Systemic acquired resistance is induced by *R* gene-mediated responses independent of cell death

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SUMMARY

On infection by pathogens, plants initiate defence responses that are able to curtail infection locally. These responses are mediated either by receptor-like proteins that recognize pathogen-associated molecular patterns or by the protein products of disease resistance (*R*) genes. At the same time, primary defence responses often result in the generation of signals that induce what is known as systemic acquired resistance (SAR), such that defence responses are enhanced on secondary pathogen challenge in distal tissues. R protein-mediated SAR induction is normally accompanied by a type of programmed cell death known as the hypersensitive response (HR) and, in some instances, cell death alone has been implicated in the induction of SAR. This has raised the question of whether R protein-mediated signalling *per se* induces SAR or whether SAR is an indirect result of the induction of HR. Using the *Rx* gene of potato, which confers resistance to Potato Virus X in the absence of cell death, we have shown that the HR is dispensable for R protein-mediated induction of SAR and that Rx-induced SAR is mediated by the same salicylate-dependent pathway induced by other R proteins.

INTRODUCTION

Plants have evolved multiple mechanisms to recognize pathogens and to induce both local and systemic responses. One of the first lines of defence is mediated by the recognition of highly conserved pathogen-associated molecular patterns (PAMPs) associated with large groups of pathogens, such as lipopolysaccharides (LPSs) and highly conserved bacterial proteins, e.g. flagellin and Ef-Tu. For the most part, this PAMP-triggered immunity (PTI) appears to be mediated by transmembrane receptor-like proteins, and is presumed to be sufficient to protect plants

against most bacterial, fungal and oomycete pathogens (Chisholm *et al*., 2006). However, the best-characterized basal defence against viral pathogens is RNA silencing, which targets viruses based on the physical characteristics of their RNAs (Ding and Voinnet, 2007). However, host-adapted pathogens are able to interfere with and overcome PTI and/or RNA silencing (Chisholm *et al*., 2006; Diaz-Pendon and Ding, 2008), necessitating a second line of defence based on much more specific recognition of pathogens or pathogen isolates. This recognition is mediated by the products of plant disease resistance (*R*) genes and results in a much more dramatic defence response, often culminating in a type of programmed cell death known as the hypersensitive response (HR) (Mur *et al*., 2008). Many R proteins recognize so-called effector proteins (pathogen proteins secreted into the host cell) and, as such, *R* gene-mediated responses are often referred to as effector-mediated immunity (ETI) (Chisholm *et al*., 2006). When a given effector protein is recognized by an R protein, it is referred to as an avirulence (Avr) protein, as it renders the pathogen non-virulent. However, most Avr proteins are thought to play a role in virulence on plants that do not possess a matching *R* gene, in part by suppressing PTI. R proteins also recognize Avr proteins from viruses. Like effector proteins, in the absence of a cognate *R* gene, viral Avr proteins also play roles in virulence by interfering with host processes and/or by playing structural or enzymatic roles (Diaz-Pendon and Ding, 2008; Kang *et al*., 2005; Schoelz, 2006). The most common type of *R* gene encodes nucleotide-binding and leucine-rich repeat (NB-LRR) proteins. There are two major classes of NB-LRR proteins that are distinguished by the domains present at their N-termini: those that possess a TIR (Toll, and interleukin-1 receptor homology) domain and those that do not (Meyers *et al*., 1999). Many non-TIR-NB-LRR proteins possess an N-terminus that is predicted to be α-helix rich and often, but not always, encodes a predicted coiled-coil (CC) motif. As such, non-TIR-NB-LRR proteins are often referred to as CC-NB-LRRs. Plant NB-LRR proteins have been shown to confer resistance to viruses, bacteria, oomycetes, fungi and arthropods (Martin *et al*., 2003). Given that R proteins are able to counter several different types of pathogen, they probably activate multiple signalling

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pathways. These induced responses stop pathogen infection locally, but also induce signals that enhance defence responses to pathogens in distal systemic tissues, a phenomenon known as systemic acquired resistance (SAR) (Vlot *et al.*, 2008). SAR responses are associated with the transcriptional up-regulation of a number of genes, including those encoding pathogenesis-related (PR) proteins in both local and systemic tissues (Durrant and Dong, 2004).

Although classical SAR experimental systems are associated with cell death induced either as a symptom of infection or by the activation of an R protein (Durrant and Dong, 2004), it has recently been reported that PTI responses can also induce SAR in *Arabidopsis* in the absence of cell death (Mishina and Zeier, 2007). As such, it is unclear whether SAR induced by R protein activation is a direct consequence of R protein signalling or whether it is an indirect consequence induced by dying cells which might, in turn, release signals that activate PTI-like mechanisms in neighbouring cells.

The *N* gene of *Nicotiana glutinosa*, encoding a TIR-NB-LRR protein, confers resistance to tobacco mosaic virus (TMV) and has been introgressed into commercial tobacco (*N. tabacum*) (Whitham *et al.*, 1994). Infection of *N* genotype tobacco leads to a typical HR-type resistance response which, in turn, induces SAR, such that secondary TMV infections will induce HR lesions significantly reduced in size, consistent with a faster resistance response. This well-established model system is highly robust and has been used to demonstrate the requirement for salicylate (SA) in the induction of SAR, and the involvement of methyl salicylate (MeSA) as a mobile signal in SAR in tobacco (Delaney *et al.*, 1994; Gaffney *et al.*, 1993; Park *et al.*, 2007; Vernooij *et al.*, 1994).

The potato *Rx* gene encodes a typical CC-NB-LRR protein and confers resistance to potato virus X (PVX) (Bendahmane *et al.*, 1999). Unlike the *N* gene, however, *Rx* confers what is known as extreme resistance (ER) in potato and, as a transgene, in tobacco (Bendahmane *et al.*, 1999). Extreme resistance is seen with a number of *R* genes conferring resistance to viruses; it is manifested as a complete lack of both macroscopic and microscopic HR lesions and is accompanied by the absence of detectable virus accumulation (Kang *et al.*, 2005). However, many *R* genes that induce ER, including *Rx*, also induce HRs depending on the strain of virus, host genetic background and environmental conditions (Baures *et al.*, 2008; Bendahmane *et al.*, 1999; Kang *et al.*, 2005). Thus, it is likely that there is no qualitative difference in the responses induced by *Rx*, but rather that the response is very rapid and efficient, such that virus accumulation is inhibited before the onset of secondary responses, such as HR.
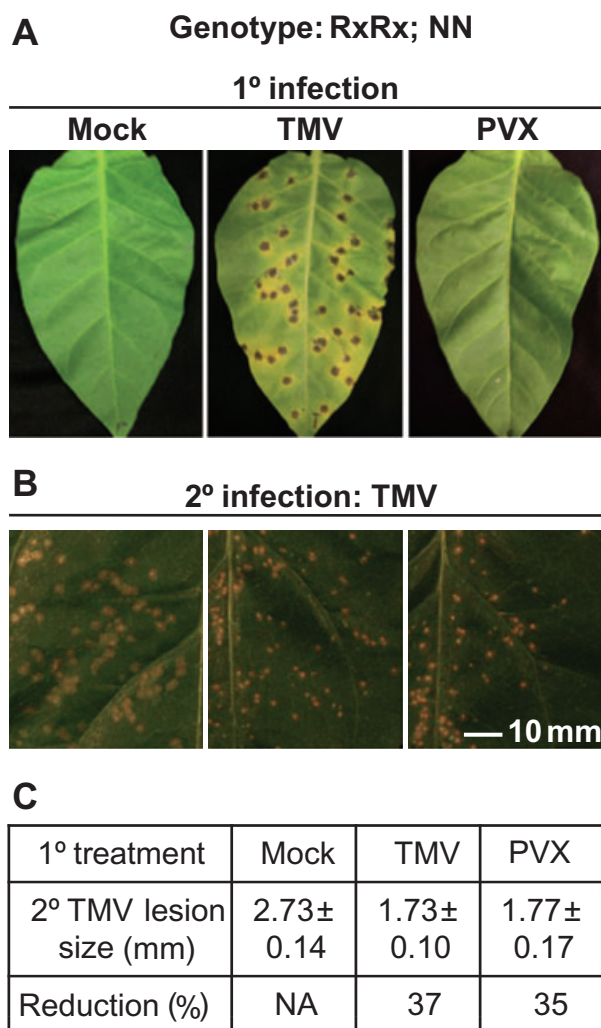
We found that *Rx*-mediated defence responses leading to ER ware able to induce SAR against subsequent TMV infections, and that this response showed the hallmarks of classic SAR responses. We conclude that the activation of *Rx* leads to the direct induction of SAR-inducing signals and that *R* protein-mediated SAR is not a by-product of HR.

RESULTS AND DISCUSSION

To determine whether HRs are required to induce SAR, we made use of tobacco plants expressing the *Rx* gene from its own promoter in the cultivar Samsun (NN) (Bendahmane *et al.*, 1999). The lower leaves of plants were subjected to either a mock treatment or infected with TMV or PVX. As expected, TMV induced HR lesions on inoculated leaves, whereas PVX did not (Fig. 1A). All plants were subsequently infected with TMV on the upper leaves 5 days later. As expected, plants previously infected with TMV showed a reduction in HR lesion size compared with mock-inoculated plants, indicating the induction of SAR (Fig. 1B,C). Likewise, plants that had been infected previously with PVX showed similarly reduced lesion sizes (Fig. 1B,C), indicating that the *Rx*-mediated resistance response is able to induce SAR to a degree similar to that induced by *N*.

Previous studies have shown important roles for SA and MeSA in the induction and signalling of SAR in the tobacco–TMV pathosystem (Vlot *et al.*, 2008). To determine whether the *Rx*-mediated induction of SAR is similar in mechanism to that induced by *N*, we investigated the requirement for these molecules in *Rx*-induced SAR. To this end, *Rx* transgenic tobacco was crossed to transgenic tobacco (cultivar Xanthi nc [NN]) expressing either the bacterial *nahG* gene (*NahG*) or an RNA hairpin targeting the tobacco SA-binding protein 2 (*SABP2*) transcript for degradation by RNAi (*RNAi::SABP2*), or to wild-type plants. *NahG* encodes a salicylate hydroxylase that metabolizes SA, whereas SABP2 is an esterase required for the conversion of the SAR signal MeSA to SA in healthy systemic tissue (Forouhar *et al.*, 2005; Kumar and Klessig, 2003; Park *et al.*, 2007). The *RNAi::SABP2* transgene reduces the *SABP2* transcript by greater than 75% (Kumar and Klessig, 2003). As all three transgenes are dominant, the resulting F1 progeny were used for SAR analysis. Plants were subjected to a primary infection (mock, TMV or PVX) followed by a secondary infection with TMV as described above. Tobacco plants expressing *nahG* can generate the SAR-inducing signal in primary infected tissue, but are unable to perceive or process the signal in systemic tissue (Delaney *et al.*, 1994; Gaffney *et al.*, 1993; Vernooij *et al.*, 1994). As expected, *Rx/NahG/NN* genotype plants failed to develop SAR after a primary infection with TMV, as the size of the lesions formed after secondary infection with TMV were as large as those formed in plants that had received a mock primary infection (Fig. 2A). Likewise, plants subjected to primary infection with PVX showed no reduction in HR lesion size on secondary infection with TMV compared with those receiving mock primary infection (Fig. 2A). Infection of F1 progeny of crosses between Rx and *RNAi::SABP2* plants with TMV following a primary infection

A   Genotype: RxRx; NN

1° infection

| Mock | TMV | PVX |

B   2° infection: TMV

—— 10 mm

C

1° treatment	Mock	TMV	PVX
2° TMV lesion size (mm)	2.73± 0.14	1.73± 0.10	1.77± 0.17
Reduction (%)	NA	37	35

Fig. 1 Extreme resistance induces systemic acquired resistance (SAR) signals. (A) Tobacco plants expressing both the *N* and *Rx* genes were rub-inoculated on three lower leaves with buffer (Mock), tobacco mosaic virus (TMV) or potato virus X (PVX). Representative leaves from this primary (1°) infection are shown 5 days post-infection (dpi). (B) Three systemic leaves immediately above the 1° infections were challenged with TMV (2° infection) 5 days after the 1° infection. Five days after the 2° infection, leaves were photographed and lesion sizes were determined. (C) Lesions sizes in millimetres (±standard deviation) from the experiment shown in (B). Reduction (%) is the percentage reduction in the size of 2° lesions on plants that received a 1° infection of TMV or PVX vs. mock 1° infected plants. N/A, not applicable. Experiments were performed at least four times with similar results.

degree of SAR-induced HR lesion size reduction as the *Rx* transgenic parent (Fig. 2C).

One of the hallmarks of *N*-induced SAR is the rapid induction of SA-responsive *PR* genes in both inoculated and healthy systemic leaves (Durrant and Dong, 2004). Cell death is not required for the local induction of *PR* genes in another plant–virus interaction. Plants resistant to cauliflower mosaic virus, which respond to infection with chlorotic lesions, have been shown to induce PR proteins in inoculated leaves, although SAR has not been tested in this system (Cole *et al*., 2001). As an additional demonstration that *Rx*- and *N*-mediated responses are similar in mechanism, we assessed the expression of the tobacco acidic *PR-1* gene in *Rx* transgenic plants inoculated with PVX. Although there was little *PR-1* expression in uninfected (day 0) or mock-infected plants, expression was significantly induced in inoculated leaves 1 day after PVX infection and in systemic leaves 5 days after PVX infection (Fig. 3). Thus, *Rx*-mediated induction of SAR appears to utilize the same SA signals and induces the same type of defence-related genes as in *N*-induced SAR, suggesting a similar mode of action.

As with other types of resistance responses, SAR is likely to be inducible by multiple stimuli and signalling pathways. Infection with *Pseudomonas syringae* can induce SAR by inducing an HR which is mediated by the products of *R* genes. At the same time, *Pseudomonas* can also induce SAR by inducing necrosis during the normal course of infection (Durrant and Dong, 2004). The latter is presumably initiated not by R proteins, but by other, as yet undefined, types of signalling pathway. In addition, SAR is induced in plants exposed to non-host bacteria as well as the purified PAMP LPS and the flagellin-derived peptide flg22 (Mishina and Zeier, 2007). Thus, both ETI- and PTI-mediated signalling are capable of inducing SAR, although the latter may often be suppressed by pathogens that employ effector proteins to disrupt PTI.

ETI and PTI are mediated by different types of receptor-like protein, and the physiological responses they induce differ greatly in magnitude. Both mechanisms appear to utilize at least some common components, such as SA signalling, and both induce *PR* gene expression (Mishina and Zeier, 2007). However, the fact that cell death is not required for PTI-mediated SAR induction does not rule out a role for HR in SAR induction by ETI-mediated responses. Whether ETI-induced SAR is a direct consequence of R protein signalling or an indirect consequence of HR remains an open question. Indeed, it is possible that ETI responses may induce SAR indirectly in a manner analogous to a paradigm known in animal immunity as the 'danger model', whereby PAMP receptors induce inflammatory responses in response to self-molecules released by dying cells (Matzinger, 2002). As such, R protein signalling could activate SAR indirectly by inducing an HR which, in turn, activates PTI-like mechanisms that respond to molecular patterns released by dying cells. Indeed, plants with lesion mimic phenotypes

with either TMV, PVX or mock treatment showed that the Rx-induced SAR is similarly compromised in the absence of SABP2 (Fig. 2B). The lack of SAR development in the F1 progeny described above was not caused by differences in genetic background or gene dosage, as the progeny of *Rx* transgenic plants crossed with wild-type Xanthi nc (NN) plants showed a similar

A   Genotype: Rx; NN; NahG

1° infection

Mock	TMV	PVX

2° infection: TMV

— 10 mm

1° treatment	Mock	TMV	PVX
2° TMV lesion size (mm)	3.26± 0.14	3.29± 0.14	3.26± 0.14
Reduction(%)	NA	- 1	0

B   Genotype: Rx; NN; RNAi::SABP2

1° infection

Mock	TMV	PVX

2° infection: TMV

— 10 mm
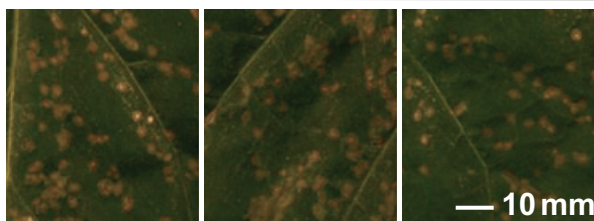
1° treatment	Mock	TMV	PVX
2° TMV lesion size (mm)	2.76± 0.12	2.62± 0.14	2.75± 0.09
Reduction (%)	NA	5	1

C   Genotype: Rx; NN

1° treatment	Mock	TMV	PVX
2° TMV lesion size (mm)	3.24± 0.24	1.82± 0.26	2.11± 0.22
Reduction(%)	NA	44	35

Fig. 2 The systemic acquired resistance (SAR) signal induced by extreme resistance is dependent on salicylate (SA)-mediated signalling. F1 progeny from the crossing of *Rx* transgenic tobacco with *NahG* (A) or *RNAi::SABP2* (B) transgenic tobacco or wild-type tobacco (C) were assessed for their ability to develop SAR. Plants were rub-inoculated on three lower leaves with buffer (Mock), tobacco mosaic virus (TMV) or potato virus X (PVX). Three systemic leaves immediately above the 1° infections were challenged with TMV (2° infection) 5 days after the 1° infection. Five days after the 2° infection, leaves were photographed (A and B) and lesion sizes were determined. Lesions sizes in millimetres (±standard deviation) are shown in table form (A–C). Reduction (%) is the percentage reduction in the size of 2° lesions on plants that received a 1° infection of TMV or PVX vs. mock 1° infected plants. N/A, not applicable. Experiments were performed at least three times with similar results.
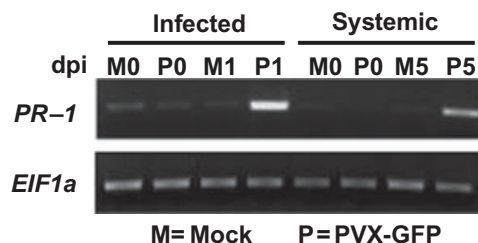
Fig. 3 Extreme resistance induces expression of the *PATHOGENESIS-RELATED-1* (*PR-1*) gene. Leaves of *Rx* transgenic tobacco were either mock-inoculated (M) or infected with potato virus X (PVX) (P). RNA was extracted from 1° inoculated leaves at 0 (M0 and P0) and 1 (M1 and P1) day post-infection (dpi), and from systemic leaves at 0 (M0 and P0) and 5 (M5 and P5) dpi. RNA was subsequently used for reverse transcription followed by polymerase chain reaction (PCR) using primers targeting the tobacco acidic *PR-1* gene. The constitutively expressed translation elongation factor 1α gene (*EF1α*) was used as a control. Two independent experiments yielded similar results.

resulting from mutation or transgene expression often possess SAR-like enhanced disease resistance, suggesting that certain types of cell death alone are sufficient to induce non-cell autono-mous defences (Lorrain *et al.*, 2003; Mittler and Rizhsky, 2000). As Rx induces SAR but not HR, this indicates, for the first time, that cell death is not intrinsically necessary for SAR induced by NB-LRR proteins. Thus, although we do not rule out the possible existence of SAR-inducing danger signals generated by dying cells, it would appear that NB-LRR protein signalling leads directly to the generation of SAR signals.

The induction of systemic signals by Rx must also be very strong. In the case of the N–TMV interaction, a relatively large number of cells are eventually infected within individual infection foci. This would allow for a relatively large base of production either in cells undergoing a resistance response and/or in cells adjacent to dying cells. However, PVX infection in *Rx* plants results in the infection of only isolated individual cells, such that only several hundred cells would be infected on a given inoculated leaf (see Experimental procedures). Nonetheless, Rx

induced the same level of systemic resistance as N, as measured by the reduction in lesion size after secondary infection by TMV.

Although we have not addressed the exact nature of the SAR-inducing signals generated by the *Rx* response, it would appear that they are similar to other *R* protein-mediated responses, in that SA and MeSA are both required in healthy systemic tissue (Fig. 2). The primary resistance response to PVX mediated by Rx, however, is not affected by NahG and thus appears to be independent of SA (Bendahmane *et al*., 2000) (data not shown). In contrast, NahG affects the primary defence responses mediated by the tobacco *N* and tomato *Tm2²* genes, as TMV inoculation results in large, spreading necrotic lesions in *nahG* transgenic plants (Brading *et al*., 2000; Delaney *et al*., 1994). In virus–*R* gene interactions, distinct HR lesions result because the initially infected individual cells are not able to contain the virus, which subsequently spreads to neighbouring cells. However, cells at the outer edge of the infection front undergo a stronger defence response, which enables containment of the virus (Wright *et al*., 2000). Thus, rather than having an intrinsically antiviral activity, SA serves to potentiate defence responses both systemically and locally (Cordelier *et al*., 2003; Ryals *et al*., 1996). This suggests that R proteins probably initiate bifurcating signalling pathways that lead to cell-autonomous antiviral resistance on the one hand and the production of SA on the other, with the latter leading to the potentiation of the former in adjacent cells and systemic tissues.

Our data are in agreement with previous studies indicating that ER induced by Rx is not qualitatively different from HR-type antiviral responses (Baures *et al*., 2008; Bendahmane *et al*., 1999), and provide an explanation for a lack of necessity for SA for Rx-mediated resistance. That is, SA is not required because the rapid response induced by Rx does not need to be potentiated in adjacent cells. Like other R proteins, however, Rx initiates the same SA-mediated signalling pathway(s) leading to the production of SAR signal(s). However, our results definitively rule out a requirement for cell death in SAR induction induced by NB-LRR proteins, and show that *R* protein signalling alone is sufficient for SAR induction. The question of whether NB-LRR proteins induce SAR by the same mechanism as PAMP receptors, however, requires further investigation.

EXPERIMENTAL PROCEDURES

Plant materials

Nicotiana tabacum (tobacco) expressing the *Rx* gene from its genomic promoter in the cultivar Samsun (NN), as well as the SABP2-silenced line 1-2 (*RNAi::SABP2*) and NahG-10 (*NahG*) transgenic lines in the tobacco cultivar Xanthi nc (NN), have been described previously (Bendahmane *et al*., 1999; Gaffney *et al*., 1993; Kumar and Klessig, 2003). The experiments in Figs 1 and 3

were performed using *Rx* transgenic plants. Pollen from SABP2-silenced line 1-2, NahG-10 and non-transgenic Xanthi nc (NN) was used to pollinate *Rx*-transgenic flowers and the resulting F1 hybrid seeds were used for the experiments shown in Fig. 2.

Pathogen preparation and experiments

PVX inoculum was prepared from *N. benthamiana* plants previously agro-infected with PVX-green fluorescent protein (GFP) (Peart *et al*., 2002). Infected systemic leaves were collected and macerated in 0.1 M sodium phosphate buffer, pH 5.2, approximately 1 week post-infection. The suspension was centrifuged at 13 000 g for 15 min at 4 °C. The supernatant was collected and frozen at –80 °C. The concentration of PVX was determined by checking the GFP fluorescent foci under UV illumination on infection of non-transgenic tobacco, and found to result in approximately 150 PVX infections per 25 cm². Uninfected *N. benthamiana* plants were similarly processed for mock infections.

To induce SAR, primary TMV, PVX or mock inoculations were carried out on approximately 6-week-old tobacco plants, as described previously (Guo *et al*., 2000). Five days after primary infection, three systemic tissues of all plants were challenged with TMV infection. The secondary lesion sizes were measured 5 days after primary infection, at which point the establishment of SAR was determined by measuring and comparing the lesion sizes of TMV-infected leaves with a vernier caliper. In each experiment, 50 lesions were measured on each of three leaves of a plant and the standard deviation was calculated. The percentage reduction in lesion size was calculated as the difference in diameter between the secondary lesions on plants that received a primary infection of TMV or PVX vs. mock primary infected plants, divided by the diameter of the secondary lesions on plants that received a mock primary infection. Each experiment was performed on at least three separate occasions, with both control (mock) and experiment (TMV and PVX) performed at the same time on any given genotype.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from leaves using Trizol reagent (Invitrogen; Carlsbad, California) according to the manufacturer's instructions. DNase-treated total RNA (2 µg) was used for reverse transcription with a SuperscriptII kit (Invitrogen). The RT product was subjected to PCR using primers (5′-TAGTCATGGGATTTGTTC TC-3′ and 5′-TCAGATCATACATCAAGCTG -3′) designed to amplify the tobacco *acidic PR-1* gene (accession number X06361) using the following conditions: one cycle at 94 °C (4 min), one cycle at 80 °C (2 min), touchdown cycles (94 °C for 15 s, 62 °C → 56 °C for 15 s and 72 °C for 30 s) (one cycle for each temperature) and 25 cycles at 94 °C (15 s), 55 °C (15 s) and 72 °C (30 s), followed by extension at 72 °C (7 min). The same samples were used

in PCR using primers (5′-AAGTATGCCTGGGTGCTTG -3′ and 5′-AGGGACAGTACCAATTCCACC-3′) designed to amplify the elongation factor 1α gene (*EF1α*) employing essentially the same conditions as above, except for the temperatures for the touchdown cycles (67 °C → 61 °C for 15 s) and the 20-cycle amplification (94 °C for 15 s, 60 °C for 15 s and 72 °C for 30 s).

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