

# Investigating trehalose synthesis genes after cold acclimation in the Antarctic nematode *Panagrolaimus* sp. DAW1

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## Summary statement

Functional genomics was used to investigate trehalose synthesis genes after cold acclimation in *Panagrolaimus* sp. DAW1, an Antarctic nematode with the ability to survive intracellular freezing.

## Abstract

*Panagrolaimus* sp. DAW1 is a freeze-tolerant Antarctic nematode which survives extensive intracellular ice formation. The molecular mechanisms of this extreme adaptation are still poorly understood. We recently showed that desiccation-enhanced RNAi-soaking can be used - in conjunction with qPCR - to screen for phenotypes associated with reduced expression of candidate genes in *Panagrolaimus* sp. DAW1. Here we present the use of this approach to investigate the role of trehalose synthesis genes in this remarkable organism.

Previous studies have showed that acclimating *Panagrolaimus* sp. DAW1 at 5 °C before freezing or desiccation substantially enhances survival. In this study the expression of *tps-2* and other genes associated with trehalose metabolism, as well as *lea-1*, *hsp-70* and *gpx-1* in cold-acclimated and non-acclimated nematodes was analyzed using qPCR. *Pd-tps-2* and *Pd-lea-1* were significantly up-regulated after cold-acclimation, indicating an inducible expression in the cold-adaptation of *Panagrolaimus* sp. DAW1. The role of trehalose synthesis genes in *Panagrolaimus* sp. DAW1 was further investigated by RNAi. Compared to the controls, *Pd-tps-2a(RNAi)*-treated and cold-acclimated nematodes showed a significant decrease in mRNA, but no change in trehalose content or freezing survival. The involvement of two other trehalose synthesis genes (*tps-2b* and *gob-1*) was also investigated.

These findings provide the first functional genomic investigation of trehalose synthesis genes in the non-model organism *Panagrolaimus* sp. DAW1. The presence of several trehalose synthesis genes with different RNAi sensitivities suggests the existence of multiple backup systems in *Panagrolaimus* sp. DAW1, underlining the importance of this sugar in preparation for freezing.

## Introduction

The Antarctic nematode, *Panagrolaimus* sp. DAW1 (PaDAW1) is the only multicellular organism known to tolerate intracellular freezing on a routine basis (Wharton and Brown, 1991; Wharton and Ferns, 1995). PaDAW1, formerly known as *Panagrolaimus davidi* CB1, is also tolerant of cryoprotective dehydration (Wharton et al., 2004a; Wharton et al., 2017; Wharton et al., 2003). These characteristics are adaptations to the nematodes' habitat – in and around Antarctic penguin colonies (Raymond et al., 2013a; Wharton and Brown, 1989). During the summer, and perhaps somewhat surprisingly, soils in these regions can warm to above 25 °C (Raymond et al., 2013a; Sinclair et al., 2006), and nutrient-rich liquid guano provides an ideal medium for the growth of bacteria on which PaDAW1 feed (Raymond et al., 2013a). PaDAW1 reproduces only when the temperature is above about 6.8 °C (Brown et al., 2004) suggesting that breeding is possible for only a limited time in each season when solar irradiation warms the soil. However, for much of the year, these habitats are both cold and dry with winter temperatures as low as -40 °C and very low humidity. PaDAW1 might be considered a temperate nematode with the capacity to tolerate long-term freezing and desiccation.

Survival of freezing and of desiccation may not be obviously related, but both involve the removal of water and concentration of solutes, and tolerance of these states may share similar mechanisms (Storey and Storey, 2013; Teets and Denlinger, 2014). Although PaDAW1 survives both intracellular freezing (Wharton and Ferns, 1995) and cryoprotective dehydration (Wharton et al., 2003), little is known of the mechanisms by which this is achieved. It is clear that survival of intracellular freezing is related to the pattern and distribution of ice formation (Raymond and Wharton, 2016) and that in turn is likely to be associated with osmoregulation within the pseudocoelomic fluid (Wharton, 2010) as well as the presence of ice-active proteins (Barrett et al., 2005). Cryoprotective dehydration seems to occur if freezing rates are slow, but extra- and even intracellular freezing can occur if freezing rates are faster. The increase in freezing rate produces a shift from cryoprotective dehydration to extracellular to intracellular freezing, accompanied by a decrease in survival (Wharton and Ferns, 1995; Wharton et al., 2003). However, many questions remain as to exactly what metabolic changes are responsible for freezing and desiccation tolerance.

The accumulation of the disaccharide, trehalose ( $\alpha$ -D-glucopyranosyl-(1 $\rightarrow$ 1)- $\alpha$ -D-glucopyranoside), is one of the best characterized metabolic changes during acclimation of anhydrobiotic organisms (Argüelles, 2014; Storey and Storey, 2013). Trehalose protects membranes and proteins from desiccation by replacing structural water (Carpenter et al., 1987; Crowe et al., 1984) and by forming cellular glass (Crowe et al., 1998). Trehalose accumulation has been associated with anhydrobiosis in nematodes such as *Aphelenchus avenae* (Madin and Crowe, 1975), *Anguina tritici* and *Ditylenchus dipsaci* (Womersley and Smith, 1981). In some species, such as *A. avenae*, trehalose accumulation seems to be essential, but not sufficient, for anhydrobiosis (Browne et al., 2004; Higa and Womersley, 1993). In others, such as some anhydrobiotic rotifer and tardigrade species, trehalose accumulation is apparently not essential (Hengherr et al., 2008;

Lapinski and Tunnacliffe, 2003). In PaDAW1, a period of acclimation at ~5 °C is associated with both the accumulation of trehalose and a significant increase in survival after subsequent exposure to freezing (Wharton et al., 2000), suggesting that trehalose might play a role in freezing tolerance (Feofilova et al., 2014; Storey and Storey, 2013; Tapia and Koshland, 2014).

Recent work looking at genes expressed during acclimation, freezing and cryoprotective dehydration in PaDAW1 (Thorne et al., 2014) identified a number of trehalose synthesis (*tps*) genes, late embryogenesis abundant (*lea*) proteins, heat shock proteins (*hsp*) and genes associated with antioxidant production that showed evidence of specific up-regulation in these conditions (and were distinct from general responses to stress). In the PaDAW1 dataset, two trehalose synthesis genes (*tps* and *gob*), six aquaporin genes and three desaturase genes as well as nine different *lea*-type genes and 20 *hsp-70* like genes were identified as potential candidates to be involved in cryoprotective dehydration (Thorne et al., 2014). Broadly similar patterns of expression were seen in the Antarctic nematode *Plectus murrayi* during freezing (Adhikari et al., 2009).

This study investigates expression of selected genes – trehalose-6-phosphate synthase 2 (*tps-2*), trehalose-6-phosphate phosphatase (*gob-1*), late embryogenesis abundant 1 (*lea-1*) protein, glutathione peroxidase 1 (*gpx-1*) and heat shock protein 70 (*hsp-70*) – in cold-acclimated and non-acclimated nematodes using qPCR. Previous work has shown that, provided nematodes are properly fed (Raymond and Wharton, 2013), acclimation at 5 °C for 3–5 days improves freezing survival from about 40% to about 85% (Wharton et al., 2000). This implies that lower temperatures induce physiological and biochemical changes that assist in freezing survival and an increase in trehalose content was a marked example of such a change. Cellular trehalose was shown to increase upon cold-acclimation and to be correlated with freezing survival (Wharton et al., 2000). The enzymes trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (GOB in PaDAW1) are directly involved in trehalose synthesis in nematodes and are essential genes (Avonce et al., 2006; Behm, 1997; Farelli et al., 2014; Pellerone et al., 2003), and the enzyme trehalase is involved in trehalose breakdown (Thorne et al., 2014; Thorne et al., 2017; Łopieńska-Biernat et al., 2015).

The role of trehalose and trehalose synthesis genes in PaDAW1 during acclimation at 5 °C was further investigated. First, *tps-2a* was silenced by RNAi and the reduction of mRNA was measured by qPCR, and the amount of trehalose itself was assessed by gas chromatography. Second, the involvement of other trehalose synthesis genes (*tps-2b* and *gob-1*) was investigated using qPCR and RNAi. Third, the freezing survival of *tps-2a,b(RNAi)*-treated nematodes was compared to that of non-treated controls.

## Materials and Methods

### *Nematode culturing and cold-acclimation*

PaDAW1 was originally isolated from McMurdo Sound region, Antarctica (Wharton and Brown, 1989) and has been maintained in the laboratory for more than 25 years (Raymond et al., 2013b). Mixed nematode cultures were grown on *E. coli* seeded NGM agar plates at 20 °C and sub-cultured weekly. Nematodes used for these experiments were collected from culture plates by a modified Baermann technique (Flegg and Hooper, 1970), and sub-cultured in five replicates of exactly equal volume. They were first incubated at 20 °C for three days and then at 5 °C for another 24 h. After cold-acclimation, nematodes were re-collected, snap frozen in a mixture of dry ice and ethanol, and stored at -80 °C until analysis.

### *RNA isolation and cDNA synthesis*

RNA was extracted using TRIzol<sup>®</sup> Reagent (Ambion, Foster City, CA, USA) and RNeasy<sup>®</sup> Mini Kits (Qiagen, Hilden, Germany) and reverse-transcribed using the VILO cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA) as described previously (Seybold et al., 2016).

### *Candidate gene cloning for RNAi*

Candidate genes were selected using two different sets of data: a study of *Plectus murrayi* (Adhikari et al., 2009) and transcriptomic data from PaDAW1 (Thorne et al., 2014) (Table S2). We selected genes that showed significant up-regulation on exposure to freezing in both *P. murrayi* and PaDAW1, particularly those involving trehalose metabolism. Target genes were PCR amplified from cDNA with gene specific primers using Taq DNA Polymerase dNTPack (Roche, Basel, Switzerland) as described previously (Seybold et al., 2016) and Table S1.

Two sets of transcripts homologous to *tps* were identified in the PaDAW1 dataset. Initial analyses suggested these were two non-overlapping portions of the same gene (Thorne et al., 2014). However, a search of the genomic scaffolds indicates that they probably come from different regions of the genome and probably represent a gene duplication. These two regions were termed *tps-2a*, showing a larger alignment, and *tps-2b*, a smaller alignment (see Table S1).

### *RNAi*

Double-stranded RNA for soaking experiments was produced by *in vitro* transcription of PCR products using the MEGAscript<sup>®</sup> T7 Transcription Kit (Life Technologies, Carlsbad, CA, USA). RNA uptake was done by desiccating nematodes at 98% relative humidity and 20 °C for 24 h, prior to soaking in dsRNA solutions as described previously (Seybold et al., 2016). Nematode cultures (four replicates for each treatment) were then rehydrated in soaking buffer (plus 1mg/mL dsRNA, and minus dsRNA) for 16 h. Cultures were then incubated for 24 h at 5 °C for recovery before they were harvested and processed.

### *Quantitative polymerase chain reaction (qPCR)*

Quantitative PCR was performed using the BioRad CFX96 System (Hercules, CA, USA) and BioRad SSoFast EVA Green Supermix with Low Rox. A typical 20  $\mu$ l reaction contained a 5  $\mu$ l sample (total of 50 ng cDNA), 10  $\mu$ l SYBR green mix, 1.2  $\mu$ l primer mix and 3.2  $\mu$ l mQ water. Specificity and efficiency assays were performed for all genes. Of six housekeeping genes tested, the combination of *Pd-gpd-2* and *Pd-tba-1* were defined as the most stable and used for all qPCR experiments to normalise data from each individual assay. The genes tested were *tps-2* (trehalose synthase 2), *gob-1* (trehalose-6-phosphate phosphatase 1), *lea-1* (late embryogenesis abundant proteins 1), *hsp-70* (heat shock protein 70), and *gpx-1* (glutathione peroxidase 1). BioRad FCX Manager software was used to control qPCR settings and to analyse qPCR data as described previously (Seybold et al., 2016). Primer details for qPCR analyses can be found in supplementary Table S2 and Seybold *et al.* (2016).

Relative differences in expression of target genes were assessed using the  $\Delta\Delta C_t$  (Livak) method (Livak and Schmittgen, 2001) where the relative difference in expression of the target gene in different samples was determined. This involves normalising the  $C_t$  of the target gene to that of the reference genes, for both the test sample and the control sample (producing a normalized relative expression value  $\Delta C_t$ ). In the next step, the  $\Delta C_t$  of the test sample is normalized to that of the control sample to give a measure of relative expression. This approach produces an estimate of how test gene expression changed with respect to the control condition and corrects for differences in the RNA yield of each culture that would otherwise complicate the analysis.

### *Gas chromatography*

Sugar extraction was performed by using a technique modified from Wharton *et al.* (2000). Sugars were extracted to compare trehalose levels from both cold-acclimated and non-acclimated cultures (grown at 5 °C and 20 °C) as well as from *Pd-tps-2(RNAi)*-treated and non-treated (control) samples acclimated at 5 °C. RNAi cultures were plated out on NGM agar plates and incubated for 72 h, to allow time for changes in trehalose levels. After incubation, samples were collected as described above and snap frozen in a mixture of dry ice and ethanol before processing.

After extraction (Wharton et al., 2000), the sugars in dried samples were converted to their trimethylsilyl derivatives by adding 20  $\mu$ l Sylon (Supelco) and incubation for 5 min at room temperature. Derivatized samples (15  $\mu$ l) were injected onto the column of an Agilent 6890N Network gas chromatograph. To reduce variation between replicas caused by the injection technique, hot needle injection after Barwick (1999) was done. Briefly, after insertion into the injection zone, the needle is allowed to heat up for 5 s. The sample is then rapidly injected by pushing down the plunger and the needle quickly withdrawn from the inlet within 1 s. Sugars were identified and quantified using reference standards. Recovery of sugars was

estimated by inclusion of 240 µg of Dulcitol in the original nematode sample and analysis of the Dulcitol peak (Wharton et al., 2000).

### *Freezing survival*

The freezing survival experiment was performed after Smith *et al.* (2008). Following RNAi soaking and acclimation at 5 °C for 24h, nematodes were washed off the plates and 50 µl samples of nematode suspension were transferred into 0.5 ml microcentrifuge tubes. These were transferred to a cooling block (Wharton et al., 2004b) and cooled from +1 °C to -15 °C at 0.5 min<sup>-1</sup>. Freezing was seeded by adding a small ice crystal to each tube when the temperature was -1 °C. Samples were held at -15 °C for 30 min, then warmed to 1 °C at 0.5 min<sup>-1</sup>, and then were transferred to mQ water in watch-glasses and incubated for 24 h at 20 °C. Survival of nematodes was assessed by determining the proportion of moving nematodes. To perform statistical analysis, 3 × 100 nematodes were counted for each of the three replicates of non-treated and *Pd-tps-2(RNAi)*-treated samples. A t-test (parametric, two samples, equal variance) was used to assess the statistical significance of sample differences. Control samples consisted of 50 µl nematode suspension in 0.5 ml microcentrifuge tubes, kept at 20 °C for the duration of the experiment.

## **Results**

### *Identifying candidate genes*

In order to directly examine the relative expression of selected genes in response to acclimation at 5 °C, quantitative PCR was performed on PaDAW1 samples maintained at the physiologically significant temperatures of 20 °C (active reproduction) and 5 °C (active acclimation). This analysis showed a significantly increased expression of four of the six genes tested and a significant reduction of expression of one (Fig. 1). The final gene tested, *Pd-gpx-1*, did not vary significantly. Expression of both *Pd-tps-2a* (relative expression =  $3.9 \pm 0.7$ ,  $p = 0.017$ ) and *Pd-tps-2b* (relative expression =  $4.6 \pm 0.6$ ,  $p = 0.021$ ) was significantly increased consistent with an increase in trehalose content (Fig. 2A). Trehalose-6-phosphate phosphatase (*Pd-gob-1*), an enzyme also involved in trehalose metabolism (relative expression =  $2.3 \pm 0.2$ ,  $p = 0.005$ ) and *Pd-lea-1* (relative expression =  $3.4 \pm 0.4$ ,  $p = 0.008$ ) were significantly up-regulated in the 5 °C samples. *Pd-gpx-1* was slightly up-regulated in the 5 °C samples compared to the 20 °C samples but this was not statistically significant (relative expression =  $1.33 \pm 0.14$ ,  $p = 0.13$ ). In contrast, *Pd-hsp-70* was slightly and significantly down-regulated (relative expression =  $0.75 \pm 0.05$ ,  $p = 0.018$ ).

The up-regulation of *Pd-tps-2* expression upon cold-acclimation in PaDAW1 was correlated with an increase in total trehalose content. Our results showed that the trehalose level was significantly higher in cold-acclimated samples ( $6.8 \pm 0.5$  µg/mg at 5 °C versus  $1.3 \pm 0.5$  µg/mg at 20 °C, a 5.2-fold increase,  $p \approx 2 \times 10^{-6}$ ) (Fig. 2A). These data show a large and significant increase in trehalose content in response to

acclimation at 5 °C, as noted previously (Wharton et al., 2000). This suggests that trehalose may be one of the agents responsible for the correlation between cold-acclimation and freezing survival.

*Can RNAi efficiently silence tps-2 expression and is there any phenotype?*

Since gene expression analysis by qPCR showed a significant up-regulation of both *Pd-tps-2a* and *Pd-tps-2b* in cold-acclimated samples (see Fig. 1A), these loci were chosen for further analysis by RNAi. Expression of *Pd-tps-2a* assessed by qPCR after treatment with *Pd-tps-2a(RNAi)* showed a decrease relative to control RNAi-treated nematodes of *Pd-tps-2* (RNAi relative expression of  $0.92 \pm 0.25$ ,  $p = 0.021$  versus control relative expression  $1.26 \pm 0.26$ , overall relative expression (RNAi/Control) = 0.73; Fig. 2B). It is not clear if this difference is biologically significant even though it is statistically significant at  $p < 0.05$ . Such small differences of expression may not be enough to generate a phenotype (Fraser et al., 2000).

RNAi of *Pd-tps-2b* (RNAi relative expression of  $1.02 \pm 0.09$ ,  $p = 0.021$  versus control relative expression  $0.95 \pm 0.09$ , overall relative expression  $\approx 1.07$ ; Fig. 2C) showed no significant decrease in expression. To determine whether a small change in TPS-2 synthesis was associated with any change in trehalose content, we measured the amount of trehalose in nematodes after RNAi treatment. No significant difference in the trehalose content between *Pd-tps-2a(RNAi)*-treated ( $9.1 \pm 2.1$  µg/mg) and non-treated control samples ( $7.9 \pm 1.7$  µg/mg) was detected (Fig. 2D). We also measured freezing survival and found no difference between RNAi-treated ( $57.7\% \pm 10.3\%$ ) and control samples ( $56.8\% \pm 12.1\%$ ) (Fig. 2E).

## Discussion

### *Investigating candidate genes*

Candidate genes were selected from those thought to be associated with cold tolerance in nematodes (e.g. Adhikari et al., 2009) and using transcriptomic data from PaDAW1 (Thorne et al., 2014; Thorne et al., 2017). Three of these are associated with trehalose synthesis and this is consistent with previous findings that cold-acclimation of PaDAW1 under these conditions is correlated with a significant increase in trehalose content (Wharton et al., 2000). Synthesis of trehalose in metazoans (which include nematodes) occurs in two steps involving the formation of trehalose 6-phosphate from glucose 6-phosphate and UDP-glucose, followed by the removal of phosphate to form trehalose (Fig. 3). The enzymes involved are trehalose 6-phosphate synthase (TPS) and trehalose 6-phosphate phosphatase (GOB), and trehalase in the breakdown of trehalose (Behm, 1997). Our data shows a significant up-regulation of both *Pd-tps-2a*, and *Pd-lea-1*, whereas *Pd-gpx-1* and *Pd-hsp-70* showed no significant change in mRNA levels.

The observation that *Pd-tps-2a* and *Pd-lea-1* are significantly up-regulated upon cold-acclimation, suggests that these two genes are cold-inducible and therefore likely to be involved in cold-adaptation of PaDAW1. Late embryogenesis proteins (lea) were originally described in plants (Tunnacliffe et al., 2010) and are now known to have a role in preventing protein aggregation in a wide range of organisms including nematodes



(Hand et al., 2016; Yaari et al., 2016). Two other genes, *Pd-gpx-1* and *Pd-hsp-70*, involved in oxidative metabolism and protein folding respectively, remained nearly unchanged after cold-acclimation, indicating that they might be constitutively expressed in PaDAW1. Similar expression patterns in response to desiccation have been demonstrated in other nematode species. For example, after cold-acclimation in *P. murrayi*, *Pm-tps* and *Pm-lea* were highly up-regulated, while *Pm-gpx* was slightly up-regulated and *Pm-hsp-70* remained unchanged (Adhikari et al., 2009). Similarly, in *Panagrolaimus superbus*, *Ps-lea* was highly up-regulated, while *Ps-gpx* was slightly up-regulated and *Ps-hsp-70* remained unchanged (Tyson et al., 2012).

The gene *hsp-70* seems to be up-regulated (inducible) in only some nematode species (Choi et al., 2014) and unchanged (constitutive) in others (Tyson et al., 2012). In *P. murrayi*, *hsp-70* is constitutively expressed, contributing to enhanced stress resistance overall. Generally, *hsp* are not constitutively expressed but are expressed in response to stress due to incompatibility with normal metabolism but there are exceptions such as in notothenioid fish (Buckley and Somero, 2008; Place and Hofmann, 2005). However, *P. murrayi* may have evolved mechanisms to maintain HSP function during normal metabolism in order to survive in an unpredictable environment like Antarctica, with sudden exposure to a variety of stressors (Adhikari et al., 2009). Survival of PaDAW1 may also depend on maintaining constitutive expression of this molecular chaperone.

Oxidative stress is experienced by organisms undergoing a wide range of abiotic stressors, resulting in generation of reactive oxygen species (Reardon et al., 2010). An oxidative stress response has been defined as part of the environmental stress response (Gasch et al., 2000), as well as part of the minimal stress response (Kültz, 2005). In PaDAW1, the antioxidant *gpx-1* was slightly but not significantly up-regulated, indicating a minor role or a constitutive expression. The locus, *gpx-1*, has been shown to be up-regulated in other nematode species in response to desiccation (Adhikari et al., 2009; Reardon et al., 2010; Tyson et al., 2012). In *Aphelenchus avenae*, *gpx-1* expression was 32 times greater in response to desiccation and RNAi of *gpx-1* reduced desiccation survival in *P. superbus* (Reardon et al., 2010).

In PaDAW1, trehalose accumulation has been shown to correlate with an increase in survival after exposure to freezing. Trehalose may thus play a role in the freeze tolerance of PaDAW1 by protecting membranes against the harmful effects of freeze-induced dehydration (Wharton et al., 2000). Our results showed that the amount of trehalose was significantly higher in cold-acclimated samples than in non-acclimated nematodes. This is consistent with cold-acclimation enhancing freezing survival in PaDAW1 (Raymond:2013ej; Wharton et al., 2000).

#### *RNAi affects tps-2 expression but not trehalose synthesis*

Gene expression analysis by qPCR showed a significant up-regulation of *Pd-tps-2* in cold-acclimated samples compared to non-acclimated samples. Furthermore, the level of trehalose, the product of TPS-2

activity, is increased 4.4-fold in response to cold-acclimation. Therefore, *Pd-tps-2* was chosen for further analysis by RNAi. Gene expression analysis by qPCR showed a slight but significant down-regulation of *Pd-tps-2a(RNAi)*-treated samples compared to the non-treated samples.

However, no significant difference in trehalose content between *Pd-tps-2a(RNAi)*-treated and non-treated samples was found. The absence of any change in trehalose content could be caused by a number of things. One possibility is that the pool of trehalose is large enough that changes in synthesis over 72 h are not detectable. This seems unlikely since acclimation at 5 °C for 96 h increases trehalose content by about 4.4-fold (Fig. 1B). Alternatively, *tps-2* may not be efficiently silenced. This could be a reflection of a limited reduction in mRNA content – only ≈25% in our data (Fig. 2B) – or because there are other genes for trehalose synthesis. According to Pellerone et al. (2003), less than 100% knockdown of *tps* expression could provide enough enzyme activity to allow residual trehalose metabolism. Therefore, a significant reduction in mRNA may not alter the amount of the enzyme enough to detect a phenotype even though RNAi might have reduced the amount of the target mRNA.

There is evidence that PaDAW1 has more than one *tps* gene which could compensate for the loss of expression at one locus. In *C. elegans*, a reduction in trehalose level of > 90% (confirmed by qPCR) was achieved after a double knockdown of both *tps* genes. However, no loss-of-function phenotypes were observed (Pellerone et al., 2003). It is therefore questionable whether a phenotype, such as a decrease in survival after exposure to freezing, would be observable in PaDAW1, particularly since the trehalose level was not decreased after *Pd-tps-2a* knockdown. It is also possible, that the half-life of trehalose is significantly longer than the RNAi effect, complicating detection of a decrease in trehalose levels.

#### *PaDAW1 has multiple genes for trehalose synthesis*

In contrast to *Pd-tps-2a*, gene expression analysis of *Pd-tps-2b(RNAi)*-treated samples showed no significant down-regulation of the target mRNA compared to the non-treated samples. The fact that *Pd-tps-2a* but not *Pd-tps-2b* is sensitive to RNAi is interesting and indicates that these genes probably act differently. They have either evolved different features or are expressed in different tissues with different accessibility to environmental RNAi. Expression in different tissues has been described for trehalase genes, where membrane associated and soluble *tre* activities have been observed (reviewed in Behm, 1997).

Conant and Wagner (2004) found that mutational robustness is greatest for closely related gene duplicates. Since duplicate genes often have similar functions, the loss of one duplicate can be tolerated because other copies can buffer against this loss. They also found a positive correlation between the amino acid distance and the number of duplicates with different knockdown effects (Conant and Wagner, 2004). Thus, the more distant two duplicates are, the more likely it is that one has a stronger knock-down effect than the other. This observation might explain our data on inhibition of *tps-2a* and *tps-2b* synthesis. Symmetric divergence, which probably increases with amino acid distance and divergence time, could

explain why distantly related duplicates often show different mutational effects (Conant and Wagner, 2004).

#### *Freezing survival is not affected by tps-2 silencing*

There was no statistical difference between the proportion of moving nematodes after freezing of non-treated samples compared to that of *Pd-tps-2a(RNAi)*-treated samples (Fig. 2E). The lack of freezing sensitivity in RNAi-treated samples is not surprising given that the trehalose content was not decreased (Fig. 2D). It is also likely that genes other than those involved in trehalose synthesis are involved in freezing survival.

Taken together, the characteristics of gene duplicates explain the different RNAi sensitivity between the two *tps-2* genes in PaDAW1 as well as the lack of effects on the trehalose content. The fact that not only the two *tps-2* genes, but also *gob-1* is involved in trehalose synthesis, indicates a multiple backup system in PaDAW1, underlining the importance of this sugar as a cryoprotectant against environmental stressors common in Antarctica.

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#### **Competing interests**

No competing interests declared

#### **Author contributions**

ACS performed the experiments. CJM, MAST, DAW and ACS conceived and designed the study and experiments. ACS and MAST developed the probes and primers. ACS wrote the manuscript with input from CJM, MAST and DAW. All authors gave final approval for publication.

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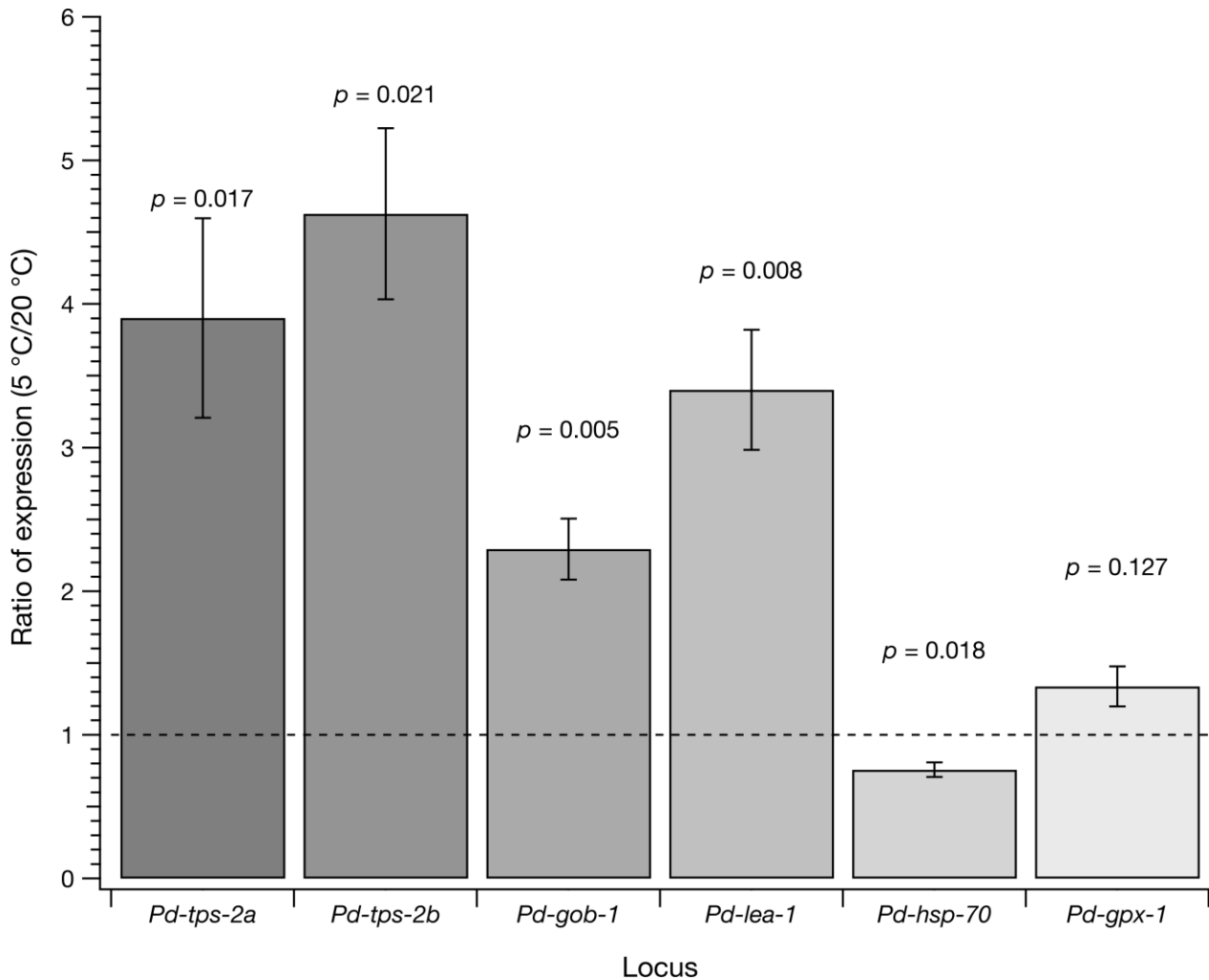
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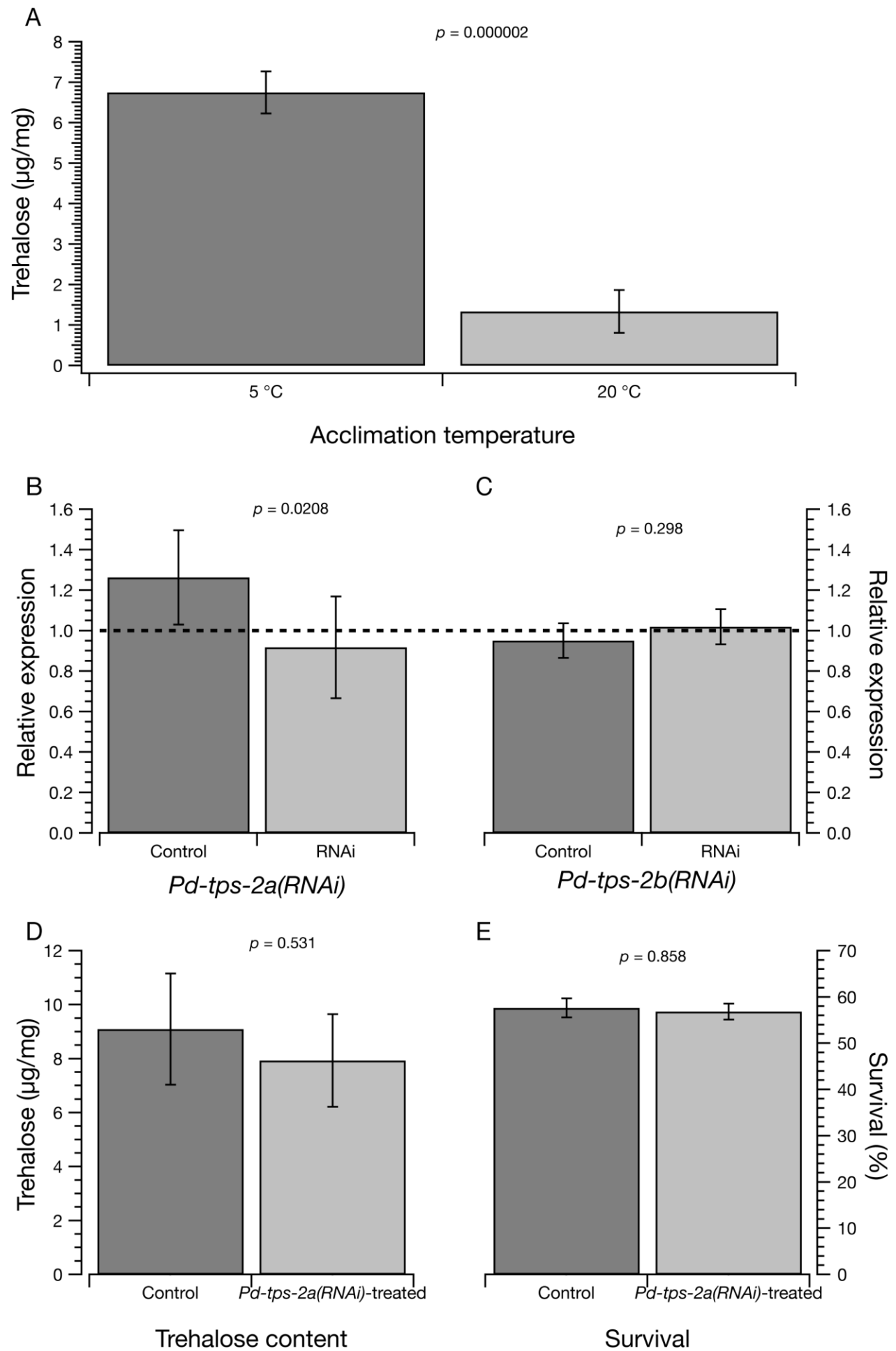
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## Figures

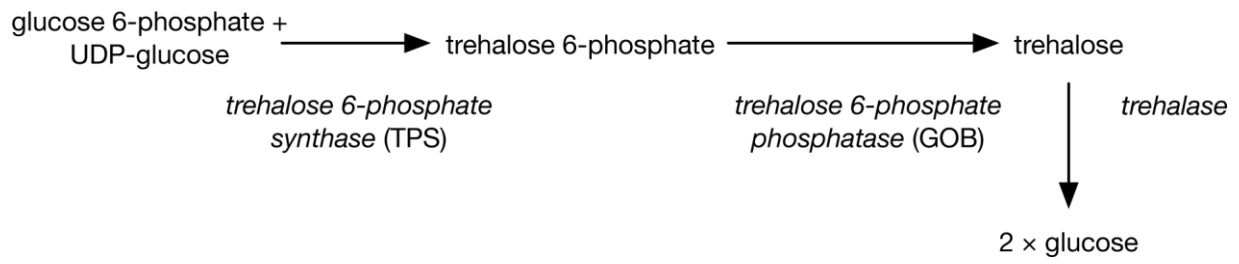


**Fig. 1: Analysis of candidate genes in PaDAW1 samples in response to cold-acclimation.** A: Gene expression analysis by qPCR of *Pd-tps-2a*, *Pd-tps-2b*, *Pd-gob-1*, *Pd-lea-1*, *Pd-hsp-70*, and *Pd-gpx-1* after acclimation at 5 °C for 24 h. Each value represents the mean $\pm$ s.d. of 5 biological replicates. Shown is the expression of the 5 °C samples relative to the 20 °C samples (control) normalized to the value of 1 (which is shown by the dashed line). The statistical significance of differential expression (assessed using t-test) is indicated for each gene. B: Trehalose level in  $\mu\text{g}/\text{mg}$  (dry weight) measured by gas chromatography in PaDAW1 samples incubated for 4d at 5 °C (acclimated) and 20 °C (non-acclimated control). Each value represents the mean $\pm$ s.d. of 4 biological replicates. Significant differential expression (assessed using t-test) is indicated by asterisks ( $P < 0.05$ ).





**Fig. 2: Analysis of *Pd-tps-2a*(RNAi)-treated and non-treated (control) samples in response to cold-acclimation.** A: Trehalose content of nematodes acclimated at 5 °C and 20 °C for 72 h were determined by gas chromatography and expressed as µg trehalose/mg of nematode dry weight. B and C: Gene expression of *Pd-tps-2a* and *Pd-tps-2b* after RNAi. Relative expression of each locus is shown as for Figure 1. D: Trehalose content of control and RNAi-treated nematodes. E: Freezing survival of control and RNAi-treated nematodes after freezing at –15 °C for 30 min as described.



**Fig. 3:** Schematic outline of trehalose synthesis and degradation in nematodes adapted from Behm (1997).

**PCR primers for amplification of genes for dsRNA production**

Primer name	Sequence (5' to 3')	Tm (°C)	Start (bp)	End (bp)	Clone size (bp)	cDNA size (bp)	Pd number
Pd-tps-2a F	TGCTTGAGTTTGTGAATCTGGG	66.2	788	1321	534	1564	PdU054198_v1.1
Pd tps-2a R	ACGCTACAAGTTTATCATCCAG	59.7					
Pd-tps-2b F	AACTCCCAATGAACATGACCA	64.1	849	1369	520	1432	PdU054346_v1.1
Pd-tps-2b R	CGTTGTTGTTGAAGAAGGTGAT	62.8					

**Table S1:** Genes and PCR primers used to amplify PaDAW1 genes for dsRNA production in *E. coli*. In addition to the gene names and primer sequences, primer melting points, clone location and size, cDNA size, and the PaDAW1 contig name [1] is shown. The cDNA size refers to the size of the gene identified at each Pd number.

## qPCR primers

qPCR primers were designed and prepared as described for PCR primers, except the product size range was set to 80-120 bp. Although the maximum temperature difference of primer pairs was set to 1 °C using Primer3web, the final temperature difference of some primer pairs exceeded this value. Sequences for all qPCR primers are shown in Table S2.

Primer name	Sequence (5' to 3')	T <sub>m</sub> (°C)	Use	Efficiency (%)
<i>Pd-gob-1</i> F	CATTCCAACCTCCATCAGCTTTATT	64.0	qPCR validation	99.4
<i>Pd-gob-1</i> R	TGATCCATCGACAGAATTAGAAGT	62.6	qPCR validation	99.4
<i>Pd-gpd-2</i> F	TGTGTTGCTGTTAATGATCCTT	61.0	Reference gene	98.4
<i>Pd-gpd-2</i> R	CAAGATTTCCACCTTCTGCTTT	63.2	Reference gene	98.4
<i>Pd-gpx-1</i> F	TGATCGTGGATTTGAAGTTGC	64.9	qPCR validation	97.8
<i>Pd-gpx-1</i> R	TATCAATTTACATGCAGGTTCC	64.6	qPCR validation	97.8
<i>Pd-hsp-70</i> F	CTGAAACTCTTACTCGTGCCA	62.0	qPCR validation	99.4
<i>Pd-hsp-70</i> R	CAGCATCTTCCATAACCTTTTGA	63.9	qPCR validation	99.4
<i>Pd-lea-1</i> F	AAAGAAAGTGCCCAAATGC	60.7	qPCR validation	108.8
<i>Pd-lea-1</i> R	CTTTATCAGAAGCATGTTGAACT	62.8	qPCR validation	108.8
<i>Pd-tba-1</i> F	CCGAGGGGATGTTGTACCTA	63.6	Reference gene	95.6
<i>Pd-tba-1</i> R	TAAAGCCAGTTGGACACCAAT	63.1	Reference gene	95.6
<i>Pd-tps-2a</i> F	AGTTTCAGAATCACCACAGACG	63.1	qPCR validation	109.3
<i>Pd-tps-2a</i> R	TGATAAAGGGCAGGGCATTG	66.9	qPCR validation	109.3
<i>Pd-tps-2b</i> F	ACCAAACCAGCAGAGGGATT	64.7	qPCR validation	103.7
<i>Pd-tps-2b</i> R	ATCGGAGTTGTCACCCCAA	66.5	qPCR validation	103.7

**Table S2:** Names, sequences, use and efficiency of qPCR primers. Primer names are formed according to the following rules: Species name (e.g. *Pd* = PaDAW1, - gene (e.g. *gob*) - primer (F = forward, R = reverse).

## Specificity and efficiency assays

Specificity assay: To ensure that primers amplify the expected product size range of 80-120 bp and that there was no amplification from any contaminating genomic DNA, primers were tested using standard PCR. PCR products were analysed via agarose gel electrophoresis for primer specificity (single band of the expected size), for genomic DNA contamination (no band in the -RT control and no or a bigger band in the gDNA control) and general contamination (water only control). Figure S1 presents an example of such an agarose gel.



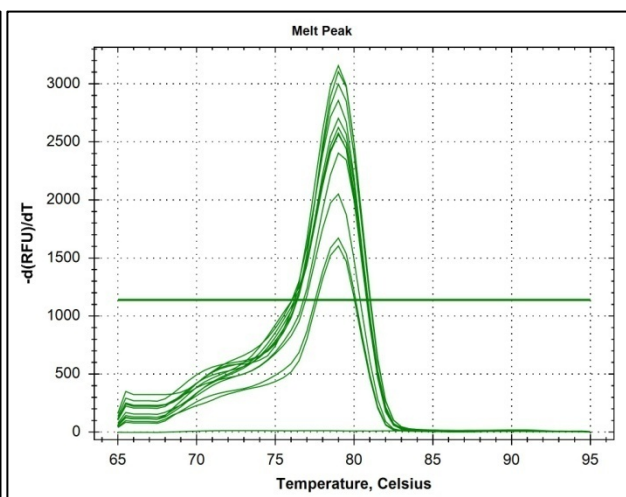
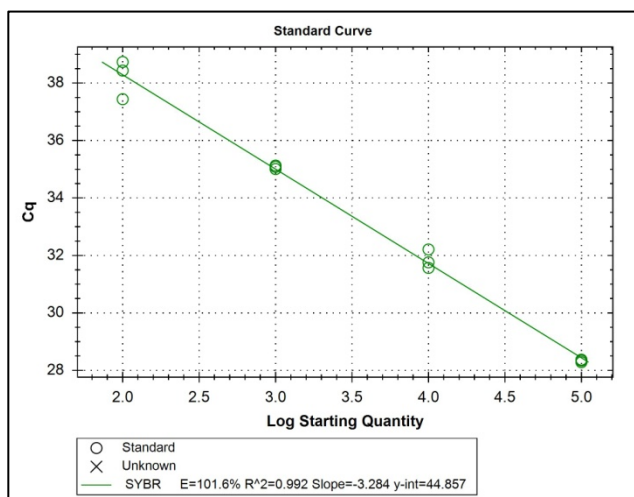
**Figure S1:** Agarose gel showing single bands of the expected size for PCR products using 8 primer pairs, no band in the -RT control, a very faint band in the gDNA control and no band in the water only control.

Melting curves (step f in the qPCR program) were also performed for each individual gene. They were analysed for primer specificity (indicated by a single melting peak) and are listed below.

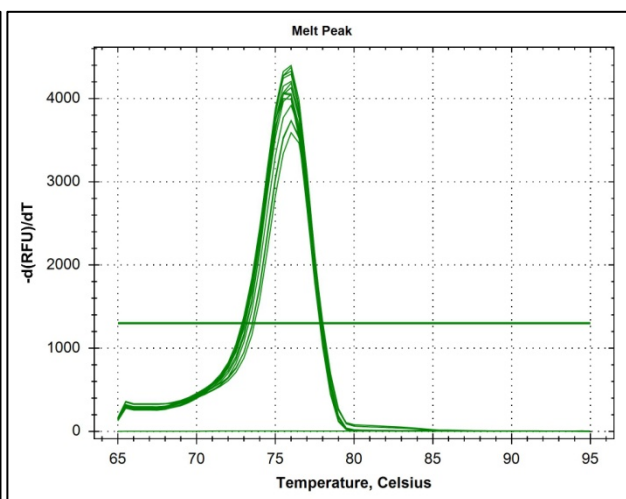
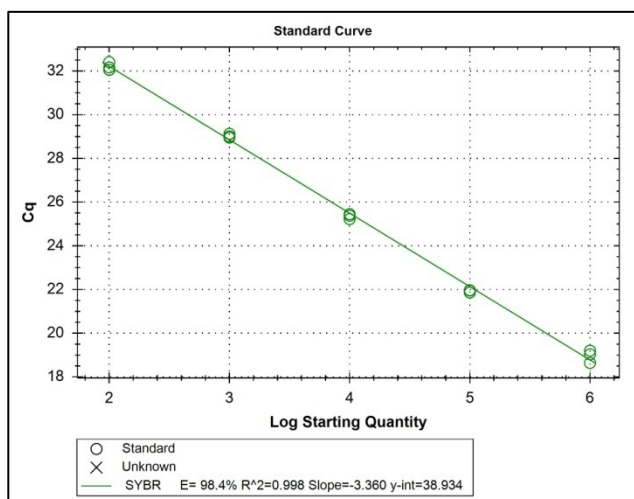
Efficiency assay: Standard curves were performed for each individual gene (technical triplicates) using a dilution series of cDNA (1×, 10×, 100×, 1000×, 10000×). For a qPCR assay the efficiency should be between 90% and 110%. This means that for every PCR cycle there is a doubling of the amount of product. Primers with efficiencies outside the acceptable range were discarded. qPCR primers and their efficiencies are listed in Table S1 and efficiency assays are listed below.

In each graph the amplification data is plotted with the log cDNA concentration on the x-axes and the threshold cycle on the y-axes. The threshold cycle is the cycle at which the fluorescence signal is first detected at higher than background levels. The software (BioRad FCX Manager) calculates efficiency (E), fit to the curve ( $R^2$ ) and slope of the curve (Slope). A  $R^2$  value between 0.95 and 1.0 indicates accurate efficiency measurement and a slope of -3.6 and -3.1 equals an efficiency of 90-100%.

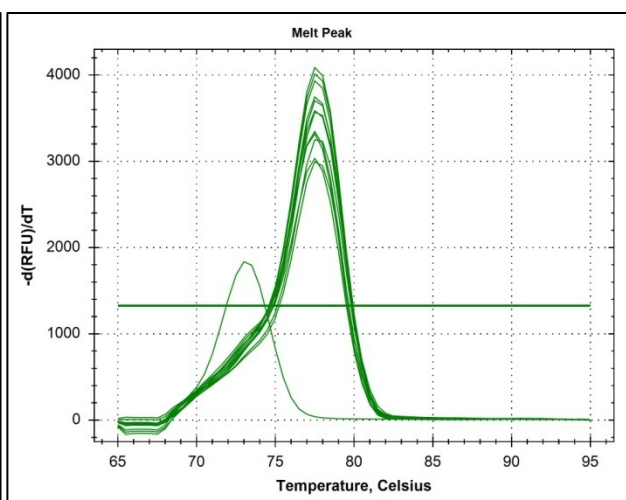
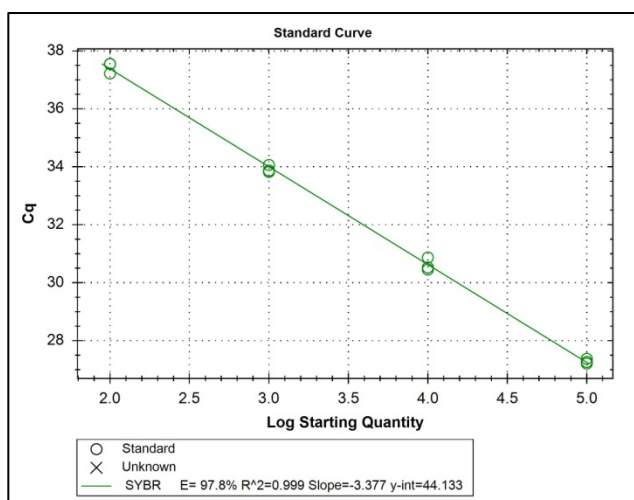
*Pd-gob-1*



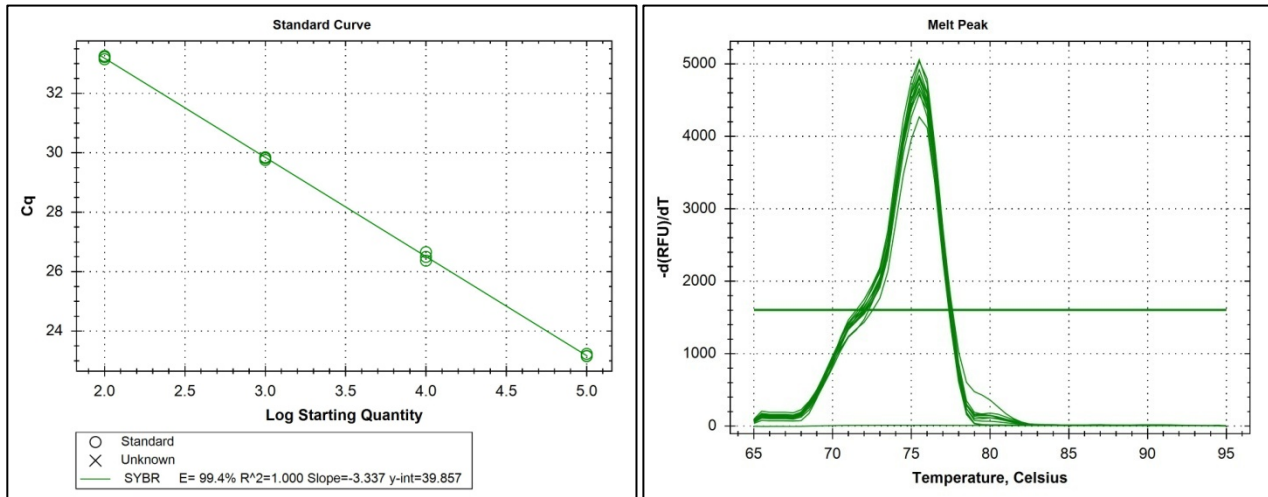
*Pd-gpd-2*



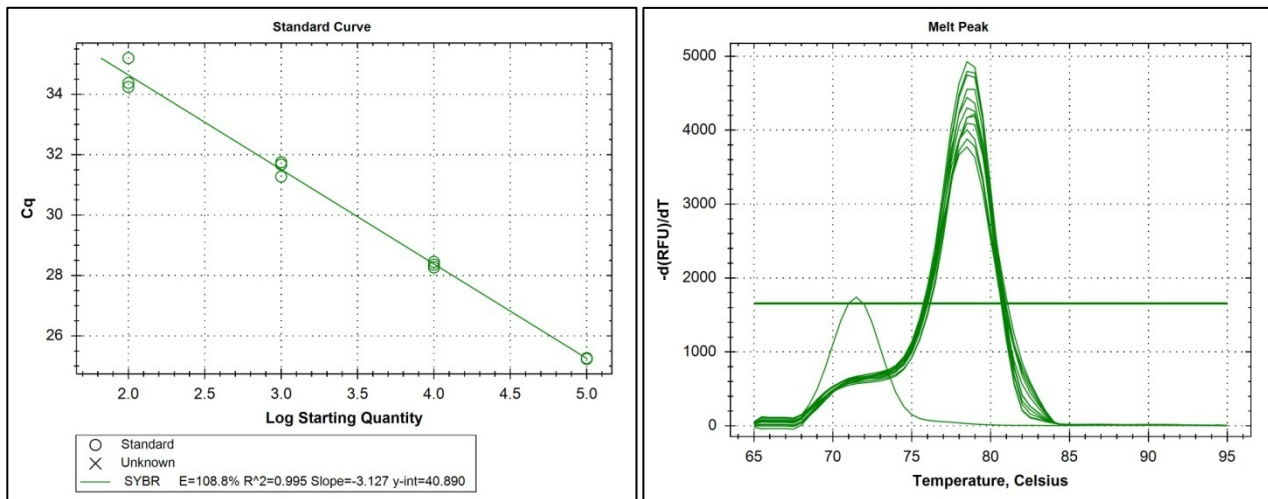
*Pd-gpx-1*



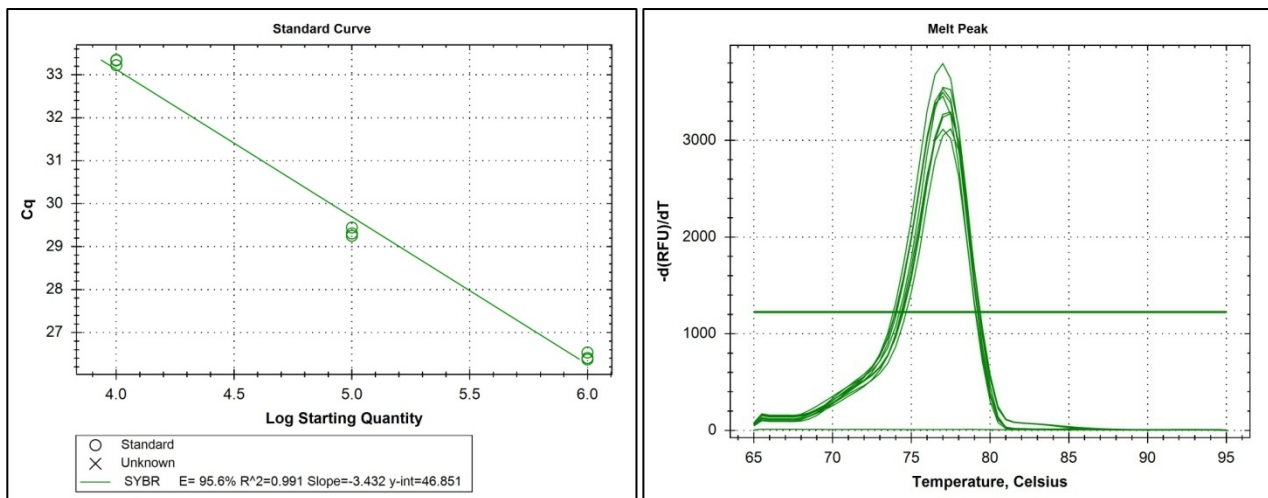
*Pd-hsp-70*



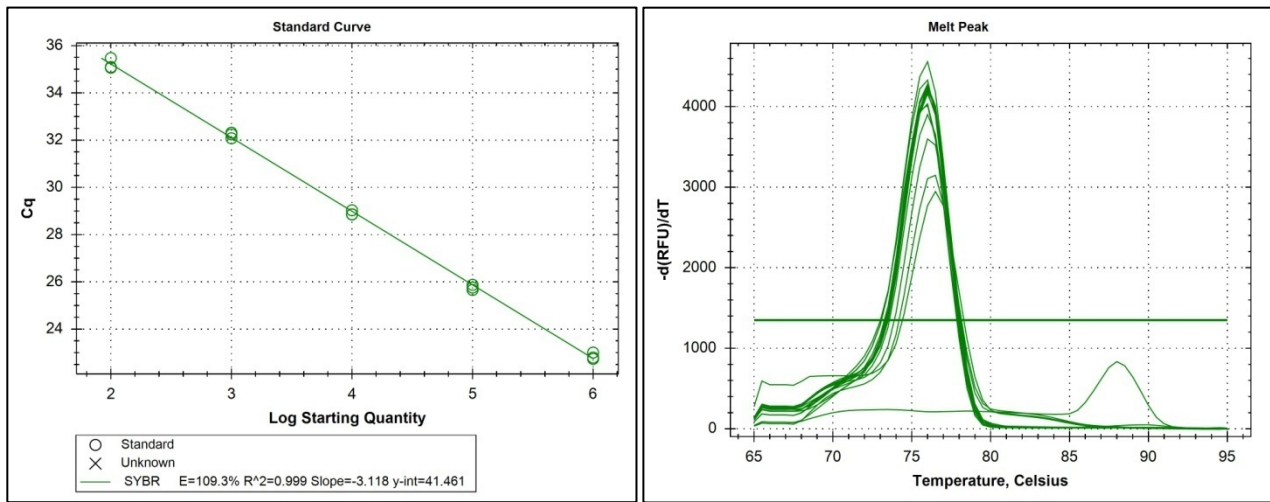
*Pd-lea-1*



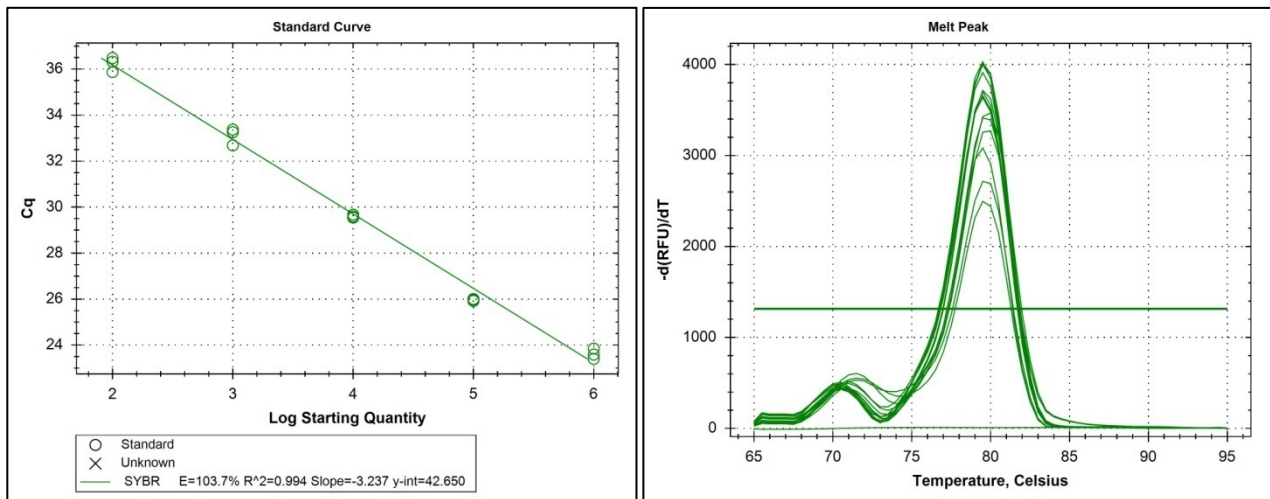
*Pd-tba-1*



*Pd-tps-2a*



*Pd-tps-2b*





## Reference genes

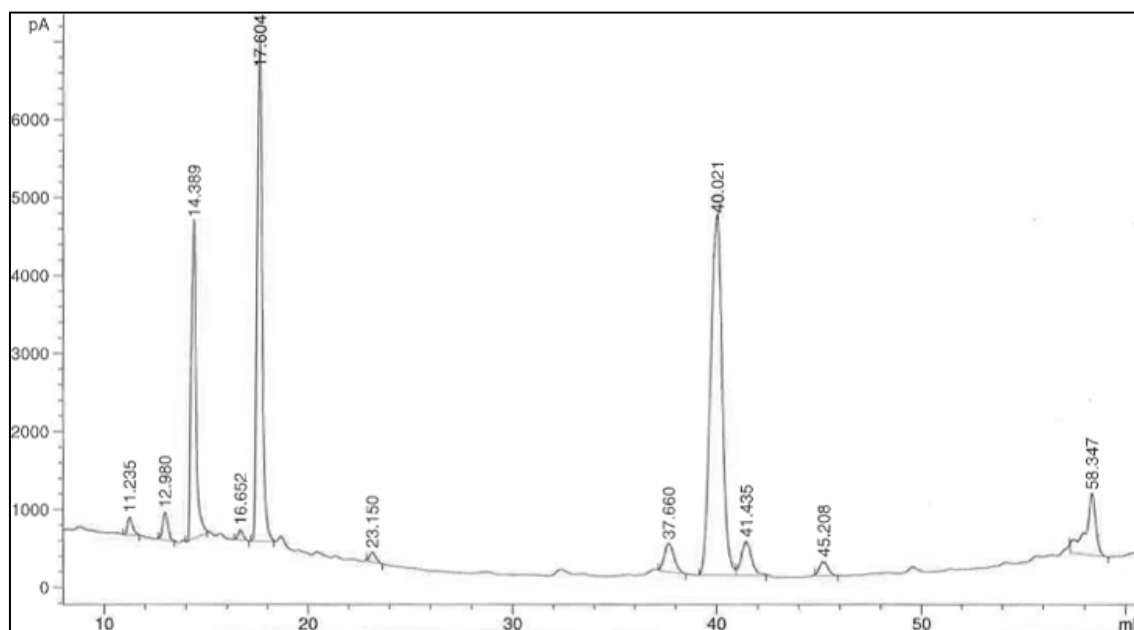
Reference genes for PaDAW1 were selected by searching the literature for stable reference genes in the closely-related *C. elegans*. Primers were designed for the following six reference genes (as described above): *Pd-act-1*, *Pd-ama-1*, *Pd-cdc-42*, *Pd-gpd-2*, *Pd-pmp-3* and *Pd-tba-1*. Of this group, the most stable combination of two reference genes were defined by using NormFinder (Table S2).

This algorithm estimates the stability of gene expression among groups, called the stability value. Genes that deviate the least from the calculated values are the most stable. With a stability value of 0.024, the combination of *Pd-gpd-2* and *Pd-tba-1* was defined as the most stable and used for all qPCR experiments. Because the suitability of selected reference genes can differ between experiments, the stability value in each experiment was analysed via NormFinder [2].

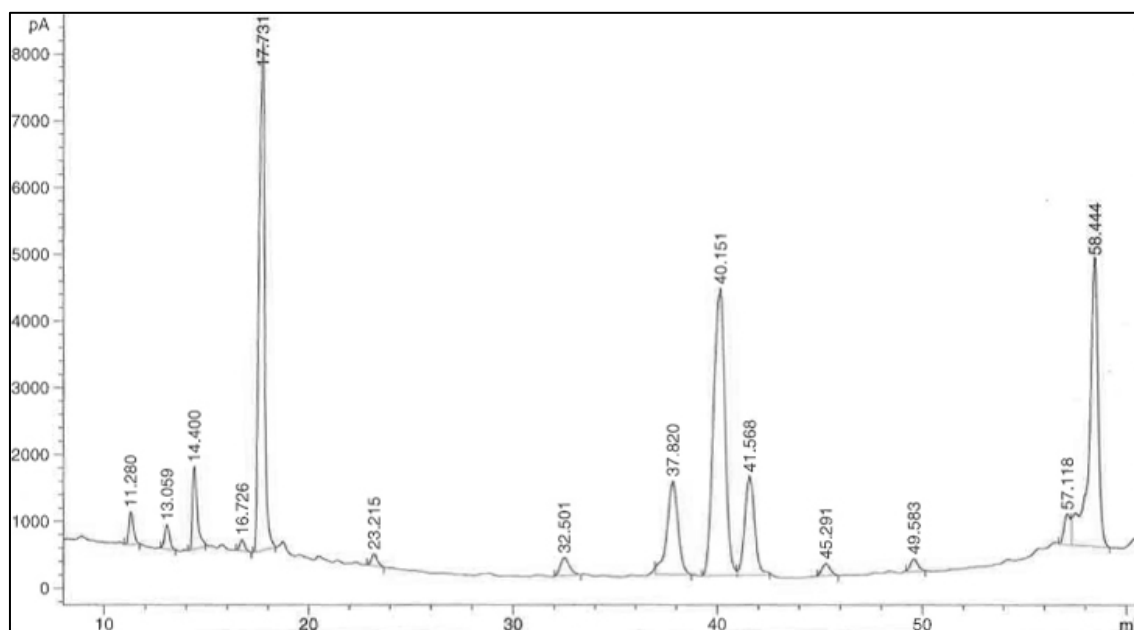
Gene name	Stability value	Best gene	<i>Pd-gpd-2</i>
<i>Pd-cdc-42</i>	0.049	<b>Stability value</b>	0.027
<i>Pd-gpd-2</i>	0.027		
<i>Pd-pmp-3</i>	0.057	<b>Best combination of two genes</b>	<i>Pd-gpd-2</i>
<i>Pd-act-1</i>	0.048		<i>Pd-tba-1</i>
<i>Pd-tba-1</i>	0.042	<b>Stability value for best combination for two genes</b>	0.024
<i>Pd-ama-1</i>	0.031		

**Table S3:** Stability value of six chosen reference genes for PaDAW1: *Pd-cdc-42*, *Pd-gpd-2*, *Pd-pmp-3*, *Pd-act-1*, *Pd-tba-1* and *Pd-ama-1*. The most stable gene is *Pd-gpd-2* (stability value 0.027) and the most stable gene combination is *Pd-gpd-2* and *Pd-tba-1* (stability value 0.024) [3,4]

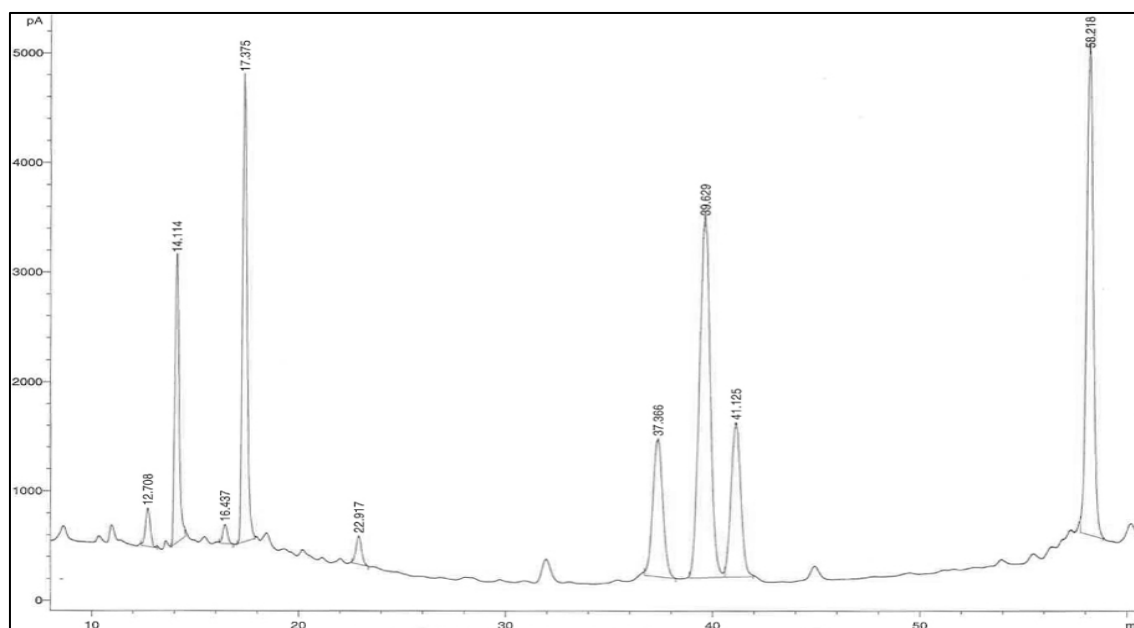
### Example chromatograms



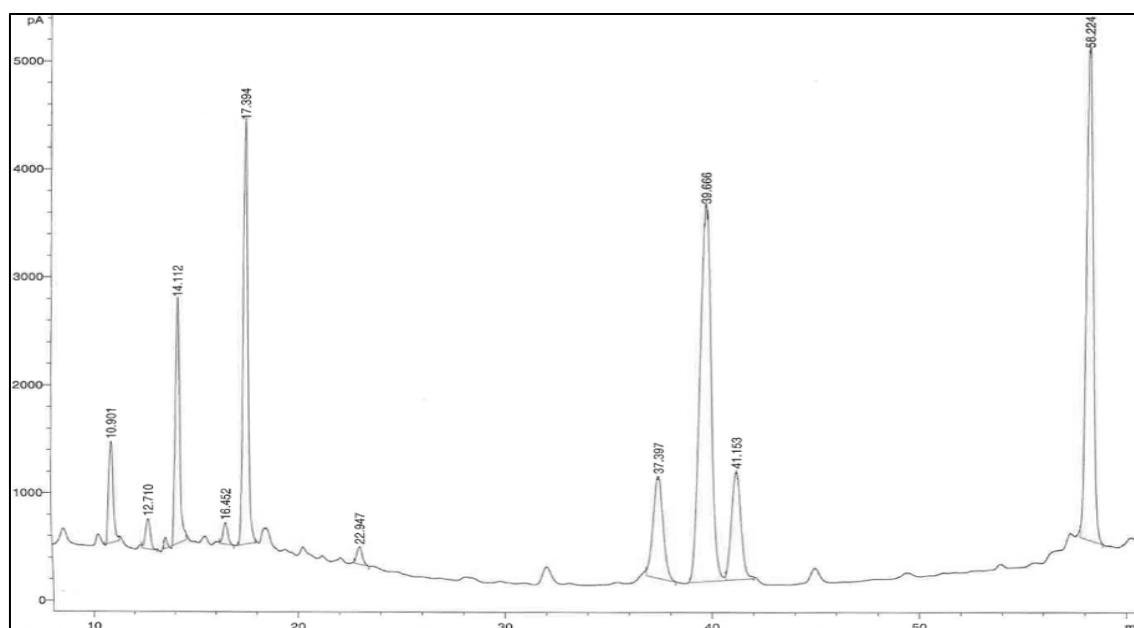
Example chromatogram of a 20°C sample (elution times: 17 = Glycerol, 40 = Dulcitol (internal standard), 58 = Trehalose)



Example chromatogram of a 5°C sample



Example chromatogram of a non-treated acclimated (control) sample (elution times: 17 = Glycerol, 39 = Dulcitol (internal standard), 58 = Trehalose)



Example chromatogram of a *Pd-tps-2a(RNAi)* treated acclimated sample

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**PCR primers for amplification of genes for dsRNA production**

Primer name	Sequence (5' to 3')	Tm (°C)	Start (bp)	End (bp)	Clone size (bp)	cDNA size (bp)	Pd number
Pd-tps-2a F	TGCTTGAGTTTGTGAATCTGGG	66.2	788	1321	534	1564	PdU054198_v1.1
Pd tps-2a R	ACGCTACAAGTTTATCATCCAG	59.7					
Pd-tps-2b F	AACTCCCAATGAACATGACCA	64.1	849	1369	520	1432	PdU054346_v1.1
Pd-tps-2b R	CGTTGTTGTTGAAGAAGGTGAT	62.8					

**Table S1:** Genes and PCR primers used to amplify PaDAW1 genes for dsRNA production in *E. coli*. In addition to the gene names and primer sequences, primer melting points, clone location and size, cDNA size, and the PaDAW1 contig name [1] is shown. The cDNA size refers to the size of the gene identified at each Pd number.

## qPCR primers

qPCR primers were designed and prepared as described for PCR primers, except the product size range was set to 80-120 bp. Although the maximum temperature difference of primer pairs was set to 1 °C using Primer3web, the final temperature difference of some primer pairs exceeded this value. Sequences for all qPCR primers are shown in Table S2.

Primer name	Sequence (5' to 3')	T <sub>m</sub> (°C)	Use	Efficiency (%)
<i>Pd-gob-1</i> F	CATTCCAACCTCCATCAGCTTTATT	64.0	qPCR validation	99.4
<i>Pd-gob-1</i> R	TGATCCATCGACAGAATTAGAAGT	62.6	qPCR validation	99.4
<i>Pd-gpd-2</i> F	TGTGTTGCTGTTAATGATCCTT	61.0	Reference gene	98.4
<i>Pd-gpd-2</i> R	CAAGATTTCCACCTTCTGCTTT	63.2	Reference gene	98.4
<i>Pd-gpx-1</i> F	TGATCGTGGATTTGAAGTTGC	64.9	qPCR validation	97.8
<i>Pd-gpx-1</i> R	TATCAATTTACATGCAGGTTCC	64.6	qPCR validation	97.8
<i>Pd-hsp-70</i> F	CTGAAACTCTTACTCGTGCCA	62.0	qPCR validation	99.4
<i>Pd-hsp-70</i> R	CAGCATCTTCCATAACCTTTTGA	63.9	qPCR validation	99.4
<i>Pd-lea-1</i> F	AAAGAAAGTGCCCAAATGC	60.7	qPCR validation	108.8
<i>Pd-lea-1</i> R	CTTTATCAGAAGCATGTTGAACT	62.8	qPCR validation	108.8
<i>Pd-tba-1</i> F	CCGAGGGGATGTTGTACCTA	63.6	Reference gene	95.6
<i>Pd-tba-1</i> R	TAAAGCCAGTTGGACACCAAT	63.1	Reference gene	95.6
<i>Pd-tps-2a</i> F	AGTTTCAGAATCACCACAGACG	63.1	qPCR validation	109.3
<i>Pd-tps-2a</i> R	TGATAAAGGGCAGGGCATTG	66.9	qPCR validation	109.3
<i>Pd-tps-2b</i> F	ACCAAACCAGCAGAGGGATT	64.7	qPCR validation	103.7
<i>Pd-tps-2b</i> R	ATCGGAGTTGTCACCCCAA	66.5	qPCR validation	103.7

**Table S2:** Names, sequences, use and efficiency of qPCR primers. Primer names are formed according to the following rules: Species name (e.g. *Pd* = PaDAW1, - gene (e.g. *gob*) - primer (F = forward, R = reverse).

## Supplementary Methods

### Specificity and efficiency assays

Specificity assay: To ensure that primers amplify the expected product size range of 80-120 bp and that there was no amplification from any contaminating genomic DNA, primers were tested using standard PCR. PCR products were analysed via agarose gel electrophoresis for primer specificity (single band of the expected size), for genomic DNA contamination (no band in the -RT control and no or a bigger band in the gDNA control) and general contamination (water only control). Figure S1 presents an example of such an agarose gel.



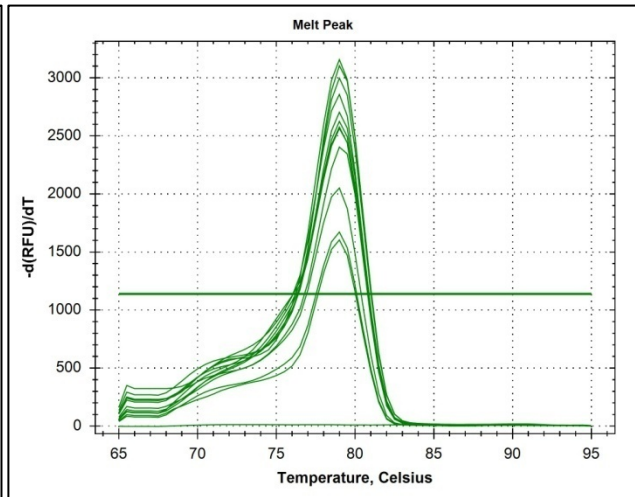
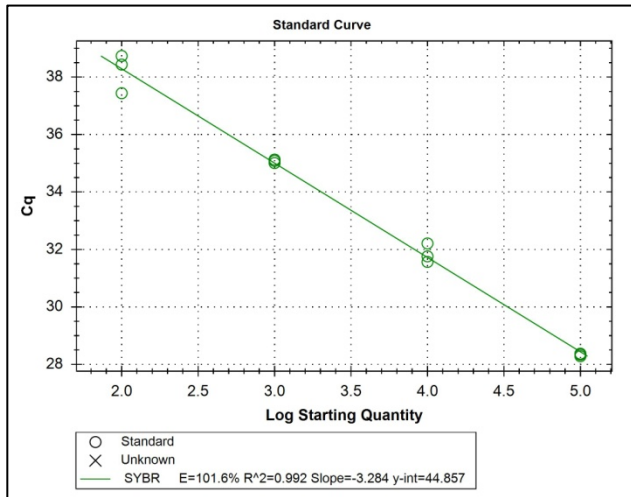
**Figure S1:** Agarose gel showing single bands of the expected size for PCR products using 8 primer pairs, no band in the -RT control, a very faint band in the gDNA control and no band in the water only control.

Melting curves (step f in the qPCR program) were also performed for each individual gene. They were analysed for primer specificity (indicated by a single melting peak) and are listed below.

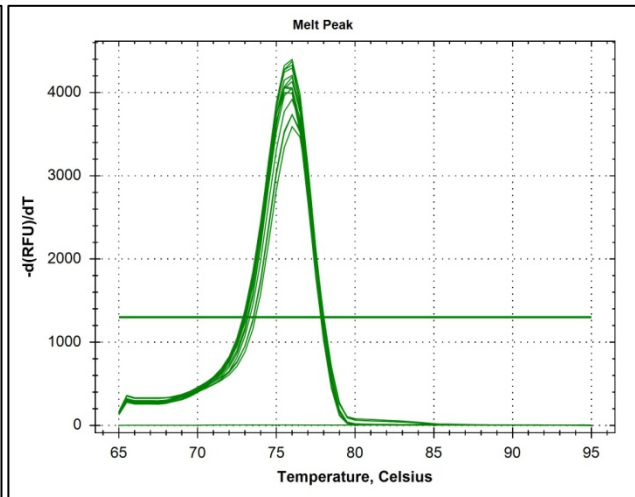
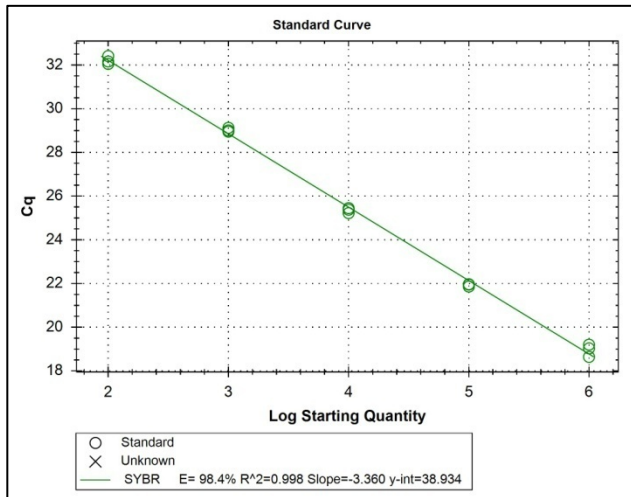
Efficiency assay: Standard curves were performed for each individual gene (technical triplicates) using a dilution series of cDNA (1×, 10×, 100×, 1000×, 10000×). For a qPCR assay the efficiency should be between 90% and 110%. This means that for every PCR cycle there is a doubling of the amount of product. Primers with efficiencies outside the acceptable range were discarded. qPCR primers and their efficiencies are listed in Table S1 and efficiency assays are listed below.

In each graph the amplification data is plotted with the log cDNA concentration on the x-axes and the threshold cycle on the y-axes. The threshold cycle is the cycle at which the fluorescence signal is first detected at higher than background levels. The software (BioRad FCX Manager) calculates efficiency (E), fit to the curve ( $R^2$ ) and slope of the curve (Slope). A  $R^2$  value between 0.95 and 1.0 indicates accurate efficiency measurement and a slope of -3.6 and -3.1 equals an efficiency of 90-100%.

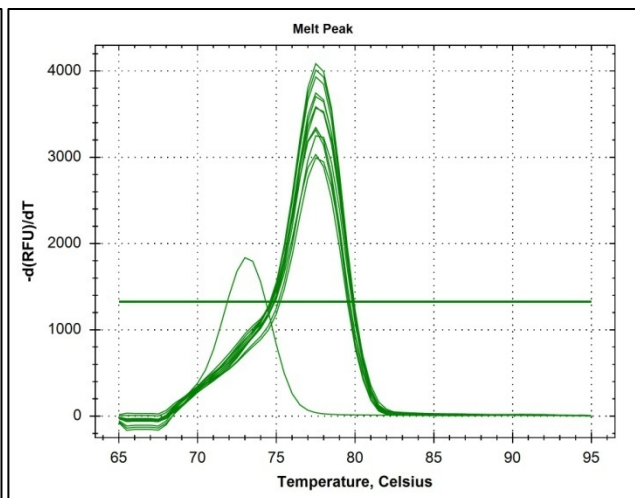
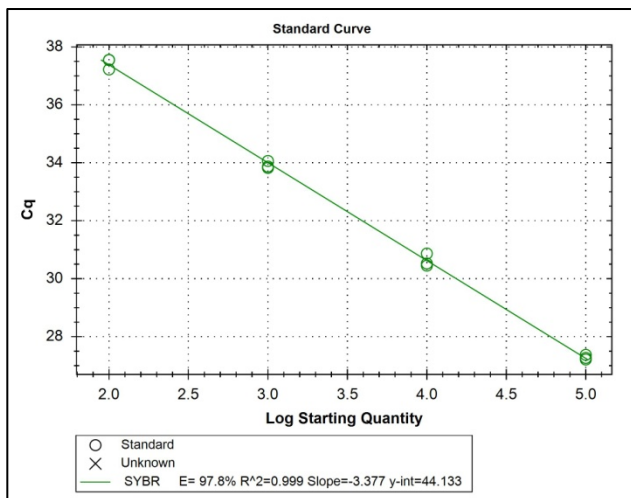
*Pd-gob-1*



*Pd-gpd-2*

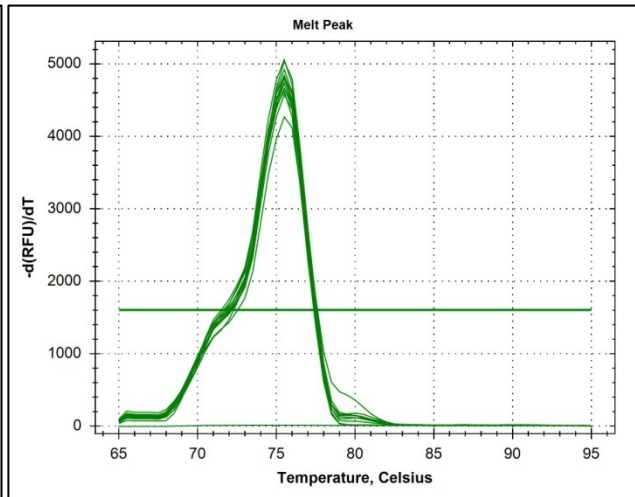
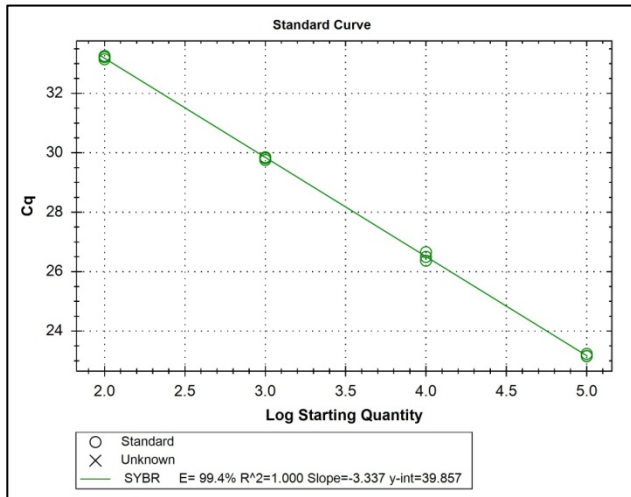


*Pd-gpx-1*

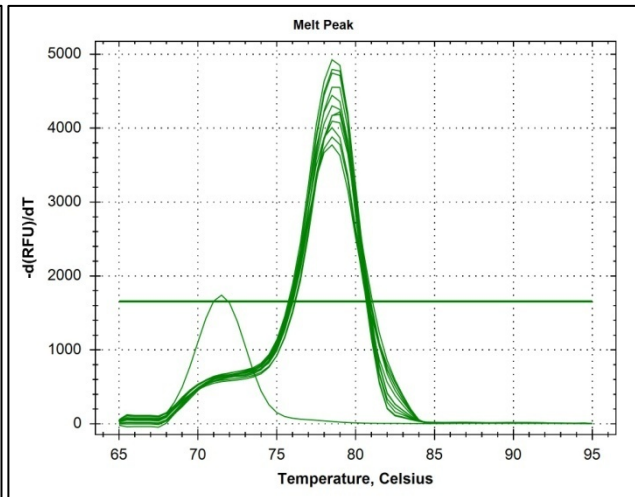
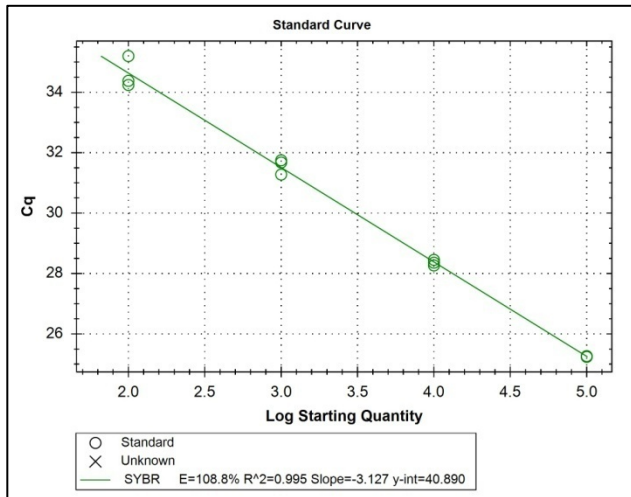




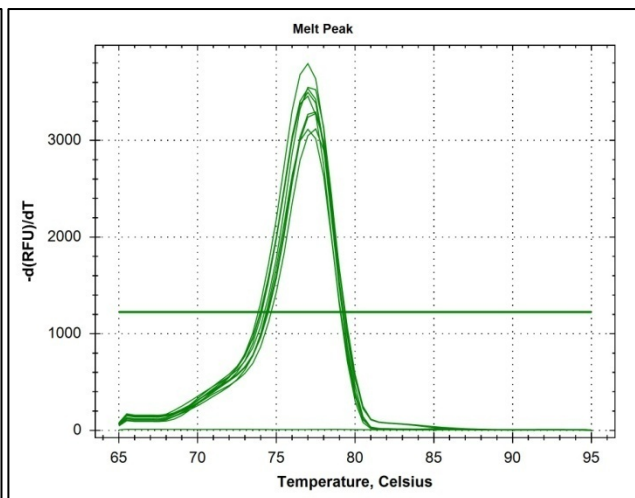
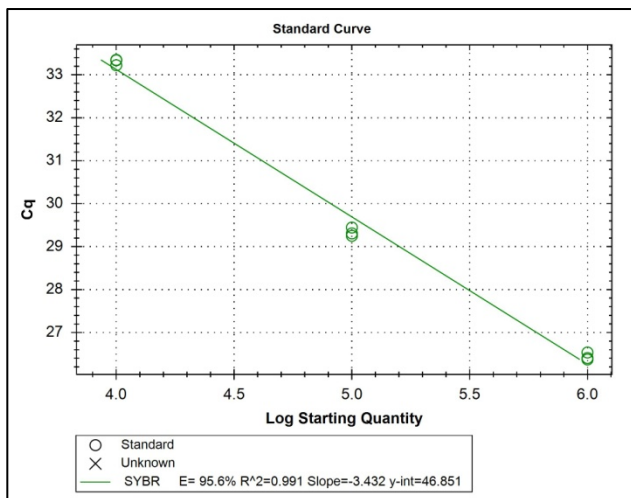
*Pd-hsp-70*



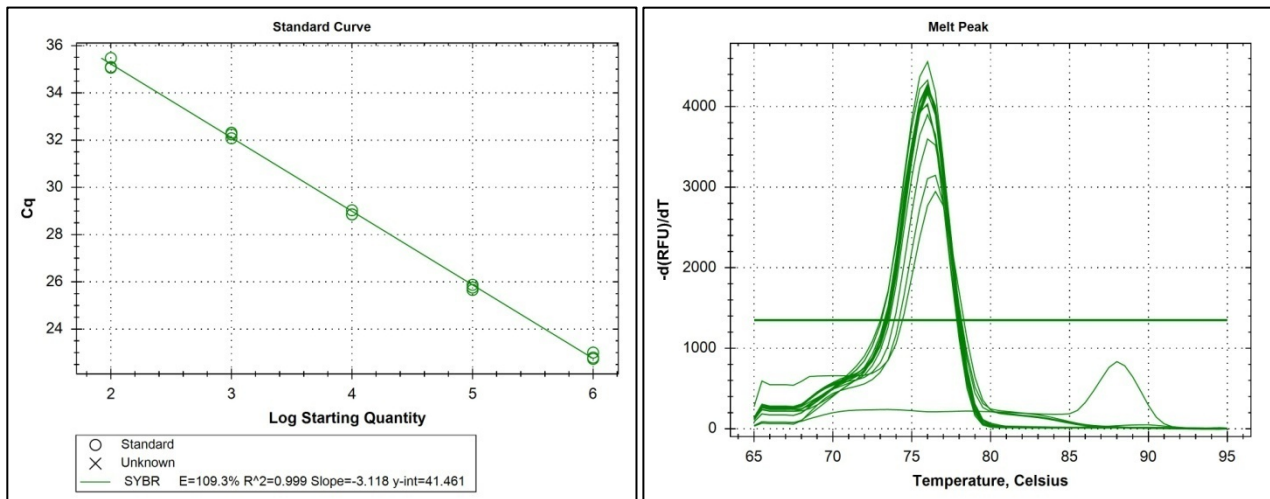
*Pd-lea-1*



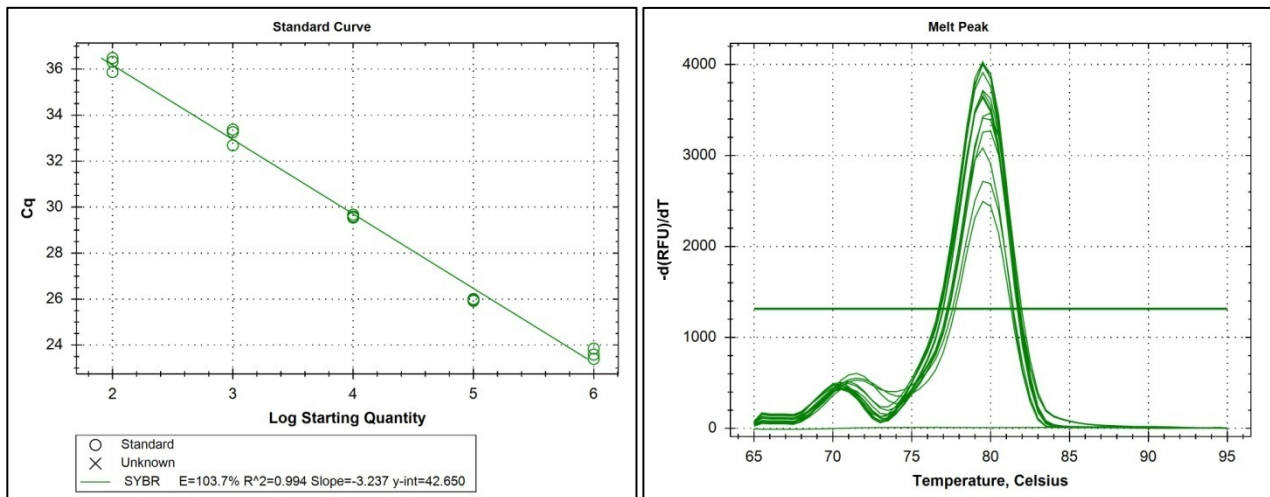
*Pd-tba-1*



*Pd-tps-2a*



*Pd-tps-2b*



## Reference genes

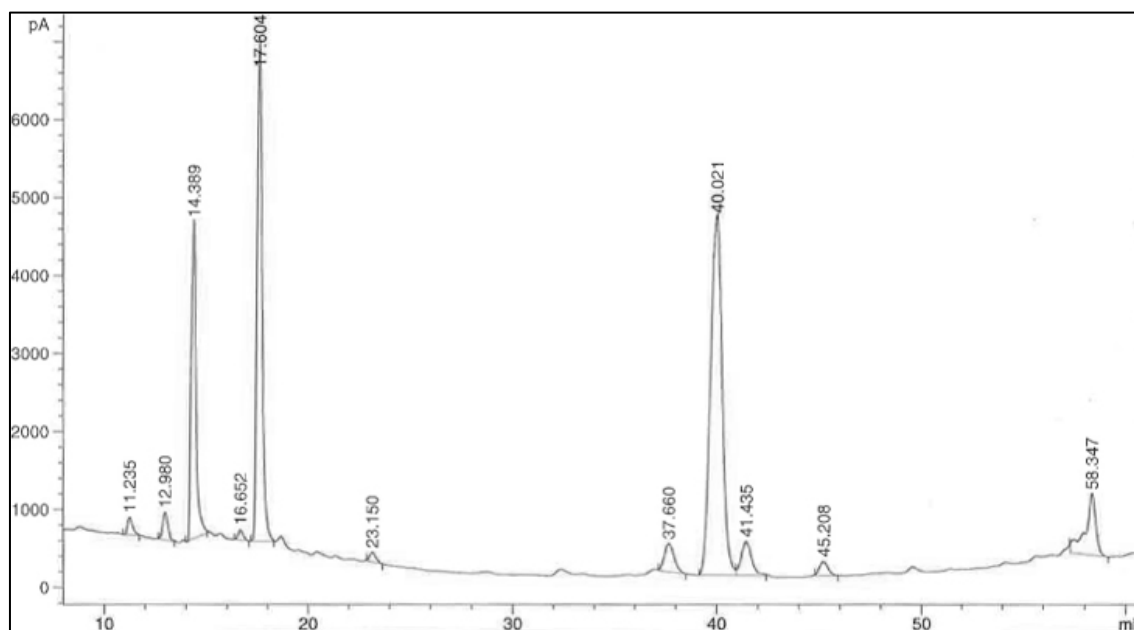
Reference genes for PaDAW1 were selected by searching the literature for stable reference genes in the closely-related *C. elegans*. Primers were designed for the following six reference genes (as described above): *Pd-act-1*, *Pd-ama-1*, *Pd-cdc-42*, *Pd-gpd-2*, *Pd-pmp-3* and *Pd-tba-1*. Of this group, the most stable combination of two reference genes were defined by using NormFinder (Table S2).

This algorithm estimates the stability of gene expression among groups, called the stability value. Genes that deviate the least from the calculated values are the most stable. With a stability value of 0.024, the combination of *Pd-gpd-2* and *Pd-tba-1* was defined as the most stable and used for all qPCR experiments. Because the suitability of selected reference genes can differ between experiments, the stability value in each experiment was analysed via NormFinder [2].

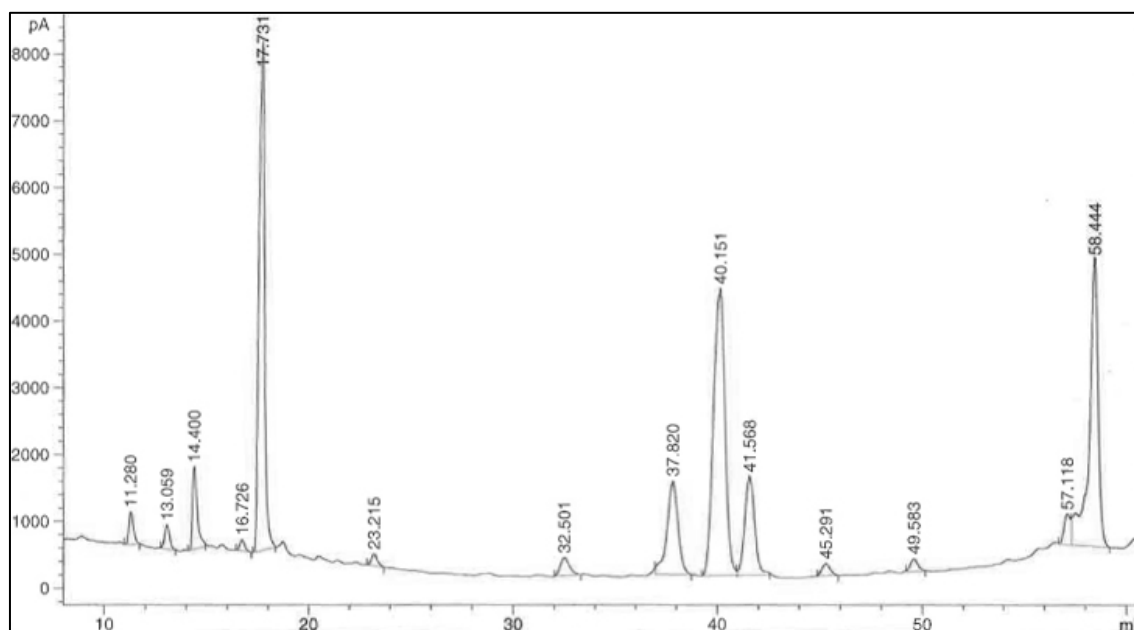
Gene name	Stability value	Best gene	<i>Pd-gpd-2</i>
<i>Pd-cdc-42</i>	0.049	<b>Stability value</b>	0.027
<i>Pd-gpd-2</i>	0.027		
<i>Pd-pmp-3</i>	0.057	<b>Best combination of two genes</b>	<i>Pd-gpd-2</i>
<i>Pd-act-1</i>	0.048		<i>Pd-tba-1</i>
<i>Pd-tba-1</i>	0.042	<b>Stability value for best combination for two genes</b>	0.024
<i>Pd-ama-1</i>	0.031		

**Table S3:** Stability value of six chosen reference genes for PaDAW1: *Pd-cdc-42*, *Pd-gpd-2*, *Pd-pmp-3*, *Pd-act-1*, *Pd-tba-1* and *Pd-ama-1*. The most stable gene is *Pd-gpd-2* (stability value 0.027) and the most stable gene combination is *Pd-gpd-2* and *Pd-tba-1* (stability value 0.024) [3,4]

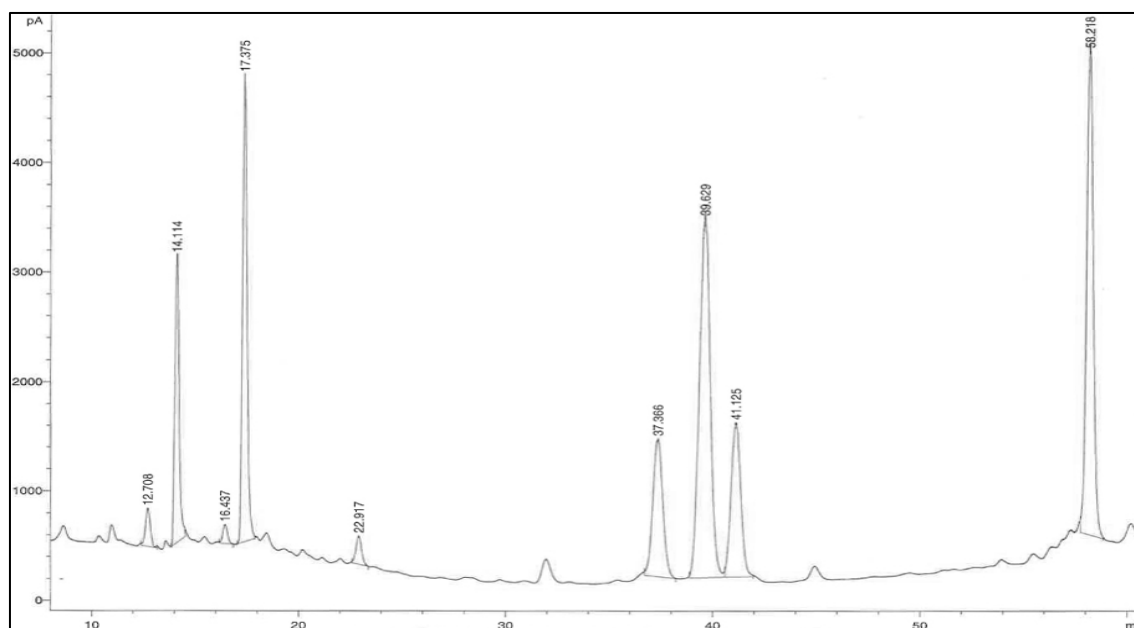
## Example chromatograms



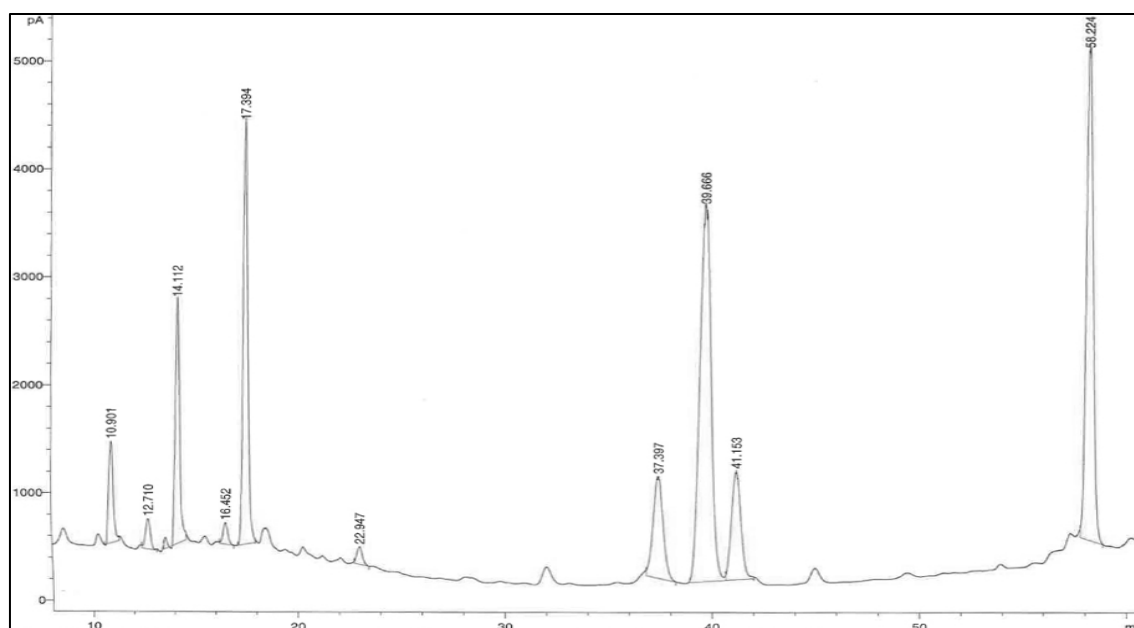
Example chromatogram of a 20°C sample (elution times: 17 = Glycerol, 40 = Dulcitol (internal standard), 58 = Trehalose)



Example chromatogram of a 5°C sample



Example chromatogram of a non-treated acclimated (control) sample (elution times: 17 = Glycerol, 39 = Dulcitol (internal standard), 58 = Trehalose)



Example chromatogram of a *Pd-tps-2a(RNAi)* treated acclimated sample

## References

1. Thorne MAS, Kagoshima H, Clark MS, Marshall CJ, Wharton DA. Molecular analysis of the cold tolerant Antarctic nematode, *Panagrolaimus davidi*. PLoS ONE. 2014;9: e104526–e104526. doi:10.1371/journal.pone.0104526
2. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem. American Association for Clinical Chemistry; 2009;55: 611–622. doi:10.1373/clinchem.2008.112797

3. Seybold AC, Wharton DA, Thorne MAS, Marshall CJ. Establishing RNAi in a Non-Model Organism: The Antarctic Nematode *Panagrolaimus* sp. DAW1. PLoS ONE; 2016;11: e0166228. doi:10.1371/journal.pone.0166228
4. Seybold A. Molecular Adaptation Mechanisms in the Antarctic Nematode *Panagrolaimus davidi*. Phd Thesis, University of Otago. 2015.