone et al. 2010). Although several studies have focused on the effects of increased atmospheric $[CO_2]$ and temperature on the susceptibility of plant species to individual viruses (Huang et al. 2012; Qu et al. 2005), there were no studies dealing with the influence of these environmental variables associated with global warming on pathogen virulence and accumulation in synergistic viral infections. We have addressed this issue in PVX/potyvirus-associated synergism in *N. benthamiana*.

Virulence of PVX was reduced in *N. benthamiana* plants grown under elevated $[CO_2]$, most likely influenced by a decrease in virus titers. Similarly, several reports focused on the systemic responses of tomato to TYLCV and of tobacco to the potyvirus PVY found that elevated $[CO_2]$ decreased disease incidence and severity (Huang et al. 2012; Matros et al. 2006). These findings suggest that host plants may benefit from future, higher atmospheric $[CO_2]$ through a reduction in damage caused by viral pathogens. In contrast, virulence of the PVX recombinant expressing the HC protein from PPV and the synergistic pair PVX-PPV were maintained under elevated $[CO_2]$. It is generally believed that most defense responses to viruses are mediated by salicylic acid (SA) signaling (Whitham et al. 2006). Indeed, treatment with SA induced resistance to PVX in *N. benthamiana* (Lee et al. 2011). It has been reported that elevated $[CO_2]$ increased the SA level in tomato and *Arabidopsis* plants (Huang et al. 2012; Sun et al. 2013; Zhang et al. 2015), suggesting that reduction in virulence of PVX under elevated $[CO_2]$ observed in this work is potentially related to changes in phytohormone levels. The lack of an effect of elevated $[CO_2]$ on virulence of PVX-HC could be connected with previous findings that suggest that HC encoded by potyviruses causes altered regulation of SA-mediated signaling (Alamillo et al. 2006; Murphy et al. 2004). Thus, interference of HC protein encoded by PVX-HC with SA signaling could counteract the augmented defenses in plants grown under elevated $[CO_2]$.

The presence of foreign inserts represents a disadvantageous load for PVX vectors, since they tend to remove the inserted sequence during infection, especially under circumstances of strong selective pressure (Barajas et al. 2006). This seemed to be the case at a temperature of 30°C, which precluded the use of PVX-HC to study virulence in plants grown under elevated temperature. Elevated temperature had a negative impact on both virulence and virus titers of the synergistic pair PVX-PPV, which implies that virulence was associated with virus accumulation to some extent. It has been reported that RNA silencing increases with increasing temperature, and there is a general trend for symptom attenuation with high temperatures,

even in viral infections expressing strong VSR (Chellappan et al. 2005; Szittyá et al. 2003). However, as judged by our transient silencing suppression assays, elevated temperature did not affect negatively the RNA silencing suppressor activity of P25 (this report) or HC (Del Toro et al. 2015), compared with standard conditions. This finding suggests that, whatever constrained accumulation of PVX and PPV at high temperatures, it was unrelated to the effectiveness of suppression of RNA silencing by the two VSRs involved in PVX/potyvirus-associated synergisms. Thus, processes other than RNA silencing may exist in plants infected by viruses that modulate weaker virulence and lower viral titers at elevated temperatures. Interactions of viral products with host factors involved in virus replication or movement or both may be less effective with rising temperatures and, as a result, there would be a reduction in virus accumulation and virulence (Chellappan et al. 2005).

Previous findings showed that P25 is the main PVX determinant involved in eliciting a systemic HR-like response in PVX-associated synergisms (Aguilar et al. 2015). Indeed, typical hallmarks of HR-type programmed cell death (Montillet et al. 2005), e.g., cell membrane damage, accumulation of reactive oxygen species (H_2O_2), and enhanced expression of lipoxygenase (LOX) activity, have been documented in both plants infected with either the synergistic pair PVX-PVY or PVX expressing the HC protein of PPV (García-Marcos et al. 2013; Pacheco et al. 2012) and plants expressing P25 in combination with VSRs (Aguilar et al. 2015). Thus, P25 behaves as a pathogenicity determinant in *Nicotiana* spp. whose function correlates with its capacity to suppress RNA silencing (Aguilar et al. 2015). Several studies have shown that some viral suppressors of RNA silencing can also act as elicitors of resistance (R) gene-mediated resistance (Li et al. 1999; Sansregret et al. 2013; Wang et al. 2014). Here, we show that the HR-like response elicited by P25 in combination with strong VSRs was significantly enhanced by a temperature of 32°C. In general, high temperatures compromise R gene-mediated disease responses including HR in different plant systems (Zhu et al. 2010), although opposite effects have also been reported (Carter et al. 2009). P25 of potexviruses has been reported to target multiple components in the antiviral RNA silencing pathway. For instance, PVX P25 interacts with Argonaute1 and leads to its degradation via a proteasome-dependent pathway (Chiu et al. 2010). The P25 of *Plantago asiatica mosaic virus*, also a member of the genus *Potexvirus*, has also been shown to interact with RNA-dependent RNA polymerase6 (RDR6) and suppressor of gene silencing3 (SGS3) and to inhibit double-stranded (ds)RNA and secondary small interfering (si)RNA synthesis (Okano et al. 2014). Interestingly, RDR6 expression was induced in *N. benthamiana* plants infected with *Turnip crinkle virus* grown at 27°C compared with infected plants grown at 21°C (Qu et al. 2005). On the other hand, an increase in growth temperature from 22 to 30°C reduced protein abundance of SGS3 in *Arabidopsis*, attenuating the formation of stable dsRNAs required for siRNA biogenesis (Zhong et al. 2013). Whether the attenuated or augmented accumulation of P25-interacting host proteins contribute to the enhancement of the HR-like response elicited by P25 at an elevated temperature needs to be further investigated.

We show that the HR-like response elicited by P25 in combination with strong VSRs was significantly ameliorated by elevated $[CO_2]$. As mentioned before, elevated $[CO_2]$ generally favors SA biosynthesis and signaling but repressed the jasmonic acid (JA) pathway (Zhang et al. 2015). It has been reported that the P25-mediated HR was correlated with an increase in LOX activity, which suggested the involvement of JA signaling in this response (Aguilar et al. 2015). Moreover, treatment with SA induced a delayed HR and a statistically

Fig. 5. Comparison of relative viral levels in *Nicotiana benthamiana* plants grown under standard (25°C) or elevated (30°C) temperatures. **A**, Quantitative reverse transcription-polymerase chain reaction was used to analyze the accumulation of *Potato virus X* (PVX) genomic RNA (g)RNA levels in plants infected with PVX or PVX plus *Plum pox virus* (PPV) at 13 days post-agroinoculation (dpa). Data represent the means $\pm$ standard errors of two replicates, each consisting of four plants that received the same treatment. Statistical comparisons between means were made by employing a Mann-Whitney *U* test for between-groups comparisons with a Bonferroni correction for multiple comparisons of α to $\alpha = 0.008$. Different letters indicate significant differences at $P < 0.008$. For each pair of bars, asterisks indicate significant differences between temperatures (Mann-Whitney *U* test, $P < 0.01$). **B**, Northern blot hybridization showing PVX RNA accumulation using PVX coat protein (CP) sequences as a probe. Expected sizes of gRNA and PVX subgenomic (sg)RNAs are indicated on the margins. Ethidium bromide-stained RNA is shown as loading control. Two independent pooled samples were analyzed for each combination. **C**, Western blot analysis of extracts derived of plants infected with PPV, PVX plus PPV, or PVX as a control at 13 dpa, using antibodies against PPV HC (helper component-proteinase). The lower panel shows the Ponceau S-stained membrane after blotting, as control of loading.

significant reduction in electrolyte leakage in leaf patches infiltrated with combinations of P25 together with HC or P19 compared with control-treated plants, which correlated with repression of JA-responsive genes (Aguilar et al. 2015). Thus, the effects of SA treatment and elevated [CO₂] on

Fig. 6. Transient expression of *Potato virus X* (PVX) P25 together with suppressors of RNA silencing in *Nicotiana benthamiana* plants kept at standard (25°C) or elevated (32°C) temperatures by agroinoculation. **A**, Leaves of *N. benthamiana* were infiltrated with combinations of *Agrobacterium* cultures containing binary constructs expressing PVX P25 plus either β -glucuronidase (GUS), *Plum pox virus* helper component-proteinase (PPV HC), PPV HCLH, or *Tomato bushy stunt virus* (TBSV) P19, and GUS plus either PPV HC, PPV HCLH, or TBSV P19, as indicated. Leaves kept at 25°C (upper row) or at 32°C (lower row) were stained with 3,3'-diaminobenzidine solution at 6, 8, and 10 days postagroinoculation (dpi). **B**, Electrolyte leakage from leaf disks infiltrated with different combinations of *Agrobacterium* cultures at 7 dpi. Data represent the means $\pm$ standard errors of three replicates, each consisting of four plants that received the same treatment. Statistical comparisons between means were made among treatments within each temperature condition by employing Scheffé's multiple range test. Different letters indicate significant differences at $P < 0.05$. For each pair of bars, asterisks indicate significant differences between temperatures (Mann-Whitney U test, $P < 0.05$). **C**, Western blot analysis of extracts derived from leaf patches infiltrated with combinations of *Agrobacterium* cultures containing P25 plus either GUS, PPV HC, PPV HCLH, or TBSV P19 at 7 dpi, using antibodies against the T7 epitope. The lower panel shows the Ponceau S-stained membrane after blotting, as control of loading.

P25-mediated HR can be attributable to the antagonistic effect of SA on JA signaling. The question remains as why elevated [CO₂] reduced the HR-like response elicited by P25 in combination with PPV HC, whereas elevated [CO₂] did not alter the virulence of either PVX-HC or PVX plus PPV compared with ambient [CO₂]. This suggests that there is either some fundamental difference at the level of phytohormone signaling between transient expression of viral pathogenicity determinants (local response) versus virus infection (systemic response) or that there is some effect also attributable to other viral genes or agrobacterium.

Members of the genus *Potyvirus* are transmitted predominantly by aphids in a nonpersistent manner, whereas transmission of PVX occurs exclusively by mechanical means (Hull 2002). In particular, aphids are expected to respond strongly to environmental changes associated with global warming because of their short generation time, low developmental threshold temperatures, and ability to survive mild winters (Harrington et al. 2007). Increases in frequency and intensity of summer storms with high winds, rain, and hail will increase wounding of plants and result in increased transmission of viruses by mechanical means. Therefore, despite the attenuating effect on virulence of PVX-PPV observed at elevated temperature, PVX/potyvirus-associated synergisms are expected to increase in importance relative to single infections under changing environmental conditions. Even more drastic

Fig. 7. RNA suppression activity of P25 in *Nicotiana benthamiana* plants grown under standard (25°C) or elevated (32°C) temperatures. **A**, Leaves of *N. benthamiana* were infiltrated with combinations of *Agrobacterium* cultures containing binary constructs expressing a free green fluorescent protein (GFP) reporter gene plus either *Potato virus X* (PVX) P25 or β -glucuronidase (GUS), as indicated. Photographs were taken with long-wavelength UV light at 4 days postagroinoculation (dpi). **B**, Western blot analysis of extracts derived from leaf patches at 4 and 7 dpi, using antibodies against GFP (upper panel) or the T7 epitope (middle panel). Below the selected lanes are densitometric analyses of protein bands. Protein levels in plants infiltrated with constructs GFP plus GUS kept at ambient temperature are given the value of 1 and other data are calculated relative to this value. It also shows the level of GFP accumulation induced by P25 compared with GUS controls. The lower panel shows the Ponceau S-stained membrane after blotting, as control of loading.

increases in these damages are expected if elevated [CO₂] makes a large contribution to augment SA-mediated defenses that are effective against single infection by PVX but not against dual infections of PVX with potyviruses. In addition, our findings on the effect of temperature on the HR-like response induced by PVX P25 suggest that the virulence caused by PVX/potyvirus-associated synergism could increase under an elevated temperature, once the temperature sensitivity of viral replication/movement processes could be overcome by specific mutations conferring heat-stable functions. Future research should consider the interactive effects of temperature and [CO₂] on virulence of PVX/potyvirus-associated synergisms.

MATERIALS AND METHODS

Binary vector constructs.

The PCR amplification and cloning of T7-tagged versions of either PVX P25 wild type or P25 mutants A104V, T117A, and K124E (Aguilar et al. 2015), PPV HC, PPV HCLH (HCL₁₃₄H) (González-Jara et al. 2005), and TBSV P19 (Uhrig et al. 2004) genes into *A. tumefaciens* binary vectors has been described previously. *A. tumefaciens* carrying pCambia1305.1 containing a gene encoding GUS was used as negative control.

The binary vector pGR107, which contains the infectious cDNA of PVX, was provided by D. C. Baulcombe (University of Cambridge, Cambridge). The binary vector pGWB2 containing a cDNA clone of PVX carrying PPV HC (pPVX-HC) sequences under the control of the PVX duplicated CP promoter was described before (Aguilar et al. 2015). pGWB_{in}PPV-3xHA is a derivative of pBinPPV (Alamillo et al. 2006), which contains the infectious cDNA of PPV and was provided by J. A. García (Centro Nacional de Biotecnología, Madrid).

Delivery of viruses and viral proteins in plants by agroinfiltration.

A. tumefaciens cultures were grown to exponential phase in Luria Bertani medium with antibiotics at 28°C. For infiltration of viral amplicons, bacterial cultures were diluted to a final optical density at 600 nm of 0.3. In the case of transient expression assays of viral proteins (leaf patch assays), each bacterial culture was diluted to a final optical density of 0.6. Equal volumes of each suspension were then combined in a 1:1 ratio, and infiltration of the mixtures was performed into leaves of *N. benthamiana* plants at lab room temperature (around 25°C). Immediately after infiltrations (no more than 15 min on the bench), plants were transferred to controlled plant growth chambers (SANYO Electronic Co.). In those cases where transient expression was to take place at higher temperatures, plants were maintained during the first 48 h after infiltration at 25°C to allow for the agrobacterium-mediated T-DNA transfer into plant tissues to take place (Del Toro et al. 2014).

In silencing-suppression assays, a free GFP reporter gene expressed from pCambia2300 was coinfiltrated with either the pCambia1305.1 vector (GUS) or with pCambia2300 expressing a T7-tagged version of P25. Leaves were then illuminated with a Blak Ray long-wave UV lamp (UVP) to visualize the fluorescence derived from the transiently expressed GFP. When possible, all of the treatment types to be compared were infiltrated on a single leaf. To minimize the effects of interleaf variability, all of the leaf disks from different plants corresponding to the same treatment type were pooled.

Plant growing conditions.

N. benthamiana plants were kept for the duration of the experiment in controlled growth chambers, with 16-h day and 8-h

night photoperiod and approximately 2,500 lux of daylight intensity. Three environmental conditions were used: i) plants grown at 25°C and ambient [CO₂] (approximately 401 ppm), ii) plants grown at 25°C and elevated [CO₂] (approximately 970 ppm), and iii) plants grown at 30°C and ambient [CO₂]. In ii, plants were kept for the 7 days prior to agroinfiltration at 25°C and approximately 970 ppm, to allow them to adapt to the elevated [CO₂] environment.

RNA and protein gel blot analysis.

Total RNA was extracted from upper leaves 10 to 13 dpa, as described (Pacheco et al. 2012). RNA samples were separated on 1% agarose formaldehyde gels and were transferred to Hybond-N membranes (Roche Molecular Biochemicals). Membrane hybridization was carried out overnight at 65°C, using digoxigenin-labeled riboprobes corresponding to PVX CP sequences (González-Jara et al. 2005).

qRT-PCR for virus detection was performed using primers that amplify a region from nucleotides 2,621 to 2,753 of the PVX sequence as described by García-Marcos and associates (2013). The relative quantification of PCR products was calculated by the comparative $\Delta\Delta C_t$ (cycle threshold) method. 18S rRNA was chosen for normalization because of its similar level of expression across all treatments.

Viral proteins were analyzed by Western blot as described by Tena Fernández et al. (2013). T7-tagged P25 was detected with horseradish peroxidase (HRP)-conjugated anti-T7 antibody (Novagen) (1:5,000 dilution). PPV HC was detected with a rabbit polyclonal antiserum (1:400 dilution) (González-Jara et al. 2005). PPV CP was detected with a rabbit polyclonal antiserum (1:2,000 dilution) (Instituto Valenciano de Investigaciones Agrarias, Valencia, Spain). For immunological detection of GFP, a rabbit anti-GFP, N-terminal antibody was used (Sigma-Aldrich) (1:3,000 dilution). An appropriate secondary antibody conjugated with HRP was used in each case. Detection was performed using the ECL system (Amersham Biosciences). Densitometric analysis of blotted protein bands were performed using the public domain software ImageJ (National Institutes of Health website, image processing and analysis in java).

Electrolyte leakage assays and 3,3'-diaminobenzidine (DAB) staining.

Cell damage was assayed by measuring electrolyte leakage. Disks ($n = 24$, 0.3 cm²) were excised from upper leaf tissue using a core borer. Disks were rinsed briefly with water and were floated on 5 ml of double distilled water for 6 h at room temperature. The conductivity of the water was measured using a Crison conductivity meter. This represented the electrolyte leakage from the leaf disks (reading 1). Then, samples were boiled for 20 min at 90°C. After the liquid cooled, the conductivity of the water was measured again. This represented the total ions present in the leaf disks (reading 2). Electrolyte leakage was represented as the percentage of total ions released (reading 1/reading 2 $\times$ 100). Reported data are means and standard errors of the values obtained in two independent experiments with three to seven independent replicates, each consisting of pools of four plants for each treatment. To clarify the graphics in each experiment, the value of control samples was set at 1 and other data was calculated relative to this value. Statistical analyses were performed using the statistical software IBM SPSS Statistics v.20 (IBM Corp.).

For DAB staining to detect H₂O₂ production, leaves were vacuum-infiltrated for 10 min with DAB solution at 1 mg/ml. Representative phenotypes were photographed with a Leica L2 stereoscope using a Leica DFC 320 camera.

ACKNOWLEDGMENTS

E. Aguilar was recipient of an FPU fellowship from the Spanish Ministry of Education, Culture and Sport (MECD). This work was supported by the Rural Development Administration (RDA) of the Republic of Korea (Prediction of the impact of climate change on the outcome of diseases caused by plant RNA viruses PJ00946102) in cooperation with the Spanish Council for Scientific Research (CSIC) and, also, by the Spanish Ministry of Economy and Competitiveness (MEC) (BIO2013-47940-R).

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