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Implementation of an artificial reaction control in a TaqMan method for PCR detection of *Ralstonia solanacearum* race 3 biovar 2

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Abstract A previously published TaqMan PCR test for *R. solanacearum* race 3 biovar 2 was modified to enable both the validation of negative results and the confirmation of positive results in a closed-tube system. Negative results were validated through the use of a reaction control plasmid, designated pRB2C2, which was designed to generate a 94bp product using the same amplimers targeting the primary diagnostic 68bp sequence in *R. solanacearum* race 3 biovar 2 DNA. SYBR Green was included in the reaction mix to facilitate the identification of post-reaction products using melt peak analysis. The 94bp reaction control had a melt peak temperature of about 90°C, while the diagnostic target amplicon had a melt peak temperature of about 83°C; thus positive results could be easily confirmed and distinguished from the reaction control product. Addition of pRB2C2 at 100 copies per reaction had no effect on the sensitivity of the TaqMan assay for *R. solanacearum* race 3 biovar 2, and the modified assay successfully detected *R. solanacearum* race 3 biovar 2 in infected, asymptomatic tomato stems and leaves as well as in potato tubers and stems.

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Abbreviations

PCR	polymerase chain reaction
RFLP	restriction fragment length polymorphism
CFBP	collection française de bactéries phytopathogènes

Ralstonia solanacearum causes bacterial wilt in a broad range of hosts and has economic significance worldwide. Race 3 biovar 2 is a subspecific taxon within the species complex that infects solanaceous plants at low temperatures, and causes brown rot of potato. Race 3 biovar 2 (equivalent to biovar 2A), has recently been reclassified as phylotype II sequevar 1, based on phylogenetic analyses of sequence data (Prior and Fegan 2005). This organism does not occur in Canada, but is a regulated, quarantine pest because of its ability to become established in temperate climates (Grousset et al. 1998; Janse 1996; Janse and Schans 1998).

The low detection limits obtainable with PCR methods, make them highly attractive for diagnosing low-level, latent infections of important pathogens like *R. solancearum*. Several conventional PCR methods have been published for the detection of *R. solancearum* (Seal et al. 1993; Glick et al. 2002; Schönfeld et al. 2003). A method utilising post-reaction RFLP analysis to distinguish infraspecific *R. solanacearum* strains at the biovar level was also

developed (Poussier and Luisetti 2000). Primer sets were also developed to enable the specific identification of divisions within the species by targeting the 16S rDNA gene (Fegan et al. 1998; Boudazin et al. 1999), and the intergenic spacer region between the 16S and 23S rDNA genes (Pastrik et al. 2000). Multiplex TaqMan methods specific for the detection of all strains of *R. solanacearum* (Weller et al. 2000) as well as race 3 biovar 2, (Weller et al. 2000), permitted analysis in real-time PCR within a closed tube system, reducing the risk of cross-contamination within the laboratory. The Weller et al. (2000) TaqMan system was specific for race 3 biovar 2, although positive results were generated with CIP430, a biovar 1 potato strain originating from Peru (Ji et al. 2007). Genetic analysis had previously demonstrated that this strain was more closely related to biovar 2 (Fegan et al. 1998).

Plant extracts may contain substances that inhibit PCR and reduce its efficiency (Bickley and Hopkins 1999). Diagnostic PCR methods used for regulatory purposes must therefore provide adequate means of validating negative results, as well as confirming positive results. Endogenous reaction controls have been previously developed for *R. solanacearum* PCR methods in multiplex systems. One method utilised high-copy number plant genes (Pastrik et al. 2000) as a reaction control in the detection of division II (biovars 1 and 2) *R. solancearum*. Another reaction control strategy targeted the cytochrome oxidase gene, a sequence common to all biovars of *R. solanacearum,* and was intended to be used with the specific detection of race 3 biovar 2 after establishing the presence of *R. solancearum* in the sample (Weller et al. 2000).

Artificial reaction controls have some advantages over endogenous controls in real-time PCR. First, a reaction control can be designed to ensure that its amplification will not compete effectively with the primary diagnostic target. Second, it can be designed so that it can be easily distinguished from the primary diagnostic target amplicon in post-reaction fluorescent melt curve analysis. Finally, since the concentration added to the reaction can be accurately controlled, threshold cycles (Ct values) can be used to determine whether the amplification efficiency has been compromised or if the reaction has failed. This provides validation of a negative result, and facilitates the establishment of maximum acceptable Ct values, eliminating the need for subjective interpretation. Previously, an artificial reaction control was implemented successfully in a TaqMan method for the detection of *Clavibacter michiganensis* subspecies *sepedonicus* (Smith et al. 2008).

The purpose of this study was to develop a reaction control to function with a previously published TaqMan detection method (Weller et al. 2000) for *R. solanacearum* race 3 biovar 2. The reaction control was designed to contain the Weller primer annealing sites at each end, with a GC content of about 65%. This would ensure that it would amplify with the race 3 biovar 2—specific primers, while generating an amplicon distinguishable from the primary diagnostic target, which had a GC content of 52%, using melt temperature analysis. The higher GC content in the reaction control would also ensure that its amplification would not overwhelm the amplification of the primary diagnostic target.

Pure culture of *R. solanacearum* race 3 biovar 2 (strain CFBP 3857) was propagated and enumerated on casamino acids (0.1%)—peptone (1%)—glucose (1%) agar plates at 28°C for 3 days. Media was purchased from BD Difco (Sparks, Maryland, USA). Cell suspensions for plant inoculum and positive control extracts were prepared from plates in sterile, purified water.

One month-old tomato seedlings (cv. Cheyenne) were potted individually in 12.5 cm pots, inoculated with *R. solanacearum* race 3 biovar 2 using the soil-soak method described by Tans-Kersten et al. (1998), and raised in a plant pest containment facility. Low, medium, and high inoculum rates were 10^2 cells g^{-1}, 10^5 cells g^{-1}, and 10^8 cells g^{-1} soil, respectively. Five plants received the high inoculum, while the low and medium inocula were given to six plants each. Five uninoculated tomato plants were also raised in the containment facility with the infected plants, and an additional four uninoculated plants were raised in a separate greenhouse facility.

Three groups of six seed potato tubers (cv. Jemseg) were each inoculated with *R. solanacearum* race 3 biovar 2 using vacuum infiltration as previously described (Bain and Pérombelon 1988). Tubers were immersed in a cell suspension containing low (10^2 cfu ml^{-1}), medium (10^5 cfu ml^{-1}) and high (10^8 cfu ml^{-1}) levels of inoculum and placed under vacuum for 5 min at 700 mbar. Tubers were air-dried overnight and each planted in a 25-cm pot. Uninocu-

lated tubers were planted in the same manner and grown with the inoculated plants in the containment facility. In addition, 55 composite samples, each comprised of 200 field tuber cores, were used as negative control material.

Inoculated and uninoculated tomato plants were grown an additional 3weeks after inoculation, while the potato plants were grown for 9 weeks after planting. Stem and leaf tissues were harvested from the tomato plants, while stems and tubers were harvested from the potatoes. Leaves were sampled randomly from various positions on the tomato plants. Approximately 5 cm to 6 cm of stem tissue above the first node above the crown was sampled from both potatoes and tomatoes. In most cases, tubers were cored from the stolon end, but very small tubers were homogenised whole.

Tissues were crushed and homogenised in 2:1 vol: wt sterile purified water in sample bags (Bioreba Ag, Reinach, Switzerland) containing a mesh insert using a Homex (Bioreba Ag, Switzerland). Stems and tubers were processed individually, while leaves were processed in composites of three. Duplicate homogenates were prepared from the tomato tissues. Total DNA was extracted from 0.5 ml of each homogenate as previously described (Smith et al. 2008).

The 94 base pair (bp) reaction control sequence (Table 1) was synthesised to order (Invitrogen, Carlsbad, California, USA), amplified in PCR with primers B2-I-F and B2-II-R, and cloned into pCR-TOPOII to generate pRB2C2. The plasmid was maintained in *Escherichia coli* strain DHα-T1. The plasmid was purified and quantified as previously described (Smith et al. 2008).

Real-time PCR was carried out using the Smartcycler (Cepheid, Sunnyvale, California, USA). The reaction mix consisted of 1X SYBR Green JumpStart Taq ReadyMix (Sigma, St. Louis, Missouri, USA), and 0.2% BLOTTO to help counteract inhibitor effects (De Boer et al. 1995). The forward and reverse primers, B2-I-F and B2-II-R (Weller et al. 2000; Table 1), respectively, were used at a concentration of 0.3 μM each, while the TaqMan probe, B2-P (Weller et al. 2000; Table 1), was used at a concentration of 0.2 μM. Linearised reaction control plasmid pRB2C2 was also included in the reaction mix at a final concentration of 100 copies per reaction. Reactions were performed in a total volume of 25 μl using 2 μl template DNA. Cycling parameters were as follows: Denaturation, 2min at 95°C; Amplification, 40 cycles of 15 s at 95°C and 60 s at 64°C; Final extension, 3 min at 72°C. Fluorescence generated by the TaqMan probe was captured in the Cy5 channel, while SYBR green fluorescence was captured in the FAM channel. The default instrument settings were used to generate the melt curves. PCR products from all plant extracts were analysed using 3% agarose electrophoresis at least once.

One-way ANOVA with Tukey's multiple comparison was used to compare the reaction control Ct values and amplicon melting temperatures generated with the different tissue types. T-tests with Welch's correction was performed to confirm significant differences in mean values when differences in variances were also detected. The D'Agostino and Pearson omnibus normality test was used to determine Gaussian goodness-of-fit for the frequency distributions, and Grubbs' test was used to detect outliers. Grubb's test was performed using an online calculator (http://www.graphpad.com/quickcalcs/Grubbs1.cfm) while all other analyses were performed using GraphPad Prism version 4.03 (GraphPad Software, San Diego, California, USA).

Pure *R. solanacearum* race 3 biovar 2 culture, serially diluted into uninfected potato homogenate,

Table 1 Oligos used for the detection of *R. solanacearum* race 3 biovar 2

Oligo	Function	Sequence (5′ to 3′)
B-I-F[a]	forward primer	tggcgcactgcactcaac
B-II-R[a]	reverse primer	aatcacatgcaattcgcctacg
B-P[a]	TaqMan probe	Cy5-5′ -ttcaagccgaacacctgctgcaag-3′- Iowa Black RQ
pRB2C2 insert	reaction control	tggcgcactgcactcaacggcgccaggagcctcgagcagttccgacgcggcgcgcatcgtggtgcaccgacgcgtaggcgaattgcatgtgatt

[a] Weller et al. (2000)

was used to evaluate the sensitivity of the TaqMan assay. The limit of detection was less than 10^3 cells ml^{-1} of potato homogenate (Fig. 1). While the addition of competing template in PCR can result in a reduction of reaction efficiency of the target (Siebert and Larrick 1992), the addition of 100 copies pRB2C2 per reaction had no effect on the Ct value generated as a function of cell concentration (Fig. 1). Correlation coefficients (r^2) of the regression lines generated with zero and 100 copies pRB2C2 per reaction were 0.996 and 0.992, respectively, while the respective slopes were −3.166 and −3.349. The two slopes were not significantly different ($P = 0.185$). This indicated that the reaction control did not adversely affect the amplification efficiency of the diagnostic target, and suggested that the limit of detection of the TaqMan assay would not likely be affected.

Ralstonia solanacearum race 3 biovar 2 was not detected in either leaves or stems from the uninoculated tomato plants raised in the greenhouse, or was it detected in any of the leaves harvested from the uninoculated plants raised in the containment facility. *Ralstonia solanacearum* race 3 biovar 2 was also undetected in the stems from four out of the five uninoculated tomato plants raised in the containment facility (Table 2).

Five of the six uninoculated potato plants had at least one stem that tested positive, although none of the stems displayed wilting symptoms. Since the negative extraction controls and the no template reaction controls were negative, this may have been

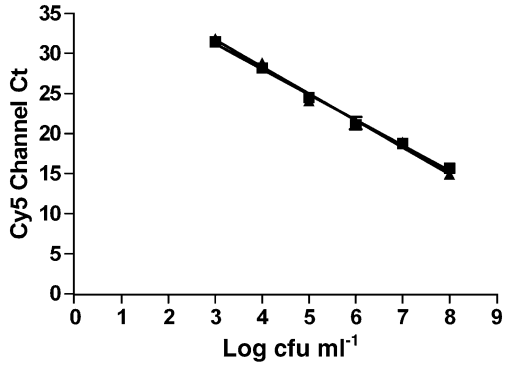

Fig. 1 Ct values generated in the Cy5 channel as a function of the concentration of the diagnostic target, *Ralstonia solanacearum* race 3 biovar 2, with zero (■) and 100 (▲) copies of pRB2C2 added per reaction. Points represent the mean of duplicate observations. Standard deviations were too narrow to display effectively as error bars

Table 2 *Ralstonia solanacearum* race 3 biovar 2 analysis from tomato stem and leaf tissues using TaqMan real-time PCR

Inoculum level	Total plants	Symptom rating[a]	Number of Plants	TaqMan results			
				Stems		Leaves	
				No. plants positive	Ct range	No. plants positive	Ct range
Zero	5	0	5	1	34.20–34.99	0	–
Zero (Greenhouse)	4	0	4	0	–	not done	not done
Low 10^2 cells g^{-1} soil	6	0	6	1	35.48–>40	3	34.36–>40
Medium 10^5 cells g^{-1} soil	6	4	1	1	14.11–15.11	1	16.89–34.30
		2	1	1	14.56–14.75	1	24.59–31.44
		0	4	4	27.23–29.96	4	31.19–31.92
High 10^8 cells g^{-1}	5	4	3	3	12.78–14.86	3	13.24–23.41
		3	1	1	13.42–13.99	1	23.95–25.27
		2	1	1	14.08–14.98	1	24.15–27.25

[a] Symptoms were rated on a scale of 0–4; 0, no symptoms; 1, chlorosis or flagging; 2, up to 50% wilted; 3, 50–75% wilted; 4, >75% wilted or dead

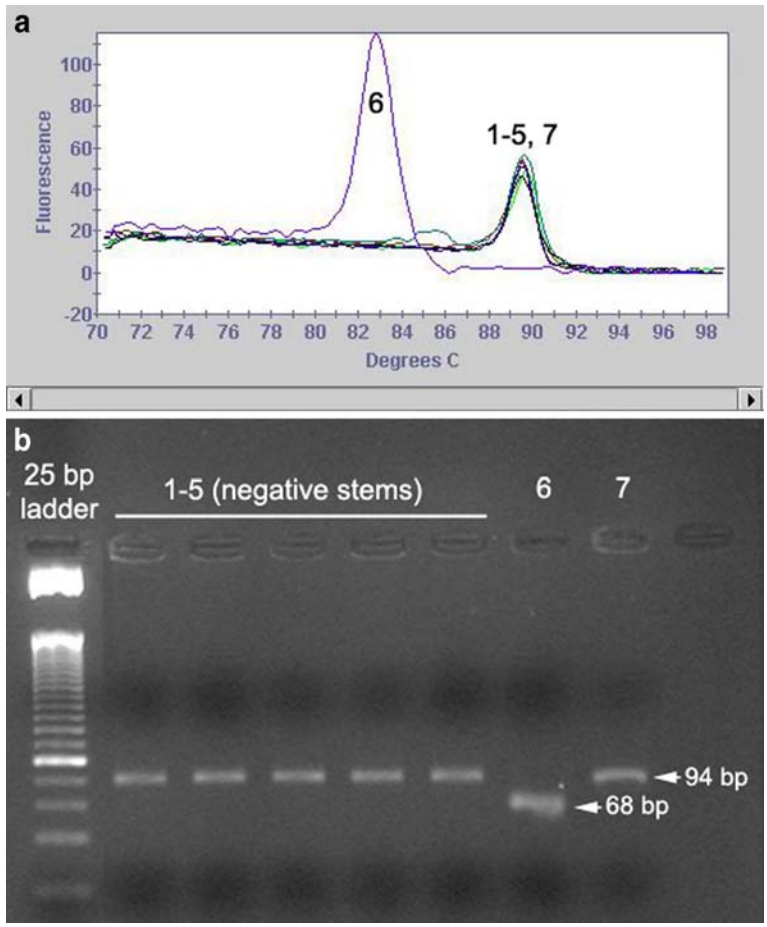

Fig. 2 Melt curve (**a**) and electrophoretic analysis (**b**) of reaction products typically generated in the TaqMan test for *R. solanacearum* race 3 biovar 2 (R3B2) by negative samples containing the reaction control pRB2C2 (Lanes 1–5) compared with the positive diagnostic control (6). *Lane and peak 7* represent the amplicon generated from pRB2C2 alone

the result of cross-infection between plants within the close confines of the containment facility. Likewise, all the tubers harvested from the inoculated and uninoculated plants raised in the containment facility tested positive.

In all the negative stem and leaf samples, the reaction control was successfully amplified, generating a signal in the FAM Channel. A melt peak of approximately 90°C was generated, and was associated with the expected 94 bp amplicon. Some examples are shown in Fig. 2.

Fifty-five diagnostic field tuber extracts were used as negative control material. Fifty confirmed negative results resembling those shown in Fig. 2 were returned. Five extracts yielded high FAM channel Cts that were determined to be Grubb's outliers ($P < 0.05$), and four out of these five reactions failed to generate a reaction control melt peak, indicating that these reactions were likely to have failed. There were no confirmed positive results.

Positive results were generated with all stem and leaf extracts harvested from both potato and tomato plants displaying bacterial wilt symptoms. In addition, all the plants receiving the medium or high inoculum generated positive results in stem and leaf tissues, whether or not they had displayed symptoms. Positive results generated a signal in the Cy5 channel and a melt peak of approximately 83°C easily distinguished from the 90°C reaction control melt peak. The 83°C melt peak was also associated with the expected 68 bp amplicon (Fig. 3).

There were 55 tubers harvested from the potato plants raised in the containment facility, and all were positive for *R. solanacearum* race 3 biovar 2, regardless of whether or not the plants or the tubers displayed any symptoms. The generation of positive results in uninoculated plants may have been the result of cross-infection between plants within the close confines of the containment facility. As with the other positive extracts, all the tuber extracts that tested positive

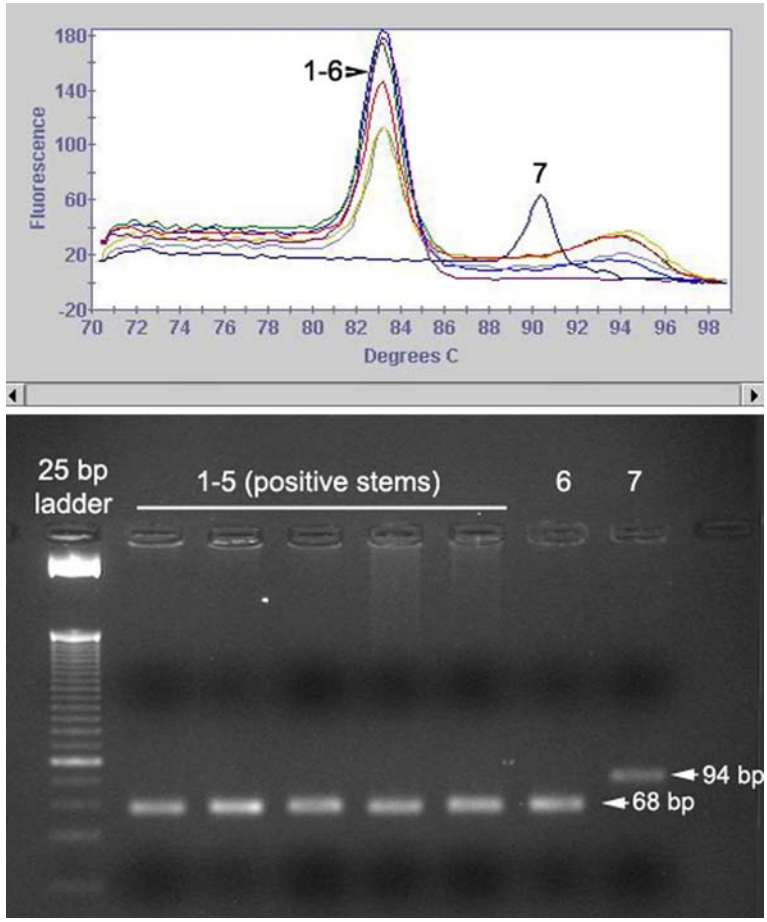

Fig. 3 Melt curve (**a**) and electrophoretic (**b**) analyses of reaction products typically generated by the TaqMan test for *R. solanacearum* race 3 biovar 2 by positive samples (1–5) compared with the positive control (6) and the reaction control pRB2C2 (7)

generated a melt peak of about 83°C as well as the 68 bp amplicon, resembling the results shown in Fig. 3.

The Ct values generated with the asymptomatic plants were generally higher than those displaying symptoms for both tomato (Table 2) and potato tissues, suggesting that the asymptomatic plants contained less bacteria. This result indicated that this assay can detect subsymptomatic concentrations of the pathogen, supporting the previous conclusion that inclusion of the reaction control did not adversely affect the limit of detection.

With statistical outliers removed, reaction control Ct values were normally distributed for each of the tissue types tested ($P > 0.18$). There was no significant difference in Ct values generated in the tomato leaves and potato stems ($P > 0.5$), but there were differences in both the means and variances of these distributions for the remaining tissue types (Fig. 4a). The Cts generated with the tomato stems displayed the greatest variablility (mean Ct 29.4+0.9), while the potato tubers generated a mean Ct, 30.8+0.5, significantly higher ($P<0.001$) than all the others (Fig. 4a). This suggested that the tissue matrix type had an influence on the reaction control Ct value. Since five reaction failures were also noted with the negative control field tuber extracts, it is likely that the potato tuber extracts exerted an inhibitory effect on the reaction. Potato starch, acidic polysaccharides, polyphenolics, fungal polyphosphates and humic acid from soil may be present in tuber extracts, and are known to inhibit DNA polymerase reactions (Bickley and Hopkins 1999). In contrast, there were no differences detected between the mean melt temperatures of the reaction control in each of the tissue types ($P>0.05$), although differences between the variances ($P=0.0002$) were detected (Fig. 4b).

The TaqMan test for *R. solanacearum* race 3 biovar 2 with the reaction control performed well in

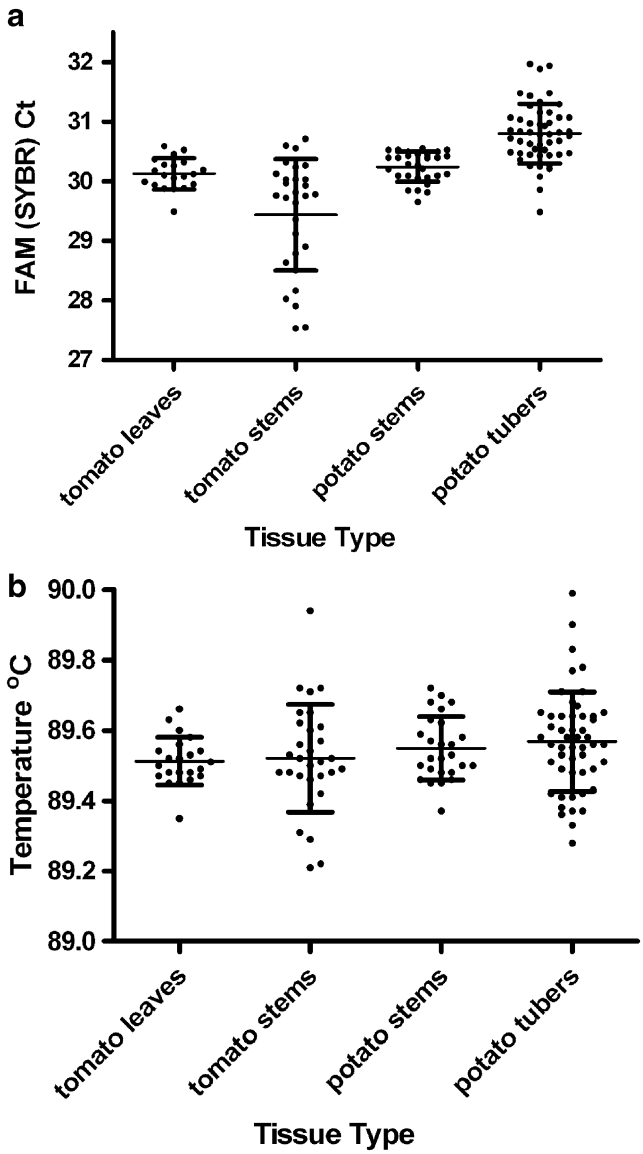

Fig. 4 Scatter plots of the pRB2C2 reaction control Ct values (**a**) and melt temperatures (**b**) measured in negative samples from different tissue types. The *error bars* represent the standard deviation around the mean, which is represented by the horizontal line

a closed tube system. The 68 bp and 94 bp diagnostic and reaction control amplicons, respectively, were easily identified and distinguished using melt analysis, permitting confirmation of results within a closed tube system. This decreased the time required for analysis considerably, compared with conventional PCR. While the matrix type had no overall effect on the reaction control amplicon's melting temperature, it did appear to affect its FAM / SYBR Ct value. In diagnostic work, it may be desirable to set a maximum Ct threshold for the reaction control as a validation criterion for a negative result (Smith et al. 2008). A reasonable maximum Ct value could be set three standard deviations above the mean. For example, with tomato leaves the reaction control generated a mean Ct of 30.13 + 0.26 (Fig. 4a), while in potato tubers, it generated a mean Ct of 30.8 + 0.50. A reasonable maximum Ct value for tomato leaves would therefore be about 30.9, while for potato tubers, it would be higher, about 32.3. The effect of the sample tissue type on the expected Ct generated should therefore be considered when determining thresholds for validation of negative results.

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