Effect of short term high temperature exposure on gene expression in raspberry cultivars

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Running head-'*Temperature stress and gene expression in raspberries*'

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SUMMARY

Effect of high temperature stress (27 and 37°C for 24 h) on gene expression profiles of annual-fruiting raspberry cultivars 'Autumn Bliss', 'Autumn Treasure', 'Erika' and 'Polka' at the flower initiation stage were evaluated using a custom *Rubus* microarray. Significant genes were obtained by pairwise t-tests using volcano plots for each cultivar by treatment. An elevation of temperature (Δ10°C) altered the expression of 40 genes (38 were down- and two up-regulated) amongst the four cultivars. Volcano filtering identified 12 common candidate genes that were modulated differentially in 'Autumn Bliss' and 'Erika' at 37°C compared to 27°C. Two aquaporin genes (PIP1 and TIP2) were down-regulated in 'Autumn Bliss' but up-regulated in 'Autumn Treasure', 'Polka' and 'Erika'. Down-regulated genes included those encoding major latex-like protein (MLPs), plasma membrane proteins (PMPs), cysteine proteins and other stress-related proteins. Validation by real-time quantitative RT-PCR indicated subtle gene expression changes suggesting a mild response to heat stress. This study has used molecular tools to increase our understanding and identify candidate genes involved in the heat stress response of raspberry cultivars.

37 Most raspberry (*Rubus idaeus* L) production is concentrated in cold temperate areas of the
38 world. However, annual-fruiting raspberries tend to be grown in warmer regions such as
39 Southern parts of Europe where the summer temperature is relatively high (Graham *et al.*,
40 2007; Graham and Jennings, 2009). The optimum temperature for annual-fruiting raspberry
41 cultivation ranges from 16 to 24°C but some cultivars like 'Polka' grow successfully even at
42 30°C (Sonsteby and Heide, 2009). Existing raspberry cultivars are poorly adapted to high
43 temperatures that may occur during the summer months and in protected cultivation (e.g.,
44 greenhouse and tunnels) (Ballington and Fernandez, 2008). A study by Gotame *et al.* (2013)
45 indicated that temperatures above 32°C generally reduced maximum photosynthetic
46 efficiency in annual-fruiting raspberries. The outdoor temperature is expected to fluctuate
47 more and increase further due to climate change. Elevated temperatures have adverse effects
48 on plant physiology, biochemistry and metabolism (Wahid *et al.*, 2007), modulate gene
49 expression, proteins and metabolites (Ahuja *et al.*, 2010), and cause protein aggregation and
50 denaturation (Berry and Bjorkman, 1980). In strawberry, total protein content decreased and
51 peroxidase activity increased when leaves were exposed to temperatures above 25°C (Gulen
52 and Eris, 2004). As with other abiotic stresses, growing plants at a high temperature also
53 triggers defence mechanisms by changing the levels of gene transcripts through physical
54 changes within the plant system and by creating signals for metabolic adjustment (Mittler *et*
55 *al.*, 2012). Heat stress causes alterations in the expression of genes for osmoprotectants,
56 detoxifying enzymes, transporters, and regulatory proteins. For example, an analysis of
57 grapevine leaves reported that heat stress up-regulated by two-fold, genes of important traits
58 including cell rescue, protein fate, primary and secondary metabolism, transcription factors
59 and signal transduction compared to recovered leaves (Liu *et al.*, 2012).
60 Microarrays are powerful tools for measuring the expression of many genes of interest
61 in different tissues and stages of development (Slonim and Yanai, 2009). Gene expression

62 profiling using microarrays have increased our understanding of several important biological
63 questions in many crops including berries (e. g. strawberry, blackcurrant, raspberries etc.)
64 during specific physiological conditions (Aharoni and O'Connell, 2002; Mazzitelli *et al.*,
65 2007; Chen *et al.*, 2010; Hedley *et al.*, 2010). Moreover, they have also been used to evaluate
66 the changes in gene patterns and transcriptome levels affected by high temperature in
67 horticultural crops e.g., Chinese cabbage (Yang *et al.*, 2006), tomato (Frank *et al.*, 2009),
68 sunflower (Hewezi *et al.*, 2008), potato (Ginzberg *et al.*, 2009) and grapes (Liu *et al.*, 2012).
69 Microarrays were also used to identify candidate genes associated with bud dormancy release
70 in raspberry (Mazzitelli *et al.*, 2007) and blackcurrant (Hedley *et al.*, 2010). These studies
71 related the expression of specific genes to physiological changes during normal growth
72 conditions.

73 Gene expression in response to high temperature conditions in leaf tissues of
74 raspberry, particularly at the flower initiation stage has not yet been reported. In this study, we
75 used a *Rubus* microarray to study the effect of high temperature conditions (27 and 37°C) for
76 a short period (24 h) on gene expression levels in four annual-fruiting raspberry cultivars
77 ('Autumn Bliss', 'Autumn Treasure', 'Erika' and 'Polka'). In terms of heat tolerance and
78 susceptibility, 'Autumn Bliss' was the most susceptible and 'Autumn Treasure' and 'Erika'
79 showed some degree of tolerance based on chlorophyll fluorescence and chlorophyll pigment
80 analysis (Gotame *et al.*, 2013). Therefore in this study, we selected and compared the most
81 susceptible to more tolerant cultivars to identify gene expression changes in raspberry leaves
82 under high temperature stress.

83

84 MATERIALS AND METHODS

85 *Plant material and treatment*

Rootstocks of four annual-fruiting raspberry cultivars, 'Autumn Bliss', 'Autumn Treasure', 'Erika' and 'Polka' were propagated in 3.5 L pots in duplicate and grown under greenhouse conditions at 20 +5°C with a photoperiod of 14 h until flower initiation. Irrigation was supplied once a day to achieve pot capacity with a fertilizer solution with an EC of 2.16 mS cm^{-1} containing 40 mg L^{-1} NH_4-N, 165 mg L^{-1} NO_3-N, 44 mg L^{-1} phosphorus (P) and 257 mg L^{-1} potassium (K). When plants reached the stage of flower initiation (usually seven weeks after root sprouting), they were transferred to climate chambers (MB-teknik, Broendby, Denmark) set at 27 and 37°C with a day length of 14 h. In the climate chambers, the irradiance was constant at 350 µmol m^{-2} s^{-1} photosynthetic active radiation and relative humidity was 60% ±5%. All plants were watered by hand with a complete nutrient solution to pot capacity at constant times of day of 08:00 am and 05:00 pm. The youngest leaf at the top of the shoot from each plant was collected after 24 h exposure to high temperature and immediately frozen in liquid nitrogen and stored in -80°C before freeze dried (Christ Gamma 1-20 LSC , UK) for RNA extraction.

RNA isolation

Total RNA was extracted from freeze dried leaf (0.1 g) samples collected after 24 h heat exposure using the RNeasy® Plant Mini Kit (Qiagen) according to the manufacturer's recommendations including the addition of 10% v/v RNA Isolation Aid (Ambion Life Technologies Ltd, UK) and 1% v/v β-mercaptoethanol (Sigma-Aldrich Company Ltd., UK) to extraction buffer RLT. The concentration and purity of the RNA was analyzed spectrophotometrically at 230, 260, and 280 nm using a NanoDrop® ND-1000 Full-spectrum UV-Visible Spectrophotometer (ThermoFischer Scientific, Epsom, UK) and an A_{260}/A_{280} ratio of 1.8-2.0 indicated good quality RNA. The integrity of the RNA was also checked by

both agarose gel electrophoresis (2%) and an Agilent Bio-analyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) which reports an objective RNA integrity indicator value (RIN) ranging from one (degraded) to ten (intact). The RNA extracted from the leaf material of all four raspberry cultivars had A_{260}/A_{280} ratios in the range of 1.8-2.0 and RIN values were usually >7.0 indicationg a high quality. The RNA samples were aliquoted in batches and stored at -80°C.

Microarray processing and data analysis

A simple pairwise microarray experimental plan was devised to utilise the *Rubus* Agilent 60 K microarray developed at the James Hutton Institute, UK and designed from a unigene set derived from RNAseq datasets. In total 16 samples were processed: 4 cultivars; 2 temperatures (27 and 37°C); 2 biological replicates, in a two-colour microarray design (27°C *vs* 37°C for each sample, including dye-swaps). RNA was labelled using standard recommended procedures (Agilent Low-Input QuickAmp RNA Labelling Kit) with 100 ng total RNA per sample. Labelling efficiencies were checked following purification on the NanoDrop® ND-1000. Hybridisation of samples to the *Rubus* array was performed overnight as recommended using the Two-Color Microarray-Based Gene Expression Analysis protocol (Agilent v 6.5). Microarrays were scanned for data acquisition at two wavelengths (each for Cyanine3 and Cyanine5 dye) using the Agilent Microarray scanner G2505B, resulting in single tiff images for each array.

Raw data were extracted from each array using Agilent Feature Extraction (v 10.7.3.1) software. Data were imported into Agilent GeneSpring software (v 7.3.1; Agilent Technologies) for subsequent quality control (QC) filtering and analysis. Data were normalised using the LOWESS (Locally Weighted polynomial regression) algorithm to balance for dye and signal intensity within and between arrays. Data were reimported into

GeneSpring as single-colour data to allow more flexible analysis. From the total dataset (55,708 probes), consistently low expressed genes were filtered out, leaving 39,049 probes with signal (>50) in at least one replicate. Comparisons were subsequently made between temperature treatments (27 and 37°C) for each cultivar by using volcano plots with threshold of a two-fold change and a Student's *t*-test (two-fold differences in expression level with a *P* value less than 0.05). Probes from the volcano filtering were clustered using a gene tree and Pearson correlation to generate a heat map. Unique and overlapping genes between the lists were selected using a Venn diagram. Sequences related to the array probes were obtained by a BLASTn search of the non-redundant nucleotide (nr/nt) databases against the National Centre for Biotechnology Information (NCBI; http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Real-time quantitative RT-PCR

Reverse transcription of a standard amount (1000 ng) of RNA per sample was performed using the QuantiTect® Reverse Transcription kit (Qiagen) including oligo d(T) and random hexamers as primers according to the manufacturer's instructions. The cDNA synthesised was diluted to a volume of 100 µL with sterile distilled water (10 ng μL^{-1}). Four candidate genes (plasma membrane protein, aquaporin, cysteine protein, and a major latex like protein; Table I) were selected for further study on the basis of showing a significant difference in expression levels between cultivars from the array experiment. Several reference genes for transcript normalization in *Rubus* were also selected from the literature (Czechowski *et al.*, 2005) and the stability of the expression profiles of the equivalent genes was examined from the array, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was selected as an appropriate internal control. Several sets of primers/probes were designed for both conventional PCR and real-time qRT-PCR using either Primer3 (Rozen and Skaletsky, 2000) or the UPL (Universal Probe Library) Assay Design Centre and recommended

160 parameters from Roche Diagnostics Ltd., UK (www.roche-applied-
161 science.com/shop/CategoryDisplay?catalogId=10001&tab=&identifier=Universal+Probe+Lib
162 rary&langId=-1).

163 Conventional PCR reactions were performed initially to confirm that each primer set
164 amplified a single product of the correct size and 1 μL cDNA (10 ng μL^{-1}) was added to a 24
165 μL master mix consisting of: 1 x Go Taq® Reaction Buffer (Promega, UK), 0.2 mM each
166 dNTP (Promega, UK), 0.3 μM each primer (Eurogentec Ltd., UK) and 1.0 Unit Go Taq®
167 DNA Polymerase (Promega, UK). The PCR amplification was based on an initial
168 denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s,
169 annealing at 60°C for 30 s, extension at 72°C for 30 s, and a final elongation at 72°C for 3
170 min using a Veriti™ 96-Well Thermal Cycler (Applied Biosystems Ltd., Warrington, UK).
171 PCR products (10 μL) were analysed on 2.0% agarose gels containing 0.5 μg mL^{-1} ethidium
172 bromide in 0.5x TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA) buffer.

173 In an effort to produce consistent and high quality ('gold standard') data from qPCR
174 studies, the minimum information for publication of quantitative real-time PCR experiments
175 (MIQE) guidelines recently published (Bustin *et al.*, 2009) were followed. qRT-PCR
176 reactions were performed with 2 μL cDNA (at 10 ng μL^{-1}) added to 23 μL FastStart
177 TaqMan®Probe Master mix (with Rox reference dye) and run on the automated ABI 7500
178 Fast Real-Time PCR System (Applied Biosystems, UK) using a standard 7500 run mode and
179 three-step cycle: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min.
180 Primers were included at an optimal final concentration of 900 nM per reaction, and UPL
181 probes used at 100 nM. In cases where UPL probes could not be used, the same
182 volumes/concentrations and cycle conditions were used with the Power SYBR® Green PCR
183 Master Mix (Applied Biosystems, UK) including melt curve analysis to detect any

nonspecific amplification. The PCR reactions were repeated in triplicate with independent cDNAs.

Relative gene expression levels were calculated by the method of Pfaffl (2001) to determine the relative quantification of a target gene in comparison to the reference gene (GAPDH). This method was necessary since the relative efficiencies of all assays were not equal, and the model incorporated the reaction efficiencies of both the candidate gene and reference gene assays. Real-time qRT-PCR efficiencies were calculated from the given slope value for each assay standard curve (C_T values *vs* log [cDNA]) (Vaerman *et al.*, 2004) by testing a pooled cDNA mixture (10 ng μL^{-1}) comprising of 'Autumn Bliss' and 'Erika' samples (exposed at 27 and 37°C) over a five-fold dilution series (20, 4, 0.8, 0.16, 0.032, and 0.0064 ng) under the PCR conditions described above. The corresponding PCR efficiency (E) was calculated according to the equation: $E = 10^{[-1/s]} - 1$; efficiencies between 80% and 110% are considered acceptable. All PCR assays subsequently used produced a standard curve within the dilution range with a high linearity (Pearson correlation coefficient, r values > 0.99). The amplification of single PCR products was also verified for a random selection of samples after each real-time qRT-PCR run by agarose gel electrophoresis. All relative gene expression levels were compared to the samples collected from 'Autumn Bliss' at 27°C and statistical analysis using General Analysis of Variance was performed by GeneStat 15.1 (GenStat V10 VSNi, Hemel Hempstead, UK).

Genomic DNA extraction, PCR and sequencing

Genomic DNA was extracted from fresh leaf material by the following optimized manual protocol for raspberry for eight raspberry cultivars ('Autumn Bliss', 'Autumn Treasure', 'Erika', 'Glen Ample', 'Glen Fyne', 'Octavia', 'Polka' and 'Tulameen') for DNA

208 sequencing. The DNA extraction buffer was prepared by dissolving 2 g CTAB (2%), 1.2 g
209 100 mM Tris-HCL (pH 8.0), 8.2 g 1.4 M NaCl and 0.74 g 20 mM EDTA (pH 8.0) in 100 mL
210 water and autoclaved. Dithiothreitol (DTT; Sigma-Aldrich Company Ltd., UK) (0.1%) was
211 immediately added to the buffer before commencing DNA extractions. Fresh leaf (1 g) was
212 ground with a mortar and pestle in liquid nitrogen and transferred to a 15 mL sterile tube. A
213 spatula tip of PVP (polyvinylpyrrolidone; Sigma-Aldrich Company Ltd., UK) followed by 5
214 mL of extraction buffer were added and mixed on a vortex before incubation at 65°C for 30
215 min. A 7.5 mL volume of chloroform/IAA (24:1) (Sigma-Aldrich Company Ltd., UK) was
216 added and samples were shaken for 15 min at room temperature followed by centrifugation
217 for 15 min at 2300 x g (4°C). The aqueous layer was filtered through muslin cloth and an
218 equal volume of ice cooled isopropanol was added before incubation for 15 min at room
219 temperature followed by 30 min at 0°C. DNA was pelleted by centrifuging for 15 min at 2300
220 x g (4°C) and re-suspended in 0.75 mL sterile water.

221 Various primer combinations (Table I) for candidate genes (i.e., plasma membrane
222 protein and aquaporin (TIP2)) were initially tested on DNA samples extracted from the eight
223 raspberry cultivars in order to generate products for sequencing for the identification of
224 sequence polymorphisms and associated markers for heat stress. Conventional PCRs were
225 performed with 100-150 ng μL^{-1} of gDNA as described previously and PCR products were
226 separated on a 2% agarose. Single PCR products were treated with ExoSAP-IT (USB®
227 Products Affymetrix, Inc., Ohio, USA) according to the manufacturer's instructions and
228 sequenced in both directions by Sanger sequencing. The primer sets successfully used for
229 generating products for sequencing were the combinations of PMPF1 and PMPR1 (product
230 size 475 bp) and Aqua1F1 and Aqua1R2 (product size 663 bp) for the plasma membrane
231 protein and aquaporin genes, respectively (Table I). Sequences were analyzed and edited
232 manually using Sequencher 4.9 (DNA Codes Corp., Ann Arbor, MI, USA) and aligned using

233 the ClustalW2 multiple sequence alignment program (www.ebi.ac.uk/Tools/msa/clustalw2/)
234 to identify any sequence polymorphisms.
235
236 *Linkage map construction of the aquaporin candidate genes (PIP1) and (TIP2)*
237 An updated marker map was produced to include both the aquaporin genes (PIP1 and
238 TIP2) using Join Map 3.0 (Van Ooijen and Voorrips, 2001) after PCR amplification (primers
239 Aqua1F1/Aqua1R1 and Aqua1F2/Aqua1R2, respectively; Table I) and scoring in the 188
240 individuals of the 'Latham' x 'Glen Moy' mapping population. Details of the map construction
241 are given in Graham *et al.* (2009).
242
243 RESULTS AND DISCUSSION
244 *Expression of microarray probe sets responsive to heat stress*
245 Gene expression variation in the harvested leaves of four annual-fruiting raspberry
246 cultivars in response to high temperatures was assessed using an Agilent *Rubus* 60 K
247 microarray with 55,708 probes. Initial quality control steps left 39,049 probes with signal
248 (>50) in at least one replicate for each sample. Statistical analyses (Student's *t*-test) using
249 volcano plots to combine strict selection criteria (two-fold change in expression level with a *P*
250 value < 0.05) was carried out between temperature treatments (27°C *vs* 37°C) for 'Autumn
251 Bliss' and 'Erika' due to the significant differences in chlorophyll fluorescence and
252 chlorophyll content between these cultivars when grown at 27 and 37°C (Gotame *et al.*,
253 2013). Since 'Autumn Treasure' and 'Erika' showed a similar physiological response to heat
254 stress which differed from the response in 'Autumn Bliss' and 'Polka', 'Autumn Treasure'
255 and 'Polka' were removed from further gene expression analysis. Moreover, 'Erika' and
256 'Autumn Bliss' were selected as they are genetically related; 'Erika' being a selection from
257 open-pollinated 'Autumn Bliss' (Nikki Jennings, personal communication). Pairwise *t*-tests

using volcano plots identified 427 differentially expressed genes for 'Autumn Bliss' and 229 genes for 'Erika', and revealed 12 candidate genes were common and down-regulated at 37°C compared with 27°C (Figure 1). This shared expression response may indicate that these two cultivars can have repression of the same set of genes, probably via common signalling pathways. This gene down-regulation seems to be a common adaptive response that enables plants to cope with new environmental conditions, possibly in order to conserve energy and used to activate heat tolerance responses.

Array probes from the volcano filtering step were clustered using a gene tree and Pearson correlation to generate a heat map to aid in the selection of differentially expressed candidate genes amongst the cultivars during heat treatment (Figure 2). From the heat map, a total of 644 genes were significantly differentially expressed between temperatures in at least one cultivar, and 'Erika' and 'Autumn Treasure' showed elevated expression of 38 genes compared to 'Autumn Bliss' and 'Polka' (Figure 3). Potential candidate genes were selected on the basis of specific gene expression patterns in the gene tree in a region of significantly changing profiles (Figure 2), and using the volcano filtering criteria, a total of 40 genes were significantly affected by high temperature exposure for 24 h and a graphical view of these expression profiles for each cultivar are represented in Figures 3 and 4. Among these probe sets showing differential expression during heat treatment, 38 were down-regulated in all cultivars and two genes (PIP1 and TIP2) were up-regulated in 'Polka', 'Autumn Treasure' and 'Erika' but not in 'Autumn Bliss' and there were significant differences in expression levels between treatments (supplementary file S1).

A BLASTn search of sequences closely related to the array probes indicated that the list of potential candidate genes belonged to different functional categories and included several examples each of major latex-like proteins (MLPs), plasma membrane proteins (PMPs), aquaporins and other stress related proteins (supplementary file S1). The list of

283 down-regulated genes contained many of the genes known to encode for metal binding
284 (metallothionein-like proteins), ubiquinone reduction (electron transfer flavoprotein-
285 ubiquinone oxido-reductase), flower development (latex-like proteins), solute transport
286 (PMPs) and Rubisco turn over in leaves (cysteine proteins). Moreover, based on current
287 knowledge, the 12 genes that were down-regulated in both cultivars (as identified by array
288 annotation) encode the same function, for example, major latex-like proteins (MLPs). The
289 MLPs are implicated specifically with fruit and flower development and in a pathogen
290 defence response (Lytle *et al.*, 2009). Similarly, genes encoding electron transfer
291 flavoprotein-ubiquinone oxidoreductase were also down-regulated. Electron transfer
292 flavoprotein-ubiquinone oxidoreductase links the oxidation of fatty acids and some amino
293 acids to oxidative phosphorylation in the mitochondria (Ishizaki *et al.*, 2005). It has been
294 reported that electron transfer flavoprotein-ubiquinone oxidoreductase catalyses the transfer
295 of electrons from electron transferring flavoprotein (ETF) to ubiquinone, reducing it to
296 ubiquinol. Oxidative phosphorylation involves the reduction of O_2 to H_2O with electrons
297 donated by NADH (i.e. transfers electrons from NADH to oxygen) and $FADH_2$ which
298 releases free energy and synthesises ATPs that finally completes the oxidation of sucrose
299 (Taiz and Zeiger, 2006). Metallothionein-like genes are metal binding, cysteine rich metal
300 binding proteins involved in metal ion detoxification (Cobbert and Goldsbrough, 2002) and
301 have also been implicated in the regulation of ATP production to reduce metal-induced
302 oxidative stress (Thomas *et al.*, 2005). Down-regulation of a zinc finger (C_3HC_4-type ring
303 finger) family protein gene was also observed at 37°C and also reported by Liu *et al.* (2012)
304 in grapevine with a B-box type zinc finger-containing protein and was also down regulated
305 during high temperature conditions. Moreover, a BLASTn search also showed five of the
306 genes responsive to the heat stress did not match any genes of known functions.

307 Interestingly, two aquaporin genes (PIP1 and TIP2) from the volcano list were down-
308 regulated in 'Autumn Bliss' but up-regulated in 'Autumn Treasure', 'Polka' and 'Erika'. The
309 plasma membrane aquaporin and aquaporin homologs are termed PIPs (plasma membrane
310 intrinsic proteins), whereas tonoplast aquaporin and aquaporin homologs are named TIPs
311 (tonoplast intrinsic proteins) (Johanson *et al.*, 2000; Johansson *et al.*, 2001). It was reported
312 that five aquaporin subfamilies were identified in plants based on DNA similarities.
313 Aquaporins facilitate the efficient transport of water molecules across membranes, play a role
314 in controlling intercellular water movement and facilitate passive exchange of water,
315 compatible solute distribution and gas transfer across membranes (Johanson *et al.*, 2001). A
316 few PIPs have also been reported to be involved in CO_2 permeability of cells. The plasma
317 membrane determines the internal mesophyll CO_2 conductance (g_m) due to the mesophyll
318 cells imposing resistance to CO_2 diffusion (as reviewed by Katsuhara *et al.*, 2008). Aharon *et*
319 *al.* (2003) also observed an increase in photosynthetic rate of transgenic tobacco plants up-
320 regulating *Arabidopsis* PIP1.
321 Expression of aquaporin isoforms are known to be affected by environmental stresses
322 (as reviewed by Jang *et al.*, 2007). Stress may either increase the transcription of aquaporin
323 genes or it may increase the activity of existing aquaporins. Studies have reported the
324 expression of aquaporin genes under a wide range of biotic and abiotic stresses including heat
325 and drought stresses (Hewezi *et al.*, 2008). For example, PIPs are involved in the regulation
326 of g_m as a rapid response in drought conditions (Flexas *et al.*, 2002). Jang *et al.* (2007)
327 reported that overexpression of PIP1;4 or PIP2;5 isoforms supressed plant growth under
328 drought stress but no effect was reported under normal conditions. Aharon *et al.* (2003) also
329 showed a negative role of an aquaporin during drought stress. Under heat stress at day time,
330 there is an increase in transpiration which induces water deficit in plants and causes a
331 decrease in leaf water potential (Tsukaguchi *et al.*, 2003). Changes in aquaporin expression

may also be related to the need to control water movement between storage tissues and rapidly growing and expanding tissues. Mazzitelli *et al.* (2007) observed a down-regulation of expressed sequence tags with similarity to an aquaporin gene in raspberry buds during the transition from endo- to para-dormancy. An influence of aquaporin expression on photosynthetic performance of plants was also reported (Aharon *et al.*, 2003; Flexas *et al.*, 2006). Aharon *et al.* (2003) performed chlorophyll measurement in tobacco plants overexpressing an *Arabidopsis* PIP1b gene and reported a positive correlation with expression of PIP1b and maximum quantum efficiency of dark adapted leaves. Flexas *et al.* (2006) also reported that *Nicotiana tabacum* L. aquaporin (NtAQP1) contributes to CO_2 conductivity of mesophyll cells (g_m) in tobacco. The effects of drought and heat stress on cereals are also interlinked and suggesting a common mechanism for heat, drought and other osmotic stress (Barnabas *et al*, 2008). Up- or down-regulation of aquaporins was reported to affect the leaf cell water permeability, water loss rate, stomatal conductance and overall leaf function (Heinen *et al.*, 2009). TIPs are a major component of the tonoplast and provide a quick equivalence of osmotic balance between cytosol and vacuolar lumen to prevent plasmolysis under hypertonic conditions (Katsuhara *et al.*, 2008). Down-regulation of TIPs may be related to storage of water in vacuoles in drought tolerant cultivars or to low water stress conditions and vice versa.

Real-time quantitative RT-PCR validation

In order to validate results obtained from the microarray experiment, specific qRT-PCR assays were designed for selected candidate genes representing different biological functions (Table I) and showing a relatively higher-fold change in expression. These assays were initially tested in the more genetically related but contrasting cultivars, 'Autumn Bliss' and 'Erika'. From the 40 selected array probe sets, the four candidate genes selected were:

357 plasma membrane protein, aquaporin (TIP2), cysteine protein and major latex like protein.

358 UPL Design Centre software (Roche) was used to design UPL probe-based assays for
359 real time qRT-PCR and ensure common thermal cycling parameters. Although all primer sets
360 successfully amplified a single product for all four candidate genes during conventional PCR
361 (Figure 5), the assays designed for the plasma membrane protein, cysteine protein and major
362 latex-like protein gene sequences all failed to produce a signal in the real-time qRT-PCR
363 format. Further investigation using outer primer sets (Table I) and sequencing of products for
364 each assay and cultivar revealed sequence variation in the region of the UPL probe design that
365 explain the absence of a signal (data not shown). Moreover, the microarray probes were
366 designed to a single *Rubus* cultivar 'Glen Moy' and since distantly related genotypes were
367 used in this study, this explains the absence of an amplification signal. This was not the case
368 for the aquaporin or the GAPDH reference gene assays which worked successfully with a
369 UPL probe in the real-time qRT-PCR format. Additional optimisation with the SYBR Green
370 master mix (see materials and methods) was subsequently tested in order to use the same
371 primer sequences and this was successful for the plasma membrane protein gene, but not for
372 the cysteine and major latex-like protein genes; the latter two assays failed to operate
373 efficiently (E value < 65%) and were removed from the validation procedure.

374 This highlighted the need to be aware that the occurrence of small sequence variation
375 amongst different raspberry genotypes can result in failure to detect signals in the real-time
376 qRT-PCR format. The occurrence of SNPs or indels in annealing regions of primers or probes
377 may hamper efficient annealing of the primer/probe or prevent amplification of a variant
378 allele.

379 Normalized gene expression levels of aquaporin (TIP2) and the plasma membrane
380 protein in 'Autumn Bliss' and 'Erika' grown at 27 and 37°C from real-time qRT-PCR were
381 calculated by the method of Pfaffl, (2001). This determined the relative quantity of the

382 candidate gene in comparison to the GAPDH reference gene for normalization of the data.
383 The normalized expression levels of the aquaporin (TIP2) increased by 1.3-fold in 'Autumn
384 Bliss' and three-fold in 'Erika' grown at 37°C compared to 27°C. However, in contrast, the
385 expression levels of the plasma membrane protein gene decreased by two-fold in 'Autumn
386 Bliss' and three-fold in 'Erika' grown at 37°C compared to 27°C (Figure 6). Analysis of
387 variance of the data of qRT-PCR analysis revealed that 'Autumn Bliss' and 'Erika' were
388 significantly different in expression of the two genes ($p<0.05$) and the interaction between
389 candidate genes and temperature was significant.
390 These aquaporin (TIP2) and plasma membrane protein candidate genes were initially
391 studied due to a significant differential expression in 'Autumn Bliss' and 'Erika' detected
392 from the array analysis. This microarray assessment of differential gene expression was
393 subsequently compared and validated with the alternative technique of real time qRT-PCR,
394 and there was agreement by both procedures apart from the expression signals of aquaporin
395 (TIP2) in 'Autumn Bliss' at 27 and 37°C (Figure 6). This slight discrepancy may be explained
396 by the different normalization procedures and/or the measurement of different but related
397 aquaporin genes in 'Autumn Bliss' by both methods. Although the magnitude of fold-change
398 differed in both 'Autumn Bliss' and 'Erika', the direction of fold-change (down-regulated)
399 was similar in PMPs (Figure 6). The differences in heat tolerance in 'Autumn Bliss' and
400 'Erika' may be associated with multiple processes and mechanisms involving heat response
401 proteins, transcription factors and other stress related genes.
402
403 *Candidate gene mapping and sequencing to identify heat stress markers*
404 An updated linkage group map was produced to include both the aquaporin genes
405 (PIP1 and TIP2) after scoring in the 188 individuals of the 'Latham' x 'Glen Moy' mapping
406 population (Figure 7). Both aquaporin genes mapped at almost identical positions on linkage

407 group 3 of the 7 *Rubus idaeus* groups, indicating that these related genes in terms of sequence
408 homology (60%) may also be members of the same gene family. Linkage group 3 contains
409 many genes associated with fruit quality traits such as flavour, colour, softening and ripening
410 (Graham *et al.*, 2009), and we can speculate that the aquaporin genes may play an additional
411 role in fruit quality as well as for a heat stress response.

412 Sequences generated from the PCR products of two heat stress candidate genes (i.e.,
413 plasma membrane protein and aquaporin (TIP2)) were successfully obtained from the eight
414 raspberry cultivars for the identification of sequence polymorphisms and potential markers for
415 heat stress. However, although sequence polymorphisms (indels or SNPs) were observed in
416 the portion of both gene sequences examined after alignment (data not shown), none were
417 associated on this occasion with either the heat tolerant or susceptible groupings for the eight
418 raspberry cultivars. It is clear that an additional portion of both candidate gene sequences will
419 need to be examined in order to identify potential heat stress polymorphic markers and further
420 validation in additional germplasm, which was outside the scope of this study.
421 Overall, we report the first use of a *Rubus* array to analyse high-throughput gene expression to
422 determine relative abundance of nucleic acid sequences expressed among heat stressed
423 raspberry cultivars. The microarray revealed that a major response of rapsberry genes to high
424 temperature for a short period involved down-regulation of many defence related genes and
425 up-regulation of aquaporin related genes. This research used the latest generation of molecular
426 tools to increase the understanding and identify potential candidate genes involved in the heat
427 stress response in annual-fruiting raspberries which merits further research.

428 We gratefully acknowledge the financial support from Interreg IVB North Sea Region
429 Programme Project ID: 35-2-05-09 ('ClimaFruit') to carry out this experiment. We

430 acknowledge to J. McNicol, Biomathematics & Statistics Scotland BioSS Office, Scotland,
431 UK) for data analysis.
432
433 REFERENCES
434 AHARON, R., SHAHAK, Y., WININGER, S., BENDOV, R., KAPULNIK, Y. and GALILI,
435 G. (2003). Overexpression of a plasma membrane aquaporin in transgenic tobacco
436 improves plant vigour under favorable growth conditions but not under drought or salt
437 stress. *The Plant Cell*, **15**, 439-447.
438 AHARONI, A. and O'CONNEL, A. P. (2002). Gene expression analysis of strawberry achene
439 and receptacle maturation using DNA microarrays. *Journal of Experimental Botany*,
440 **53** , 2073-2087.
441 AHUJA, I., DE VOS, R. C. H., BONES, A. M. and HALL, R. D. (2010). Plant molecular
442 stress responses face climate change. *Trends in Plant Science*, **15**, 664-674.
443 BALLINGTON, J. R. and FERNANDEZ, G. E. (2008). Breeding raspberries adapted to
444 warm humid climate with fluctuating temperature in winter. *Acta Horticulture*, **777**,
445 87-90.
446 BARNABAS, B., JAGER, K. and FEHER, A. (2008). The effect of drought and heat stress
447 on reproductive processes in cereals. *Plant Cell and Environment*, **31**, 11-38.
448 BERRY, J. and BJORKMAN, O. (1980). Photosynthetic response and adaptation to
449 temperature in higher plants. *Annual Review of Plant Physiology*, **31**, 491-543.
450 BUSTIN, S. A., BENES, V., GARSON, J. A., HELLEMANS, J., HUGGETT, J., KUBISTA,
451 M., MUELLER, R., NOLAN, T., PFAFFL, M. W., SHIPLEY, G. L.,
452 VANDESOMPELE, J. and WITTWER, C. T. (2009). The MIQE guidelines:
453 Minimum information for publication of quantitative real-time PCR experiments.
454 *Clinical Chemistry*, **55**, 611-622.

455 CHEN, X., NIKS, R. E., HEDLEY, P. E., MORRIS, J., DRUKA, A., MARCEL, T. C.,
456 VELS, A. and WAUGH, R. (2010). Differential gene expression in nearly isogenic
457 lines with QTL for partial resistance to *Puccinia hordei* in barley. *BMC Genomics*, **11**,
458 629 pp.
459 COBBERT, C. and GOLDSBROUGH, P. (2002). Phytochelatins and metallothioneins: roles
460 in heavy metal detoxification and homeostasis. *Annual Review of Plant Biology*, **53**,
461 159-182.
462 CZECHOWSKI, T., STITT, M., ALTMANN, T., UDVARDI, M. K and SCHEIBLE, W. R.
463 (2005). Genome wide identification and testing of superior reference genes for
464 transcript normalization in *Arabidopsis*. *Plant Physiology*, **139**, 5-17.
465 FLEXAS, J., BOTA, J., ESCALONA, J. M., SAMPOL, B. and MEDRANO, H. (2002).
466 Effects of drought on photosynthesis in grapevine under field conditions: an
467 evaluation of stomatal and mesophyll limitations. *Functional Plant Biology*, **29**, 461-
468 471.
469 FLEXAS, J., RIBAS-CARBO, M., HANSON, D. T., BOTA, J., OTTO, B., CIFRE, J.,
470 MCDOWELL, N., MEDRANO, H. and KALDENHOFF, R. (2006). Tobacco
471 aquaporin NtAQP1 is involved in mesophyll conductance to CO_2 in vivo. *Plant*
472 *Journal*, **48**, 427-439.
473 FRANK, G., PRESSMAN, E., OPHIR, R., ALTHAN, L., SHAKED, R., FREEDMAN, M.,
474 SHEN, S. and FIRON, N. (2009). Transcriptional profiling of maturing tomato
475 (*Solanum lycopersicum* L.) microspores reveals the involvement of heat shock
476 proteins, ROS scavengers, hormones, and sugars in the heat stress response. *Journal of*
477 *Experimental Botany,* **60**, 3891-3908.
478 GINZBERG, I., BAREL, G., OPHIR, R., TZIN, E., TANAMI, Z., MUDDARANGAPPA, T.,
479 DE JONG, W. and FOGELMAN, E. (2009). Transcriptomic profiling of heat-stress

response in potato periderm. *Journal of Experimental Botany*, **60**, 4411-4421.

GOTAME, T. P., ANDERSEN, L., PETERSEN, K. K., PEDERSEN, H. L., OTTOSEN, C.-O. and GRAHAM, J. (2013). Chlorophyll fluorescence and flowering behaviour of annual-fruiting raspberry cultivars under elevated temperature regimes. *European Journal of Horticulture Science,* **78**, 193-202.

GRAHAM, J. and JENNINGS, S. N. (2009). Raspberry breeding. (JAIN, S. M. and PRIYADARSHAN, M., Eds.). *Breeding Tree Crops*. IBH and Science Publication, Oxford, UK, 233-248.

GRAHAM, J., HACKETT, C. A., SMITH, K., WOODHEAD, M., HEIN, I. and McCALLUM, S. (2009). Mapping QTL for developmental traits in raspberry from bud break to ripe fruit. *Theoretical and Applied Genetics*, **118**, 1143-1155.

GRAHAM, J., HEIN, I. and POWELL, W. (2007). Raspberry. *Genome mapping and molecular breeding in plants.* (KOLE, C., Ed.). Springer-Verlag Berlin Heidelberg, 207-216.

GULEN, H. and ERIS, A. (2004). Effect of heat stress on peroxidase activity and total protein content in strawberry plants. *Plant Science*, **166**, 739-744.

HEDLEY, P. E., JOANNE, R. R., JORGENSEN, L., GORDON, S., MORRIS, J. A., HACKETT, C. A., CARDLE, L. and BRENNAN, R. (2010). Candidate genes associated with bud dormancy release in blackcurrant (*Ribes nigrum* L.). *BMC Plant Biology*, **10**, 202-215.

HEINEN, R. B., YE, Q. and CHAUMONT, F. (2009). Role of aquaporins in leaf physiology. *Journal of Experimental Botany*, **60**, 2971-2985.

HEWEZI, T., LEGER, M. and GENTZBITTEL, L. (2008). A comprehensive analysis of the combined effects of high light and high temperature stresses on gene expression in sunflower. *Annals of Botany*, **102**, 127–140.

ISHIZAKI, K., LARSON, T. R., SCHAUER, N., FERNIE, A. R., GRAHAM, I. A. and LEAVER, C. J. (2005). The critical role of *Arabidopsis* electron-transfer flavoprotein: ubiquinone oxido-reductase during dark-induced starvation. *The Plant Cell,* **17**, 2587-2600.

JANG, J. Y., LEE, S. H., RHEE, J. Y., CHUNG, G. C., AHN, S. J. and KANG, H. (2007). Transgenic *Arabidopsis* and tobacco plants overexpressing an aquaporin respond differently to various abiotic stresses. *Plant Molecular Biology*, **64**, 621-632.

JOHANSON, U., KARLSSON, M., JOHANSSON, I., GUSTAVSSON, S., SJOVALL, S., FRAYSSE, L., WEIG, A. R. and KJELLBOM, P. (2001). The complete set of genes encoding major intrinsic protein in Arabidopsis provides a framework for a new nomenclature for major intrinsic proteins in plants. *Plant Physiology*, **126**, 1358-1369.

JOHANSSON, I., KARLSSON, M., JOHANSON, U., LARSSON, C. and KJELLBOM, P. (2000). The role of aquaporins in cellular and whole plant water balance. *Biochimica et Biophysica Acta*, **1465**, 324-342.

KATSUHARA, M., HANBA, T. Y., SHIRATAKE, K. and MAESHIMA, M. (2008). Expanding roles of plant aquaporins in plasma membranes and cell organelles. *Functional Plant Biology*, **35**, 1-14.

LIU, G. T, WANG, J. F., CRAMER, G., DAI, Z. W., DUAN, W., XU, H.-G. WU, B.-H. FAN, P.-G. WANG, L.-J. and LI, S.-H. (2012). Transcriptomic analysis of grape (*Vitis vinifera* L.) leaves during and after recovery from heat stress. *BMC Plant Biology,* **12**, 174-183.

LYTLE, B. L., SONG, J., DE LA CRUZ, N. B., PETERSON, F. C. JOHNSON, K. A., BINGMAN, C. A., PHILLIPS, G. N. and VOLKMAN, B. F. (2009). Structures of two *Arabidopsis thaliana* major latex proteins represent novel helix-grip folds. *Proteins,* **76**, 237-243.

MAZZITELLI, L., HANCOCK, R. D., HAUPT, S., WALKER, P. G., PONT, S. D. A., MCNICOL, J., CARDLE, L., MORRIS, J., VIOLA, R., BRENNAN, R., HEDLEY, P. E. and TAYLOR, M. A. (2007). Co-ordinated gene expression during phases of dormancy release in raspberry (*Rubus idaeus* L.) buds. *Journal of Experimental Botany*, **58**, 1035–1045.

MITTLER, R., FINKA, A. and GOLOUBINOFF, P. (2012). How do plants feel the heat? *Trends in Biochemical Sciences*, **37**, 118-125.

PFAFFL, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research*, **29**, 45 pp.

ROZEN, S. and SKALETSKY, H. J. (2000). Primer3 on the www for general users and for biologist programmers. *(*KRAWETZ, S. and MISENER, S., Eds.). Bioinformatics Methods and Protocols*: Methods in Molecular Biology,* Humana Press, Totowa, NJ, 365-386.

SLONIM, D. K. and YANAI, I. (2009). Getting started in gene expression microarray analysis. *PLoS Computational Biology*, **5**, 1-4.

SONSTEBY, A. and HEIDE, O. M. (2009). Effects of photoperiod and temperature on growth and flowering in the annual (primocane) fruiting raspberry (*Rubus idaeus* L.) cultivar 'Polka'. *Journal of Horticulture Science and Biotechnology*, **84**, 439-446.

TAIZ, L. and ZEIGER, E. (2006). *Plant physiology*. Sinauer Associates, Inc., Publicers, Massachusetts. 682-688.

THOMAS, J. C., PERRON, M., LA ROSA, P. C. and SMIGOCKI, A. C. (2005). Cytokinin and the regulation of a tobacco metallothionein-like gene during copper stress. *Physiologia Plantarum*, **123**, 262-271.

TSUKAGUCHI, T., KAWAMITSU, Y., TAKEDA, H., SUZUKI, K. and EGAWA, Y. (2003). Water status of flower buds and leaves as affected by high temperature in heat

555 tolerant and heat sensitive cultivars of snap bean (*Phaseolus vulgaris* L.). *Plant*
556 *Production Science*, **6**, 4-27.
557 UPL. (2012). Universal Probe Library Assay Design Centre. [https://www.roche-
558 appliedscience.com/sis/rtpcr/upl/index.jsp?id=UP030000: retrieved on 29.07.2012.
559 VAERMAN, J. L., SAUSSOY, P. and INGARGIOLA, I. (2004). Evaluation of real-time
560 PCR data. *Journal of Biological Regulators and Homeostatic Agents*, **18**, 212-214.
561 VAN OOIJEN, J. W. and VOORRIPS, R. E. (2001). JoinMap version 3.0: software for the
562 calculation of genetic linkage maps. *Plant Research International*, Wageningen, The
563 Netherlands.
564 WAHID, A., GELANI, S., ASHRAF, M. and FOOLAD, M. R. (2007). Heat tolerance in
565 plants: An overview. *Environmental and Experimental Botany*, **61**, 199-223.
566 YANG, K. A., LIM, C. J., HONG, J. K., PARK, C. Y., CHEONG, Y. H., CHUNG, W. S.,
567 LEE, K. O., LEE, S. Y., CHO, M. J. and LIM, C. O. (2006). Identification of cell wall
568 genes modified by a permissive high temperature in Chinese cabbage. *Plant Science*,
569 **171**, 175-182.

570