Technical advance

A procedure for the transient expression of genes by agroinfiltration above the permissive threshold to study temperature-sensitive processes in plant–pathogen interactions

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SUMMARY

Localized expression of genes in plants from T-DNAs delivered into plant cells by *Agrobacterium tumefaciens* is an important tool in plant research. The technique, known as agroinfiltration, provides fast, efficient ways to transiently express or silence a desired gene without resorting to the time-consuming, challenging stable transformation of the host, the use of less efficient means of delivery, such as bombardment, or the use of viral vectors, which multiply and spread within the host causing physiological alterations themselves. A drawback of the agroinfiltration technique is its temperature dependence: early studies have shown that temperatures above 29 °C are nonpermissive to tumour induction by the bacterium as a result of failure in pilus formation. However, research in plant sciences is interested in studying processes at these temperatures, above the 25 °C experimental standard, common to many host–environment and host–pathogen interactions in nature, and agroinfiltration is an excellent tool for this purpose. Here, we measured the efficiency of agroinfiltration for the expression of reporter genes in plants from T-DNAs at the nonpermissive temperature of 30 °C, either transiently or as part of viral amplicons, and envisaged procedures that allow and optimize its use for gene expression at this temperature. We applied this technical advance to assess the performance at 30 °C of two viral suppressors of silencing in agropatch assays [*Potato virus Y* helper component proteinase (HCPro) and *Cucumber mosaic virus* 2b protein] and, within the context of infection by a *Potato virus X* (PVX) vector, also assessed indirectly their effect on the overall response of the host *Nicotiana benthamiana* to the virus.

Keywords: agroinfiltration, plant virology, plant–virus interactions, transient expression.

INTRODUCTION

Agrobacterium tumefaciens is one of the few bacteria capable of delivering DNAs into plant cells. In nature, the transferred DNA

fragment (T-DNA) of the tumour-inducing plasmids delivered by the agrobacterium into plant nuclei encodes genes required for crown gall tumour formation as part of the bacterial life cycle. However, laboratory modifications have created versatile binary vector systems in which the T-DNA, free of tumour-inducing genes, can express any desired gene or sequence fragment under the control of a eukaryotic promoter, the most common of which for research purposes is the *Cauliflower mosaic virus* 35S promoter. Some of the T-DNA molecules delivered into a plant cell can integrate stably by recombination into the plant genome, thus becoming inheritable. This constitutes the basis of current techniques for plant transformation (Gelvin, 2003). More recently, transient, localized expression of genes and their products in plants from the bulk of nonintegrated T-DNAs delivered into the plant cell by the bacterium has become a widely used tool in plant pathology and cell biology studies. This technique is commonly known as agroinfiltration or agroinjection, and ways to enhance the levels of expression or its large-scale use have been envisaged employing a variety of approaches (Chen *et al.*, 2013; Voinnet *et al.*, 2003).

Transient, steady-state levels of gene products expressed from T-DNAs delivered into the agroinfiltrated leaf patch (agropatch) are influenced by several factors, one of which is their recognition as foreign by the host, which elicits a silencing response that depresses both the transcript messenger RNA (mRNA) levels and those of their encoded protein products (Johansen and Carrington, 2001). The trigger of this silencing response is probably the presence of double-stranded transcripts derived from sense and antisense transcription of the T-DNA sequences, causing the generation of small interfering RNAs (siRNAs) to promoter sequences, or to promoterless T-DNAs, in theory expected not to be transcribed (Canto *et al.*, 2002). The co-expression of viral suppressors of gene silencing that prevent the targeting and degradation of T-DNA-derived transcripts arose as a strategy to counteract this partial silencing of T-DNA gene expression (Canto *et al.*, 2002; Johansen and Carrington, 2001; Voinnet *et al.*, 2003).

Another important factor in agroinfiltration efficiency is the type of plant host. Some plant species are not physically amenable to the efficient infiltration of their leaves with *Agrobacterium*

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cultures. The experimental plant species *Arabidopsis thaliana*, *Nicotiana tabacum* and *Nicotiana benthamiana* are all readily infiltrated, but differences in the respective transient levels of the gene products achieved can be large: the latter host expresses higher levels of transcript mRNAs and their products than the first two (Andrews and Curtis, 2005). This may be related to the fact that, in *N. benthamiana*, the salicylic acid- and virus-inducible RNA-dependent RNA polymerase 1 (RdRP1), involved in antiviral defences, is naturally truncated, possibly causing its hypersusceptibility to many different plant viruses (Yang *et al.*, 2004; Ying *et al.*, 2010). Thus, unless a study requires a specific plant species, *N. benthamiana* is the usual host of choice for agroinfiltration assays in experimental studies.

Temperature is another factor affecting the efficiency of agroinfiltration as a tool for gene expression in plants. Optimal temperatures for transient gene expression through agroinfiltration appear to be in the 25 ± 0.5 °C region in *N. benthamiana* (Chen *et al.*, 2013; Lai and Chen, 2012), although the tumour induction optimum of the bacterium may be lower, 22 °C, in other hosts (Fullner and Nester, 1996). The transfer of T-DNAs into plant cells involves a complex set of bacterial genes and the physical formation of a pilus structure to allow for the physical transfer of bacterial DNA into the plant. Temperatures of 29 °C and above completely prevent the development of tumours as certain proteins involved in the transfer machine are not functional and, critically, pilus formation also does not take place (Fullner and Nester, 1996; Gelvin, 2003). In addition, it has been shown that the strength of the plant silencing defence response that targets both viruses and T-DNA transcripts steadily increases with temperature (Chellappan *et al.*, 2005; Qu *et al.*, 2005; Szyttia *et al.*, 2003; Velázquez *et al.*, 2010). Thus, in addition to failure in the T-DNA transfer process, a stronger silencing defence response could have a further negative effect on any expression from agrodelivered T-DNAs. Therefore, agroinfiltration as a technique to transiently express genes in plants at temperatures above 29 °C would appear to be a nonviable option.

In this work, we first assessed the efficiency and viability of agroinfiltration for the expression of genes from T-DNAs at 30 °C, either transiently or as part of a viral *Potato virus X* (PVX)-based amplicon. We then devised and optimized a procedure that allows its use for gene expression at this temperature and characterized its properties and parameters. With this procedure, we assessed the performance at 30 °C of two viral suppressors of silencing in agropatch assays [*Potato virus Y* helper component proteinase (HCPro) and *Cucumber mosaic virus* (CMV) 2b protein] and found that, at least for HCPro, its suppressor of silencing activity in agropatch assays is as strong at 30 °C as it is at 25 °C. Within the context of infection by a PVX vector, we also found that the strength of the overall response of the host *N. benthamiana* to the virus at high temperatures overcomes the effect of either 2b or HCPro on the neutralization of host defences.

RESULTS

Poor steady-state levels of a reporter expressed at 30 °C from agroinfiltrated T-DNAs

A green fluorescent protein (GFP) reporter was agroinfiltrated at standard (25 °C) temperature into *N. benthamiana* leaf patches, together with empty construct pROK2 or pROK2 constructs expressing P1-hexahistidine-tagged HCPro (P1-6x-HCPro) from *Potato virus Y* (PVY), 2b protein from CMV or P19 from *Tomato bushy stunt virus* viral suppressors of silencing (Fig. 1A, agropatches labelled C, 1, 2 and 3, respectively). Within a few minutes after infiltration, plants were moved to growth cabinets at either 25 or 30 °C. After 3 days at standard temperature, GFP-derived fluorescence in the patches in which the viral suppressors of silencing were present was much more intense than in the patch in which GFP was being expressed alone under the UV lamp (Fig. 1A, left leaf, patches 1, 2 and 3 versus patch C), as partial silencing of the T-DNA-derived GFP transcript was relieved by the viral factors. Both the visual intensity of fluorescence and the amounts of GFP detected by Western blot in the presence of the three different viral suppressors were similar (Fig. 1A, left leaf; Fig. 1B, top left Western blot, compare 1, 2 and 3), indicating that 6x-HCPro (P1 and 6x-HCPro separated by proteolytic cleavage) and 2b displayed suppressor strengths comparable with that shown by tombusviral P19, usually considered to be a strong suppressor of silencing in this type of assay. Both HCPro and 2b were also detected serologically by Western blot in their corresponding patches (Fig. 1B, bottom left and right panels, respectively).

By contrast, in those plants kept for 3 days at 30 °C, GFP-derived fluorescence in infiltrated patches was visualized very poorly and unevenly (Fig. 1A, right leaf). Western blot analysis of the infiltrated patches showed the presence of very low, but comparable, amounts of GFP in all four patches, with no dependence on whether or not a viral suppressor was co-infiltrated (Fig. 1B, top right Western blot panel). GFP levels in the C patch in this leaf were lower than in the left leaf that had been kept at 25 °C (Fig. 1B, lanes labelled C in top Western blot panels). Interestingly, neither HCPro nor 2b could be detected serologically (Fig. 1B, bottom left and right panels, respectively).

We concluded that, in leaves kept at 30 °C after infiltration, transient GFP and suppressor expression, as well as suppressor function, were compromised. Given the published literature, a likely cause for this outcome could be an inefficient transfer of T-DNA. However, it could also be the case that other factors, such as host processes at that temperature having a negative effect on the steady-state levels of T-DNA transcripts or their protein products, could also influence this outcome. In this regard, we also tested whether the transfer of plants from 25 to 30 °C after infiltration could have affected negatively their efficiency in

Fig. 1 Assessment of the efficiency of agroinfiltration to transiently express a green fluorescent protein (GFP) reporter in infiltrated *Nicotiana benthamiana* patches at different temperatures at 72 h post-infiltration (hpi). The GFP reporter vector was co-infiltrated together with empty vector (patches and Western blot lanes labelled C), or together with vectors expressing P1-6x-HCPro, 2b protein or P19 suppressors of silencing (patches and Western blot lanes labelled 1, 2 and 3, respectively). (A) Visualization of GFP-derived fluorescence under a UV lamp in a leaf kept at 25 °C for 72 hpi (left leaf), in a leaf kept for 72 hpi at 30 °C (right leaf) and in a leaf kept for the first 24 hpi at 25 °C and the following 48 hpi at 30 °C (central leaf). (B) Western blot analysis using total extracts of the accumulation in the infiltrated patches of the GFP reporter (top panels) and of the suppressors 6x-HCPro and 2b protein (bottom panels) using antibodies against GFP and 6xhistidine tags, respectively. Below the selected lanes is a densitometric analysis of the protein bands (protein levels in leaf samples kept for 72 hpi at 25 °C are given the arbitrary value of 100%). Note that the same samples from the central leaf patches appear duplicated in both top left and right Western blots. This is because densitometric analysis requires bands to be in the same membrane to eliminate the effect of differences between membranes in blotting efficiency or in antibody binding. Lane M in the blots shows the molecular weight markers in kilodalton (kDa), and the small panels below the Western blots show the membranes stained with Ponceau-S after blotting, as controls of loading.

agropatch assays as a consequence of heat shock. To do this, we compared 25 °C plants with those that had been placed at 30 °C 3 days prior to their agroinfiltration, and found no difference in their performance (data not shown).

Twenty-four hours at 25 °C after infiltration allowed T-DNA transfer into plant cells and restored transient gene expression at 30 °C

We hypothesized that, if T-DNA transfer into plant cell nuclei was being prevented by a temperature of 30 °C, the provision of a window of time after infiltration at standard temperature, sufficiently long to allow for transfer to occur, but sufficiently short that most T-DNA-derived protein accumulation and functional action would take place at 30 °C, would allow us to perform studies on protein function at high temperatures using the agroinfiltration technique. For this reason, we tested keeping plants for 24 h at 25 °C after infiltration, followed by 48 h at 30 °C (Fig. 1A, centre leaf). With this procedure, at 72 h post-infiltration (hpi), we obtained levels of expression of the GFP reporter that were on the order of those seen in leaves kept all the time at 25 °C (Fig. 1A, central versus left leaf; Fig. 1B top left Western blot panel). As an example, quantification of the GFP band density corresponding to patch 2 showed that, in the central leaf patch, there was 83.7% of the protein found in the left leaf. This was in contrast with the meager 9% obtained at 30 °C in the equivalent sample (Fig. 1B, top Western blot panels). Both HCPPro and 2b proteins were also detected serologically in their corresponding patch samples in the central leaf, although in a smaller proportion relative to the protein found in the equivalent patch in the left (25 °C) leaf than for the GFP reporter (Fig. 1B; bottom Western blot panel). As an example, quantification of the 2b protein band density corresponding to patch 2 in the left and central leaves showed that the latter accumulated one-third (32.9%) of the suppressor found in the former. The reason for this disparity between the accumulation of the reporter and of the two suppressors could lie in the existence of host processes that specifically target the suppressor but not the reporter (Nakahara *et al.*, 2012).

Thus, as expected, T-DNA transfer was critically affected by the 30 °C temperature and the provision of a 24-h 25 °C window after infiltration overcame this obstacle. We tested a window of only 12 h, but the results were unsatisfactory (Fig. S1A, see Supporting Information).

We then determined the maximum proportion of protein found at 72 hpi that could have accumulated in the initial 24 h. For this purpose, we performed a time course experiment to analyse the protein accumulation at 24, 48 and 72 hpi of the GFP reporter co-infiltrated with empty vector or with vectors expressing either 6x-HCPPro or 2b protein, for which we had means of serological detection (Fig. 2). We tested the protein accumulation in plants

Fig. 2 Time course analysis by Western blot of green fluorescent protein (GFP) reporter and of suppressor accumulation in infiltrated patches at 24, 48 and 72 h post-infiltration (hpi). (A) Agroinfiltrated *Nicotiana benthamiana* leaves were kept at 25 °C for the whole 72 hpi. (B) Agroinfiltrated leaves were kept at 25 °C for the first 24 h and then at 30 °C for the following 48 h. The GFP reporter construct was co-infiltrated together with empty vector or with *Potato virus Y* (PVY) P1-6x-HCPPro or *Cucumber mosaic virus* (CMV) 2b protein suppressors of silencing. Total patch extracts were analysed by Western blot for GFP (top panels, using an antibody against GFP), 6x-HCPPro (bottom left panels, using antibodies against 6xhistidines or against HCPPro) and 2b protein (bottom right panel, using an antibody against 2b protein). Below the selected lanes are densitometric analyses of protein bands (protein levels in leaf samples collected at 72 hpi are given the value of 100%). Lane M in the blots shows molecular weight markers in kilodalton (kDa), and the small panels below the Western blots show the membranes stained with Ponceau-S after blotting, as controls of loading.

kept all the time at 25 °C (Fig. 2A) and in plants kept for the first 24 h at 25 °C and the following 48 h at 30 °C (Fig. 2B). In both cases, at 24 h, we detected the accumulation of the GFP reporter and 2b suppressor, but failed to detect the accumulation of 6×-HCPro using two separate antibodies. For the GFP reporter, accumulation in the first 24 h at 25 °C was limited to 15% or less of the total found at 72 hpi (Fig. 2A, B, top right panels). The same pattern was found for the 2b suppressor in plants kept all the time at 25 °C: 2b accumulation at 24 hpi was also less than 12% of the protein found at 72 hpi (Fig. 2A). However, in the case of plants kept for 24 h at 25 °C and the following 48 h at 30 °C, 2b accumulation declined after the initial 24 h and, at 72 hpi, it was only 35.6% of that found at 24 h (Fig. 2B, bottom right panel). We found the same pattern in a modified 2b tagged with six histidines and an HA peptide (Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala) at its N- and C-termini, respectively (construct 6×-2b-HA; Fig. S1B). By contrast, 6×-HCPro accumulation followed a similar pattern in plants kept all the time at 25 °C and in plants kept for the first 24 h at 25 °C and the following 48 h at 30 °C. No clear decline from 48 to 72 h was observed, in contrast with the observations for the 2b protein (Fig. 2B). These data clearly indicate differences between 2b and 6×-HCPro in their speed of expression and maturation, stability and turnover in a similar cellular environment.

Thus, in both situations, most of the accumulation of our GFP reporter (over 85%) and of 6×-HCPro took place at 30 °C, as did whatever functionality the latter might have had at that temperature. This allowed us to conclude that, in our particular experiment, HCPro displayed suppressor of silencing activities at 30 °C that were at least as strong as those displayed at 25 °C in the agropatch assay system. The case of 2b protein, native or tagged, was different, as its accumulation declined substantially after the initial 24 h if plants were transferred to 30 °C. Remarkably, even these lower levels of suppressor were sufficient to suppress efficient silencing of the GFP reporter [Figs 1, 2A versus 2B (top GFP Western blot panels), S1A].

Viable expression of viral amplicons at 30 °C through agroinfiltration

We tested how binary constructs expressing viral amplicons would perform in our system. For this, we used a construct expressing a PVX vector, and PVX vectors expressing either PVY P1-6×-HCPro (construct PVX-P1-6×-HCPro) or CMV 6×-2b-HA protein (construct PVX-6×-2b-HA). After agroinfiltration at 25 °C, plants were kept at either 25 °C for 7 days or 25 °C for 24 h and then transferred to 30 °C for the remaining 6 days. Plants were monitored daily for the appearance of systemic infection symptoms during this period (Table 1) and, at the end of the seventh day, samples were taken from systemic tissue to monitor viral coat protein (CP) levels. In plants kept at 25 °C, symptoms appeared on the fifth day in all three cases, and increased with time. Symptoms were more severe in the case of PVX-P1-6×-HCPro and PVX-6×-2b-HA, and particularly strong in the case of PVX-6×-2b-HA, leading, on the seventh day, to the start of necrosis in apical leaves (Table 1 and Fig. 3A). By contrast, in all three cases, plants kept at 30 °C remained visually symptomless (Table 1 and Fig. 3A). Western blot analysis of systemic tissue revealed that, regardless of the absence of symptoms, all plants were infected with the virus. Densitometric analysis of the Western blot CP bands revealed that, at 25 °C, tissue infected with PVX or PVX-6×-2b-HA contained comparable levels of protein, and greater levels than in tissue infected with PVX-P1-6×-HCPro (Fig. 3B, top panels). In the symptomless plants kept at 30 °C, CP levels in the three viruses were lower, and differences among them were less marked (Fig. 3B, top panels). This is somewhat surprising as it occurred despite the fact that two of the constructs expressed 6×-2b-HA and P1-6×-HCPro, both strong suppressors of gene silencing in agropatch assays, as shown previously (González *et al.*, 2012 and Tena *et al.*, 2013, respectively), and, at least for 6×-HCPro, functionally as active at 30 °C as at 25 °C (this work). That both suppressors accumulated at 30 °C in virus-infected tissue was confirmed serologically (Fig. 3B, bottom panels).

Table 1 Visual assessment of systemic infection symptoms in plants infected with *Potato virus X* (PVX)-based vectors (PVX, PVX-P1-6×-HCPro or PVX-6×-2b-HA) at 4–7 days post-infiltration (dpi).

	4 dpi		5 dpi		6 dpi		7 dpi	
	25 °C	25 °C (24 h)– 30 °C (48 h)	25 °C	25 °C (24 h)– 30 °C (48 h)	25 °C	25 °C (24 h)– 30 °C (48 h)	25 °C	25 °C (24 h)– 30 °C (48 h)
PVX	No symptoms	No symptoms	Curling in apical leaves, mosaic	No symptoms	Curling in apical leaves increased	No symptoms	Curling in apical leaves increased	No symptoms
PVX-P1-6×-HCPro	No symptoms	No symptoms	Curling in apical leaves, mosaic	No symptoms	Curling in apical leaves increased	No symptoms	Curling in apical leaves increased	No symptoms
PVX-6×-2b-HA	No symptoms	No symptoms	Curling in apical leaves, mosaic	No symptoms	Curling in apical leaves increased	No symptoms	Curling in apical leaves increased. Start of necrosis in leaves 3–6 above infiltrated ones	No symptoms

Fig. 3 Assessment of the efficiency of agroinfiltration to deliver *Potato virus X* (PVX)-based amplicons to *Nicotiana benthamiana* plants, and their accumulation at different temperatures, at 7 days post-infiltration. (A) Symptoms found in systemic leaves of plants infiltrated with a PVX amplicon, or with PVX amplicons expressing either P1-6x-HCPro or 6x-2b-HA protein (PVX-P1-6x-HCPro and PVX-6x-2b-HA, respectively). At 25 °C, plants showed systemic symptoms in the three cases (first, third and fifth images from the left). Symptoms were more severe if a suppressor was expressed, in particular 6x-2b-HA (third and fifth images versus first image from the left). Plants kept at 30 °C after an initial 24 h at 25 °C appeared symptomless (second, fourth and sixth images from the left). (B) Western blot analysis of viral coat protein (CP; top panels), 6x-HCPro (bottom left panel) and 6x-2b-HA (bottom right panel) accumulation in systemic tissue (fifth leaf above the infiltrated ones) of the plants shown in (A) using antibodies against PVX CP, 6x-histidines and 2b protein, respectively. CP and suppressor accumulation were lower in samples kept at 30 °C after an initial 24 h at 25 °C than in plants kept all the time at 25 °C. (C) Western blot analysis of viral CP accumulation in systemic tissue (fifth leaf above the infiltrated ones) in plants kept for 7 days after infiltration at 30 °C versus plants kept all the time at 25 °C. In both (B) and (C), CP protein accumulation was similar between virus treatments, but lower in the plants kept at 30 °C than in those kept at 25 °C. Below the selected lanes are densitometric analyses of CP bands as a measure of virus titres. Protein levels in plants kept at 25 °C infected with the PVX construct are given the value of 100%. Lane M in the blots shows molecular weight markers in kilodalton (kDa), and the small panels below the Western blots show the membranes stained with Ponceau-S after blotting, as controls of loading.

As even a very low transfer of T-DNA by the agrobacterium could be sufficient to allow the viral amplicon to replicate independently, and given that we observed a low level of expression of the GFP reporter even in plants transferred to 30 °C immediately

after agroinfiltration (Fig. 1, right leaf and right Western blot panel), we tested whether infiltration of the viral amplicons at 30 °C would cause plants to become infected. At 7 days after agroinfiltration, plants remained symptomless (data not shown),

but Western blot analysis of the viral CP in systemic tissue revealed that they were infected, although CP levels were again lower than those found in plants kept at 25 °C (Fig. 3C). Furthermore, the levels of CP in all cases were very similar to those found in plants kept for the first 24 h after infiltration at 25 °C prior to their transfer to 30 °C for 6 days (Fig. 3C versus 3B). However, repeated tests showed that, in some instances, PVX infection failed to occur in plants transferred to 30 °C immediately after agroinfiltration (Fig. S1C). Therefore, even for T-DNAs expressing viral amplicons, a 24-h window at 25 °C is advisable.

DISCUSSION

Most research in laboratories investigating the mechanisms and processes underlying plant–virus interactions takes place at the standard room temperature, usually 25 °C. One of the main tools in plant pathology for functional studies is agroinfiltration, a technique that has its optimal working temperature precisely at 25 °C (Chen *et al.*, 2013). At temperatures of 29 °C and above, it is nonfunctional (Fullner and Nester, 1996; Gelvin, 2003). However, plants interact with their environment under seasonal and daily changes in temperature that, within a crop growing season, can span from a low of 10 °C or less to a high in the upper 30 °C region. Thus, there is interest in studying plant processes at 30 °C and above, especially as agriculture in warm and highly populated regions is becoming increasingly important, and as climate models forecast future increases in both average and extreme temperatures in most areas. The use of agroinfiltration as a fast and versatile tool to express proteins in native or modified form, or to transiently silence any gene at elevated temperatures, would be very useful.

However, in plant–virus interactions, increased temperature leads, in general, to weaker infection symptoms (Hull, 2002). In this regard, there is documented evidence that gene silencing-based defence processes that are key to the infection outcome show a positive correlation with temperature: it has been reported that antiviral silencing becomes progressively stronger against RNA viruses and agroinfiltrated T-DNAs from 15 to 24 °C (Szyttia *et al.*, 2003), against positive-sense RNA viruses from 21 to 27 °C (Qu *et al.*, 2005), against negative RNA viruses from 26/18 °C to 32/26 °C (day/night; Velázquez *et al.*, 2010) and against DNA geminiviruses from 25 to 30 °C (Chellappan *et al.*, 2005). In all cases, this coincides with a decrease in the accumulation of corresponding viral titres, their mRNAs or the products they encode, and with an increase in the levels of siRNAs to these sequences. This observation has been used to advantage to transform plants at higher temperatures to constitutively express a potyvirus-based viral amplicon vector, avoiding its pathological effects. When transformation, regeneration and plant growth take place at high temperature, the amplicon fails to replicate and plants avoid virus-derived pathological effects during their regeneration and

development. Lowering the temperature in full-grown, symptomless plants allows the amplicon vector to express any potential product at high levels in some of the transgenic lines (Dujovny *et al.*, 2009).

In contrast with gene silencing, less is known about the effect of temperature on other defence processes targeting viruses, such as proteasome or autophagy routes. Temperature can, however, also affect the biological activities of viral factors involved in processes such as movement (Boyko *et al.*, 2000) and replication (Ohsato *et al.*, 2003), although, for the latter, to discriminate it from gene silencing is problematic. To date, there is little evidence regarding the temperature dependence of the activity of viral suppressors of silencing, but as this takes place through binding to nucleic acids or to host factors, within and/or interacting with physical subcellular structures/compartments, it could be the case.

Here, we have developed a procedure that allows the use of the agroinfiltration technique for the expression of genes at 30 °C in virus–plant interaction studies. We first characterized the efficiency of agroinfiltration to express proteins at 30 °C and found that, at this temperature, the accumulation of a GFP reporter and of the viral proteins tested was either very poor or undetectable (Fig. 1). By providing a 24-h period post-infiltration at 25 °C before placing the plants at 30 °C, we allowed sufficient time for T-DNA transfer and subsequent gene expression to occur, whereas a shorter window of 12 h was found to be inefficient (Figs 1 and S1). In this way, and by co-expression of our reporter with any of three viral suppressors of silencing, we were able to achieve levels of accumulation that were over 83% of those obtained in similarly agroinfiltrated plants kept at 25 °C (Fig. 1B).

In contrast with our GFP reporter, the accumulation of both viral HCPro and 2b suppressor proteins was lower than that found in plants kept all the time at 25 °C (33% in the case of 2b protein; visually lower, but not quantified, in the case of 6×-HCPro; Fig. 1B). The reason for this disparity with the reporter could be the intrinsic stability or, unlike the reporter, the viral suppressors may be differentially targeted by host processes that negatively affect their steady-state levels, such as the proteasome (Ballut *et al.*, 2005; Dielen *et al.*, 2011; Jin *et al.*, 2007; Sahana *et al.*, 2012), or in the case of both HCPro and 2b protein, through their binding to a calmodulin-related protein that may direct them to degradation via autophagy (Nakahara *et al.*, 2012).

Although 24 h is usually insufficient for protein accumulation after agroinfiltration (Voinnet *et al.*, 2003), we tested, in a time course, the levels of accumulation of proteins in our system from 24 to 72 hpi. We found that we could detect both GFP reporter and 2b suppressor at 24 hpi. They are small proteins of 25 and 16 kDa, respectively. By contrast, we failed to detect accumulation after 24 h of the much larger (50 kDa) 6×-HCPro, which was also expressed as a polyprotein downstream of the self-cleaving potyviral P1 factor. In any case, the levels of accumulation of GFP and 2b protein at 24 hpi were less than 12% of those found at

72 hpi (Fig. 2). Therefore, the vast majority of proteins were actually expressed at 30 °C, as were their biological activities, if any. Our data from 6×-HCPro and 2b (or 6×-2b-HA) show that the former requires more time to be translated and accumulated than the latter (Fig. 2B), and also suggest that its stability is greater than that of the 2b protein and its turnover is slower. It may also be possible that the 2b protein is targeted for degradation in a more efficient manner than is HCPro by autophagy. These results also suggest that not all 'strong' suppressors may perform in the same manner in our agroinfiltration procedure to express proteins at high temperatures, although, in our conditions (30 °C and 72 hpi), we observed no difference in the suppressor effect on GFP reporter levels using either 6×-HCPro or 2b protein, or P19 (Fig. 1). The fact that the steady-state levels of 2b protein decreased much more rapidly than those of 6×-HCPro (Fig. 2B) suggests that, if the experiment was performed at higher temperatures or for a longer period of time (4–5 days), differences in suppressor activity may arise, and the more stable suppressor would be preferable.

We also tested how our procedure would help express viral PVX amplicons from binary vectors. We found that, although the 24-h window at 25 °C is not strictly necessary for the amplicon to successfully take hold in a plant, it is necessary to ensure that all plants become infected. When we tested PVX and PVX expressing either 6×-HCPro or 6×-2b-HA protein, we found that, regardless of the differences between the three in the symptoms they induced and in their CP titres in plants kept at 25 °C, at 30 °C, all plants were asymptomatic and CP levels were more uniform in the three cases, roughly between 25% and 50% of those found in systemic tissue of PVX-infected plants kept at 25 °C (Table 1 and Fig. 3A, B). This may indicate that defence responses against PVX are stronger at 30 °C than at 25 °C or, alternatively, that viral replication is negatively affected by the higher temperature. Neither of the suppressors expressed by PVX-P1-6×-HCPro or PVX-6×-2b-HA increased CP levels from those found in tissue infected with PVX at 25 or 30 °C (Fig. 3B, C), despite 6×-HCPro being a strong suppressor at 30 °C. This is in agreement with the observations that PVX RNA did not increase in plants infected with a PVX recombinant virus expressing the *Plum pox virus* HCPro compared with plants infected with the PVX vector (González-Jara *et al.*, 2005).

In conclusion, we have designed a procedure that allows the use of the agroinfiltration technique for the efficient expression of genes in plants for scientific studies at 30 °C. This technical advance allows: (i) studies *in vivo* at these temperatures of, for example, fluorescently tagged proteins and their interactions with subcellular structures or other proteins, and their dynamics; (ii) the performance of biological assays, such as reported here, on the suppression of the silencing of a reporter by viral factors; or (iii) comparative studies of the synthesis and turnover of proteins. We have also shown the viability of the agroinfiltration of binary

vectors for the expression of viral amplicons at 30 °C. With this tool, we were able to monitor the effect of heterologous viral suppressors of silencing, whose activities were previously assessed at high temperatures, on the overall response of the host to the virus.

EXPERIMENTAL PROCEDURES

Binary vector constructs

Reporter and viral proteins were transiently expressed in plants from corresponding gene sequences expressed from caulimovirus 35SP-driven, pROK2-based binary vectors: vectors expressing the free GFP reporter and CMV (Fny) 2b protein have been described in González *et al.* (2010); the binary vector expressing PVY P1-6×-HCPro (construct P1-6×-HCPro) has been described in Tena *et al.* (2013); the binary vector expressing TBSV P19 suppressor has been described in Canto *et al.* (2006). To generate the pROK2 binary vector that expressed 2b protein with six histidines fused at its N-terminus and an HA peptide (Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala) fused to its C-terminus, used in Fig. S1 (construct 6×-2b-HA), the 2b coding sequence was amplified in sequential polymerase chain reaction (PCR) events with flanking oligos that contained the HA peptide and 6×histidine sequences, resulting in a 2b sequence that had attached, at its 3' end, an *Ascl* site plus that of the HA peptide in frame and a *SacI* site plus a stop codon at its end, and, at its 5' end, an *XbaI* site followed by an ATG, the sequence coding for 6×histidine and a *BamHI* site in frame with the following sequence coding for 5' end nucleotides of the 2b sequence. The fragment was cloned between the *XbaI* and *SacI* sites of pROK2.

With regard to the three PVX viral amplicons, binary vector pgR 107 was used to express infectious PVX that contains an additional CP promoter and a polylinker for the insertion and expression of foreign genes (Lu *et al.*, 2003). PVX expressing PVY P1-6×-HCPro has been described previously (Tena *et al.*, 2013). To create PVX expressing 6×-2b-HA, the tagged 2b protein was amplified by PCR with appropriate oligos and cloned into *Clal/SalI*-linearized construct pgR 107.

All PCRs were performed with Phusion™ DNA polymerase (Finnzymes, Keilaranta, Finland). All constructs were confirmed by sequencing of the inserted fragments and of the vector flanking regions.

Transient expression of genes in plants and local suppression of silencing in agroinfiltration patch assays

For transient expression assays (patch assays) of single proteins expressed from binary vectors, the latter were transferred to nononcogenic electrocompetent *A. tumefaciens* strain C58C1 derived from a single colony to prevent bacterial variability. Cultures were grown to exponential phase in Luria–Bertani medium with antibiotics at 28 °C. For infiltration, each bacterial culture was diluted to a final optical density of 0.3 at 600 nm. Different cultures harbouring different T-DNAs were then combined, and infiltration of the mixtures was performed as described by Canto *et al.* (2002). In silencing suppression assays, a free GFP reporter gene expressed from a binary vector under the control of the 35S promoter was expressed transiently in an *N. benthamiana* leaf, co-infiltrated with

either the empty binary vector pROK2 or with another vector expressing a protein to be tested for the suppression of silencing activity. Leaves were then illuminated at 72 hpi with a Blak Ray® long-wave UV lamp (UVP, Upland, CA, USA) to visualize the fluorescence derived from the transiently expressed free enhanced GFP as described by González *et al.* (2010).

In the case of PVX amplicons, the binary vectors expressing them were electroporated into electrocompetent *A. tumefaciens* strain GV3101 already containing a pJIC SA_Rep that conferred tetracycline resistance, kindly provided by Professor D. C. Baulcombe's group (University of Cambridge, Cambridge, UK). For infiltration, *Agrobacterium* cultures were grown as described in the previous paragraph.

Agroinfiltrations were performed on the laboratory bench at 25 °C. Immediately after infiltrations (no more than 15 min on the bench), plants were transferred to Controlled Environment Plant Growth chambers (SANYO Electronic Co.; Panasonic Corp., Osaka, Japan), where they were maintained in a 16-h/8-h day/night photoperiod and at temperatures of 25 °C or 30 °C, or combinations thereof, depending on the experiment. In addition to the chamber's own temperature controls, temperature accuracy was independently monitored with a PCE HT-71 datalogger sensor (PCE Instruments Ltd., Southampton, UK).

Immunoblot detection of proteins and densitometric analysis

Infiltrated fresh leaf tissue discs of 25 mm in diameter were separated from the leaf with a borer (0.05–0.1 µg of fresh leaf tissue). Total proteins were extracted by grinding the nitrogen-frozen discs with a pestle in 400 µL of extraction buffer [0.1 M tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 8, 10 mM ethylenediaminetetraacetic acid (EDTA), 0.1 M LiCl, 1% β-mercaptoethanol and 1% sodium dodecylsulphate (SDS)], and the samples were boiled and fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) in 10% (for HCPro detection) or 15% (for GFP and 2b protein detection) gels. Gels were wet blotted in Tris-glycine buffer onto Hybond-P poly(vinylidene difluoride) (PVDF) membranes (Amersham, GE Healthcare, Little Chalfont, Buckinghamshire, UK). For immunological detection of GFP, a rabbit anti-GFP, N-terminal antibody was used (Sigma-Aldrich, St Louis, MO, USA; Cat. G1544). For detection of 6×-HCPro, a mouse monoclonal antiserum to six histidines was used (Sigma-Aldrich; Cat. H1029). In addition, a mouse monoclonal antibody to PVY HCPro (Ab 1A11; Canto *et al.*, 1996) was used for its detection in the panels shown in Fig. 2, indicated as α-HCPro. For the detection of 2b protein, a mouse polyclonal serum was used (González *et al.*, 2010). For the detection of PVX by Western blot, a commercial rabbit antibody was used (No. 070375/500; Loewe Biochemica GmbH, Sauerlach, Germany). Blotted proteins were detected using commercial secondary antibodies and SigmaFast™ 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium (BICP/NBT) substrate tablets (Sigma-Aldrich). Comparative protein densitometric analysis of blotted proteins was made with Quantity One 4.6.3 1-D analysis software (Bio-Rad Laboratories, Hercules, CA, USA). The numbers shown in the figures represent the quantification of protein amounts in individual Western blot bands, and the values given are percentages relative to the value found in the internal control within the same blot. Densitometric comparisons were only made between bands within the same membrane and not between bands that corresponded to different blots or antibodies, unless internal controls were taken into account.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 (A) Assessment of the efficiency of agroinfiltration to transiently express a green fluorescent protein (GFP) reporter in infiltrated *Nicotiana benthamiana* patches at different temperatures. The GFP vector was co-infiltrated together with an empty vector (patches labelled C) or with vectors expressing *Potato virus Y* P1-6×-HCPro (patch labelled 1), *Cucumber mosaic virus* (CMV) 2b protein or a modified 2b protein with both a hexahistidine tag and an HA peptide tag at the N- and C-termini, respectively (6×-2b-HA) (patches labelled 2 and 3, respectively). The top panel shows the visualization of GFP-derived fluorescence under the UV lamp in a leaf kept at 25 °C for 72 h post-infiltration (72 hpi; left leaf), in a leaf kept at 30 °C for 72 hpi (right leaf) and in a leaf kept for the first 24 hpi at 25 °C and the following 48 hpi at 30 °C (central leaf). Note the similarity in fluorescence between the left and central leaves. The bottom panel shows the visualization of GFP-derived fluorescence under a UV lamp in a leaf kept at 25 °C for 60 hpi (left leaf), in a leaf kept for 60 hpi at 30 °C (right leaf) and in a leaf kept for the first 12 hpi at 25 °C and the following 48 hpi at 30 °C (central leaf). Note that, in this case, fluorescence in the central leaf is much weaker than that in the left leaf. (B) Time course accumulation of the 6×-2b-HA protein at 24, 48 and 72 hpi by Western blot analysis using an antibody against the 2b protein. During the first 24 h, the plant was kept at 25 °C, and afterwards transferred to 30 °C. Note that the highest accumulation of 6×-2b-HA occurred at 24 hpi. (C) Detection of the presence of the virus in plant tissues agroinfiltrated with binary vectors that expressed three *Potato virus X* (PVX) amplicons by Western blot analysis using an antibody against PVX coat protein (CP). Immediately after infiltration plants were kept at either 25 °C or 30 °C. Note that, in one case (PVX-P1-6×-HCPro), infiltration on leaves transferred to 30 °C failed to initiate infection. Lane M in the blots shows molecular weight markers in kilodalton (kDa), and the small panels below the Western blots show the membranes stained with Ponceau-S after blotting, as controls of loading.