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Ethylene and 1-MCP differentially regulate gene expression during onion (*Allium cepa* L.) sprout suppression[1]

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ABSTRACT

Onion is regarded as a non-climacteric vegetable. In onions, however, ethylene can suppress sprouting while the ethylene binding inhibitor, 1-MCP (1-methylcyclopropene) can also suppress sprout growth yet, it is unknown how ethylene and 1-MCP elicit the same response. In this study, onions were treated with 10 μL L^{-1} ethylene or 1 μL L^{-1} 1-MCP individually or in combination for 24 h at 20°C before or after curing (six weeks) at 20 or 28°C then stored at 1°C. Following curing, a subset of these same onions was stored separately under continuous air or ethylene (10 μL L^{-1}) at 1°C

Onions treated with ethylene and 1-MCP in combination after curing for 24 h had reduced sprout growth as compared with the control 25 weeks after harvest. Sprout growth following storage beyond 25 weeks was only reduced through continuous ethylene treatment. This observation was supported by a higher proportion of down-regulated genes characterised as being involved in photosynthesis measured using a newly developed onion microarray. Physiological and biochemical data suggested that ethylene was being perceived in the presence of 1-MCP since sprout growth was reduced in onions treated with 1-MCP and ethylene applied in combination but not when applied individually. A cluster of probes representing transcripts up-regulated by 1-MCP alone but down-regulated by ethylene alone or in the presence of 1-MCP support this suggestion. Ethylene and 1-MCP both down-regulated a probe tentatively annotated as an ethylene receptor as well as *EIN3*, suggesting that both treatments down-regulate the perception and signalling events of ethylene.

INTRODUCTION

Onion (*Allium cepa* L.) is traditionally classified as non-climacteric (Downes et al., 2010). Both ethylene and 1-MCP have been shown to inhibit sprout growth (Chope et al., 2007a; Bufler, 2009), which decreases bulb quality. Onion quality is dependent on the rate of internal sprout growth during storage. To eliminate the use of artificial chemicals, such as maleic hydrazide, the use of the plant growth regulator (PGR), ethylene, has been found to reduce sprout growth in onions when applied continuously throughout storage (10-15 µL L^{-1}). Bufler (2009) found 'Copra' onions held in continuous ethylene (10.6 µL L^{-1}) had reduced sprout growth compared with those held in air. Surprisingly, treatment with 1-MCP for 24 h after curing (six weeks at 28°C prior to cold storage) reduced sprout growth in SuperSweet1 (SS1) onions when stored at 4 or 12°C (Chope et al., 2007a). Although ethylene and 1-MCP have both been shown to reduce sprout growth, biochemical and physiological responses to each stimulus differ (Downes et al., 2010). Ethephon is an ethylene yielding chemical which, when applied directly to plants, can elicit a response characteristic of ethylene treatment (Yang, 1969; Warner and Leopold, 1969). Application of Ethephon to onion plants two weeks prior to harvest was found to reduce sprout incidence by 5% after 32 weeks storage at 0°C, however no significant reduction in rooting was observed (Adamicki, 2005). Unlike Ethephon treatment, continuous ethylene exposure has been found to increase shelf-life after 14 days at 20°C (Adamicki, 2005; Johnson, 2006). The combination of ethylene and 1-MCP has not been investigated in onion although it has in potato (Prange et al., 2005).

Onions are in the order Asparagales, which possess some of the largest genomes of the eukaryotes, especially in the genus *Allium* (Kuhl et al., 2004). Onion is diploid and comprises a large nuclear genome of 16,415 Mbps (over 5 times that of the human genome) spread over eight chromosomes (Havey et al., 2008; NCBI, 2008). To date, the large size has hindered plans to sequence the onion genome, however, 20,180 expressed sequence tags (ESTs) are available, mainly from a cross of inbred cultivars, 'Bringham Yellow Globe 15-23 (BYG)' x 'Alisa Craig 43 (AC)' (NCBI, 2008). These ESTs have been used to develop the first onion microarray. Although literature exists on the effect of ethylene and 1-MCP on climacteric fruits and vegetables at the molecular level, the mechanisms by which exogenously applied ethylene and 1-MCP suppress sprout growth in onions are still unknown.

Here we present novel transcriptional profiles, biochemical and physiological analyses of onions in response to short 24 h ethylene and/or 1–MCP treatments prior to storage, with or without the addition of long-term continuous ethylene during storage.

RESULTS

Ethylene and 1-MCP Treatments Reduce Sprout Development

After harvest, onions were subjected to treatment with ethylene, 1-MCP or ethylene and 1-MCP either before or after curing at either 20°C or 28°C. After curing, bulbs were placed in cold storage (1°C), with a subset of bulbs being stored under continuous ethylene supply (Figure 1). Average sprout growth at 25 and 35 weeks was 29 and 58 % of bulb height, respectively, with curing temperature affecting sprout length after 25 weeks only. Onions cured at 20°C had a mean sprout length of 38 % of bulb height whereas those cured at 28°C were 20 % of bulb height 25 weeks after harvest (Table 1). Differences between treatments were only observed after 25 weeks with the most significant reductions in sprout growth due to ethylene and 1-MCP in combination before (19 % of bulb height) or after (12 % of bulb height) curing compared to the control bulbs (45 % of bulb height). In addition, onions treated with only 1-MCP before (23 % of bulb height) or after (31 % of bulb height) curing had shorter sprouts than the control.

Interactions between pre-storage treatments and continuous storage treatments were observed in onions cured at 20°C only. Ethylene treatment throughout storage reduced sprout growth (43 % of bulb height) compared with controls held in air (59 % of bulb height) irrespective of pre-storage treatments. Nevertheless, mean sprout length of onions pre-treated with combined ethylene and 1-MCP treatment *before* curing was even shorter at 29 % of bulb height (Table 2). In contrast, onions treated with ethylene and 1-MCP *after* curing, and then treated continuously with ethylene, had longer sprouts (64 % of bulb height), yet those continuously stored in air had shorter sprouts at 38 % of bulb height (Table 2).

Continuous Supply of Ethylene During Storage Reduces Root Development

There was no main effect of curing temperature or treatment on rooting; however, the interactions between treatment and curing temperature were significant

(P = 0.028). The percentage of bulbs with roots was only significantly lower in onions treated with ethylene after curing at 28°C. However, several treatments resulted in a higher percentage of onions with roots, including bulbs treated with ethylene before curing at 20°C, bulbs treated with ethylene and 1-MCP after curing at 28°C, bulbs treated with ethylene and 1-MCP before curing at 20°C and bulbs treated with 1-MCP alone before curing at 20°C (data not shown).

The continuous supply of ethylene during storage reduced the incidence of rooting (18 %) compared to control bulbs stored in air (63 %). Less rooting was also observed in bulbs cured at 20°C (29 %) compared with 28°C (51 %). Onions treated with ethylene and 1-MCP after curing at 20°C then stored in air had no rooting (Table 3); this treatment regime also had an inhibitory effect on sprout growth (Table 2). Onions cured at 20°C then stored in continuous ethylene had almost no rooting irrespective of the pre-storage treatment. Onions cured at 28°C then stored in continuous ethylene had more rooting but this was absent in onions pre-treated with ethylene and 1-MCP before curing (Table 3).

Curing Onions Reduces Respiration

Respiration rate was measured throughout storage in onions stored in the UK only. Onion respiration rate was affected by curing with a 6-fold decrease over six weeks. Before curing, control bulbs had the lowest respiration rate and bulbs treated with ethylene had the highest respiration rate with onions treated with 1-MCP alone or in combination with ethylene lying between the two, however, this was not quite significant (Figure 2). Control bulbs cured at 20°C had the lowest respiration rate at the end of storage, compared with control bulbs cured at 28°C, which have the highest respiration rate. Treatments applied before curing at 28°C had higher respiration rates than bulbs treated after curing at 28°C.

Treatment with Ethylene and/or 1-MCP Does Not Affect Bulb Dry Matter

Onion bulb dry weight was not affected by pre-storage treatments in the onions stored in air. However, onion dry weight was affected by curing temperature and time. There was no change in dry weight of onions cured at 20°C throughout storage, but those cured at 28°C had higher dry weight before curing (116 mg g^{-1} FW), than the

mean value of all post-cured onions (110 mg g^{-1} FW). No significant differences in dry weight were found between pre-storage treatments (ethylene and/or 1-MCP), storage treatments (continuous ethylene/air) or curing temperatures (20 or 28°C) in the onions stored in continuous ethylene treatment.

Curing Temperature and Post-Curing Treatments Alter Carbohydrate Concentrations

Non-structural carbohydrates were measured in all samples to assess the impacts of treatments on carbohydrate metabolism during curing and storage. Glucose content of onions treated before curing with combined ethylene and 1-MCP was lower throughout storage, yet by 25 weeks glucose had increased in line with the control. This lower glucose content in onions treated with ethylene and 1-MCP before curing was also observed in fructose but only at 17 weeks after harvest. Onions treated after curing had higher sucrose content at 25 weeks (254 mg g^{-1} DW) than those treated before curing (236 mg g^{-1} DW), and the control (212 mg g^{-1} DW). This trend was also observed in the onions glucose content coinciding with the initiation of sprout growth. Fructose content tended not to vary much between treatments, but at the end of storage onions treated with ethylene after curing had lower fructose content (145 mg g^{-1} DW) than the control (190 mg g^{-1} DW) (Figure 3). All other treated onions had lower fructose content compared with the control at the end of storage however, those treated with ethylene after curing were the only onions with lower fructose content.

Sucrose and total fructans were the only non-structural carbohydrates affected by curing temperature (data not shown). Onions cured at 28°C had higher sucrose content (206 mg g^{-1} DW) but lower total fructans (187 mg g^{-1} DW) than those cured at 20°C (188 and 203 mg g^{-1} DW, respectively). The lower content of total fructans in onions cured at 28°C was due to lower nystose, DP5 and DP6 content. Sucrose and total fructan content was 1.2-fold and 1.5-fold higher, respectively after 25 weeks, in onions treated in combination with ethylene and 1-MCP after curing. This peak was also observed in onions treated with 1-MCP after curing but only contained higher total fructans (1.5-fold increase) not higher sucrose content. Notably, the difference in total fructan content between treatments was due to the largest fructans DP6 – DP8 (Figure 3). This peak in total fructans at 25 weeks in onions treated with ethylene and 1-MCP after curing or 1-MCP after curing did subsequently decrease by almost half during the final 10 weeks in storage. In contrast, an increase in total fructans was

observed in the control onions or onions treated before curing or with ethylene after curing in the final 10 weeks of storage. It was difficult to compare the biochemical carbohydrate data with gene expression profiles as very few genes classified as being involved in carbohydrate metabolism were differentially regulated in response to the treatments. Only one gene classified as being involved in carbohydrate metabolism was differently regulated in response to the short 24 h treatments. Cellulose synthase-like family C (CSLC9) was down-regulated in response to 1-MCP in the presence and absence of ethylene.

Ethylene and 1-MCP Elicit Unique Transcriptional Profiles

An onion microarray was utilised to characterise the transcriptional profiles of onions subjected to ethylene and/or 1-MCP treatments before and after curing, and continuously treated with ethylene during storage (Figure 1). In total, 1,228 probes representing transcripts with differential changes in expression were observed in response to ethylene and/or 1-MCP treatment as compared with the control. These probes were clustered into nine groups depending on their degree of response to each stimulus. Six of the clusters (Clusters 1, 2, 3, 4, 5 and 7), representing 1,048 probes, had similar expression profiles across all pre-curing treatments (Figure 4). The remaining 180 probes were divided into three clusters (Clusters 0, 6 and 8), which showed differential expression when treated with ethylene and/or 1-MCP (Figure 4). Cluster 0 represented 71 probes, including gibberellin 20 oxidase 2, whose transcript abundance was lower in onions treated with ethylene or 1-MCP alone, but no change was observed in their abundance in onions treated with ethylene and 1-MCP together. Cluster 6 represented 87 probes whose transcript abundance was lower in onions treated with 1-MCP whether in the presence of ethylene or not, including the gibberellin receptor GID1L2 and CSLC9. Finally, cluster 8 included 22 probes whose transcript abundance was lower in onions treated with ethylene irrespective of whether 1-MCP was present or not. These included precursors for expansin and a protease inhibitor/seed storage/LTP family protein.

Probes were classified into functional categories (Table 4) based on their similarity to rice protein sequence database. Although probes representing transcripts characterised as being related to PGRs included those associated with auxins, cytokinins and ethylene (Table S1), the only PGR probes that were differentially expressed between ethylene and 1-MCP treatments were gibberellin receptors and

gibberellin oxidase. Table 5 details the 30 most up- or down-regulated probes after short treatment pre- or post-curing with ethylene and/or 1-MCP treatments. A gene that appeared twice in the 30 most up- or down-regulated genes in response to treatment was 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) which was up-regulated in response to ethylene alone or in combination with 1-MCP applied before curing.

The abundance of transcripts represented by 574 probes were differentially regulated in response to continuous ethylene storage, with 272 having greater abundance and 302 having less abundance than the control treatment (Figure 5). Functional characterization of these probes revealed that a relatively large proportion of the down-regulated genes were classified as involved in photosynthesis (6.6 %), whereas only 0.4% of upregulated genes were in this class. Interestingly, the transcript abundances of several probes related to PGRs were greater under continuous ethylene, including gibberellin 2-beta-dioxygenase, ethylene-insensitive 3 (*EIN3*) (Table S1), auxin-responsive gene family member and importantly 1-aminocyclopropene-1-carboxylate oxidase (*ACO*) (Table S1). Other probes annotated as being related to PGRs, revealed the down regulation of an auxin efflux carrier component and a cytokinin dehydrogenase by continuous ethylene. To test the sensitivity and consistency of the microarray analysis, the expression of five probes was determined using quantitative real time PCR. There was a qualitative relationship between the qPCR and microarray data, with a correlation coefficient of $r = 0.68$ ($P < 0.001$; Figure 6), confirming the data from the microarray presented an accurate indication of transcript abundances in the onion samples.

DISCUSSION

Onions were treated before or after curing (6 weeks at 20 or 28°C) with 24 h treatments of ethylene and/or 1-MCP before being transferred to cold storage at Cranfield University (CU). A subset of these onions was removed following pre-curing treatment and six weeks curing for cold storage in continuous air or ethylene (Figure 1). Biochemical, physiological and molecular techniques were used to identify the most successful methods of onion sprout suppression and the transcriptomic changes that occurred following each treatment to help identify possible mechanisms for sprout suppression.

Sprout Suppression Through Exogenous Ethylene Down-Regulates Photosynthesis Related Genes

Onions treated with ethylene and 1-MCP after curing had the shortest sprout length after 25 weeks in storage and this was also found in the subset of onions stored in continuous air 35 weeks after harvest. The shorter sprout growth in onions treated with combined ethylene and 1-MCP after curing was also supported by the reduced utilisation of sucrose and the larger fructooligosaccharides; DP6, DP7 and DP8 that were higher at 35 weeks (Figure 3). Treatment with ethylene and 1-MCP after curing resulted in no root growth in the onions held in continuous air. Although treatment with ethylene and 1-MCP before curing and 1-MCP before curing at 28°C also resulted in shorter sprouts, this was not consistent with the subset of onions stored separately. This said, onions treated with ethylene and 1-MCP in combination before curing and 1-MCP before curing had reduced expression of a probe annotated as coding for the protein cellulose synthase-like family 9 (CSLC9) following treatment. This protein is involved in cell wall polysaccharide synthesis therefore the down regulation of this protein in response to the above mentioned treatments may have played a role in the reduction of sprout growth by suppressing the production of new growth.

Comparison between onions stored in continuous air or ethylene revealed those stored in ethylene had reduced sprout growth, which is consistent with similar previous work (Bufler, 2009). Reduced sprout growth in response to continuous ethylene storage was also supported by the microarray data. Onions treated with continuous ethylene had a higher proportion of down-regulated probes characterised as being involved in photosynthesis compared with onions stored in air. This suggests the onions stored in ethylene had not yet reached the advanced stages of sprouting when the growing sprout becomes green. The question remains whether the down regulation of probes characterised as being involved in photosynthesis in onions stored in continuous ethylene are a direct result of the ethylene or a result of the slowed sprout development. Immediately after treatment with ethylene for 24 h before curing, and therefore prior to sprout growth, only 17 probes characterised as being involved in photosynthesis were found to be differentially regulated as compared to the control. Nine of these genes were down-regulated and eight were up-regulated (0.7 and 0.6% of the total genes differentially regulated, respectively). This suggests that the greater proportion of down-regulated photosynthesis related probes following continuous

ethylene treatment are more likely to be due to the delay in sprout development rather than ethylene itself. That said, molecular response to a short 24 h treatment before curing and extended continuous ethylene treatment throughout storage are likely to differ therefore further investigation is required.

Ethylene is Perceived in the Presence of 1-MCP in Onion

Short treatments with ethylene and 1-MCP individually have both been shown to reduce sprout growth in onion (Chope et al., 2007a; Downes et al., 2010), although no work has investigated the effect of both ethylene and 1-MCP applied together. In potato 'Russet Burbank' tubers, 1-MCP has been used to reduce the reported detrimental effect of ethylene on fry colour darkening; 1-MCP did not interfere with ethylene-induced sprout suppression, and ethylene did not cause such a dark fry colour when tubers were pre-treated with 1-MCP (Prange et al., 2005). Prange et al. (2005) hypothesised that the 1-MCP may bind to the ethylene receptors and that the continuous ethylene then regulates sprout growth by binding to newly formed ethylene receptors in the sprout eyes where mitotic activity is highest. It is possible that at these sites of high mitotic activity (e.g. potato eyes), in addition to the production of new ethylene receptors, greater 1-MCP metabolism may occur since Huber et al., (2010) suggested that 1-MCP may be metabolised *in planta*.

In this study, treatment of onions with ethylene and 1-MCP together resulted in higher sucrose and fructan concentrations than found in those treated with ethylene alone (Figure 3). Also, sprout growth was reduced in onions treated with combined ethylene and 1-MCP but not in those treated with each compound separately. This study therefore suggests that ethylene and 1-MCP applied simultaneously for 24 h affects onion physiology and biochemistry differently than when applied individually, suggesting that ethylene and 1-MCP are both eliciting a response. This may be a consequence of different affinities of receptors for 1-MCP and ethylene. This is plausible since there are five known receptors identified in Arabidopsis: ETR1, ETR2, ERS1, ERS2 and EIN4 (Chang et al., 1993; Hua et al., 1995; Hua and Meyerowitz, 1998; Sakai et al., 1998) and it is unknown whether 1-MCP binds similarly to each.

Ethylene has previously been shown to increase respiration rate in onion (Ecker, 1987; Downes et al., 2010); in this study respiration rate of the treated onions was highest after treatment with ethylene and lowest after treatment with 1-MCP. The respiration rate of onions treated with both ethylene and 1-MCP together lay between

those treated with either ethylene or 1-MCP alone suggesting the physiological response of onions to ethylene in the presence of 1-MCP was not as great as when 1-MCP was absent. This increase in respiration rate in response to ethylene in the presence or absence of 1-MCP may explain the increase in expression of probes annotated as HMGR following these same treatments. In plants, HMGR may be related to sterol biosynthesis and membrane biosynthesis and its activity has been positively correlated with rapidly dividing cells in maize (Ji et al., 1992). An increase in expression of *HMGR* could suggest an increase in rapidly dividing cells and therefore sprout growth. However, ethylene alone or in combination with 1-MCP did not result in increased sprout growth as compared with the control onions. Following treatment with ethylene, respiration rate returned to levels in line with the control onions. Therefore, it would be interesting for future research to investigate whether expression of HMGR also returns to baseline levels shortly after ethylene treatment. HMGR is also involved in the production of sesquiterpenes, and like ethylene is involved in plant defence (Chappell et al. 1997). It is possible that the increase in expression of *HMGR* was a direct result of the ethylene treatment however, *HMGR* was not up- or down-regulated by continuous ethylene treatment (Table S2).

1-MCP May Not Bind All Ethylene Receptors

At the transcriptional level, three clusters representing 180 probes, showed a differential response to ethylene and/or 1-MCP suggesting that ethylene and 1-MCP probably do not elicit the same response by being perceived as the same molecule. In climacteric fruits, 1-MCP blocks the ethylene molecule from binding to the receptor preventing the perception of ethylene and it is unlikely that this mechanism differs in onion. Cluster 0 contained probes representing transcripts down-regulated by exogenous ethylene but not in the presence of 1-MCP, suggesting that these transcripts may only respond to a specific ethylene receptor or group of receptors that bind 1-MCP. In contrast, cluster 8 contained a set of probes representing transcripts only up-regulated by 1-MCP alone but down-regulated by ethylene alone or in the presence of 1-MCP. This suggests these transcripts respond to ethylene perception by a receptor or group of receptors not bound by 1-MCP. Differences in ethylene and 1-MCP concentration, treatment duration, timing and temperature may result in differential gene expression since physiological and biochemical responses differ depending on these parameters (Blankenship and Dole, 2003; Watkins, 2006; Bufler, 2009). In

addition, it is worth noting that given the differences in dormancy between various onion cultivars, it is difficult to make broad predictions of ethylene responses in onions (Yasin and Bufler, 2007).

Exogenously Applied Ethylene and/or 1-MCP Down-Regulate Ethylene Receptors

All microarray probes representing transcripts with differential expression characterised as being involved with ethylene showed a similar pattern in expression when treated with both ethylene and/or 1-MCP. Ethylene and 1-MCP both appeared to have an effect on ethylene perception by down regulating a transcript with similarity to an ethylene receptor. Other non-climacteric species, such as citrus, have low and continuous production of ethylene, which is autoinhibited following propylene treatment (Katz et al., 2004). Although citrus exhibit some climacteric-like characteristics in the early stages of development, during the non-climacteric later phase, CsERS1 (Citrus Ethylene Response Sensor 1) expression remained constant following ethylene treatment (20 µL L^{-1}). Treatment with 1-MCP was only applied after harvest, when the citrus fruits were in the climacteric-like phase, however 1-MCP was found to down-regulate CsERS1 interfering with the autocatalytic production of ethylene. The results herein suggest that ethylene may actually reduce the expression of an ethylene receptor in onion. The other citrus ethylene receptor CsETR1 (Citrus Ethylene Receptor 1) was not affected by ethylene or 1-MCP and Katz et al., (2004) concluded that this specific receptor may not be regulated by ethylene. Similarly, Rasori et al. (2002) found no change in the regulation of ETR1, but down regulation of ERS1 after treatment with 1 µL L^{-1} 1-MCP (25°C for 24 h) in climacteric peaches 'Maria Marta'.

The results in the present study show that 1-MCP down-regulated an ethylene receptor in onion yet this was also found after ethylene treatment. Taken together with previous findings this suggests that exogenously applied ethylene and/or 1-MCP may mediate ethylene perception by down regulating the production of some but not all ethylene receptors. That said, Ma et al. (2009) found that treatment of broccoli florets with 2.5 µL L^{-1} 1-MCP for 12 h decreased gene expression of the broccoli ethylene receptors ETR1 and ETR2.

Ethylene and 1-MCP Down-Regulate *EIN3* in the Early Stages of Storage

In Arabidopsis, the absence of ethylene usually results in the rapid degradation of EIN3 (Guo and Ecker, 2003), a transcription factor acting downstream of the ethylene receptors in the ethylene signalling pathway (Alonso et al. 1999). However, the results presented here have found that the presence of ethylene and 1-MCP appears to down-regulate *EIN3*. This down regulation of both an ethylene receptor and ethylene transcriptional regulators by both ethylene and 1-MCP may help to explain why both compounds result in sprout suppression (Chope et al., 2007a; Downes et al., 2010), by down regulating the perception and signalling events of ethylene. In direct contrast, gene expression analysis of onion treated with continuous ethylene for 29 weeks (plus six weeks curing), revealed a greater transcript abundance for probes annotated as *EIN3* and *ACO*, which is involved in ethylene biosynthesis. As well as an increase in the expression of these transcripts, an increase in the transcript abundance of a probe annotated as gibberellin 2-beta-dioxygenase was also observed, which is involved in gibberellin biosynthesis. The probe representing a transcript annotated as cytokinin dehydrogenase was down-regulated; cytokinin dehydrogenase is an enzyme which deactivates cytokinins through the cleavage of their side chains (Galuszka et al., 2001). Although after 35 weeks storage, sprout growth of onions stored in continuous ethylene were shorter than those held in continuous air, it is possible that, ethylene was no longer having an inhibitory effect on sprout growth at this advanced stage of storage. Chope et al. (unpublished) found that onions may become less sensitive to ethylene and produce less endogenous ethylene the longer they are in storage. This was evidenced by a consistently low transcript abundance of probes with similarity to 1-aminocyclopropene-1-carboxylate synthase (ACS), involved in ethylene biosynthesis, and EIN3, a transcriptional regulator. It would be interesting to investigate at what stage of storage the inhibitory effects that ethylene has on the transcriptional regulation of PGRS.

In conclusion, experiments showed that treating onions with combined ethylene and 1-MCP after curing for just 24 h consistently reduced sprout and root growth for 25 weeks. Long term storage over 25 weeks may require extended periods of ethylene treatment although beyond this transcriptional changes suggest that continuous ethylene no longer controlled onion PGRs. Previous hypotheses have intimated that ethylene and 1-MCP may each be able to elicit a response in potato due to the production of new ethylene binding sites (Prange et al., 2005). An alternative explanation, supported by our data, might be that ethylene and 1-MCP bind with

different affinities to different ethylene receptors in onion. It appeared that ethylene and/or 1-MCP down-regulated probes representing transcripts annotated as ethylene receptors, as well as ethylene transcriptional regulators (EIN3). Further research is required into the structure of different ethylene receptors to investigate whether 1-MCP can bind all receptors and with what affinity. Since microarray data was only gathered from onions immediately after treatment at the beginning of storage or at the end of storage in continuous ethylene, it would be interesting to further investigate the dynamic effect ethylene/1-MCP has at the transcriptional and indeed the metabolic level.

MATERIALS AND METHODS

Plant material and curing

Onion seeds 'Sherpa' (medium pungency, medium dry matter) were drilled on sandy clay loam (Alistair Findlay's, Cardington, Beds., UK; 1.2 x 0.3 ha) on 5 March 2008 at a rate of 57 seeds m^{-2} with pesticides applied as per commercial practice although remained MH-free. Plants were machine-harvested at 100% fall-down on 17 September 2009. Onions were stored in 72 large net bags (approx. 60 bulbs) and 24 half net bags (approx. 30 bulbs) buried amongst loose bulbs in one tonne wooden crates for batch curing at the Sutton Bridge Crop Storage Research (Lincs., UK). Bulbs were artificially cured at either 20 or 28°C for 6 weeks as per normal commercial practice in the UK with relative humidity controlled at 65 – 75%.

Experimental design

The experiment was a completely randomised design with three replicates taken from three sections of the field. There were seven postharvest treatments per replicate; 1. 1 µL L^{-1} 1-MCP before curing (MB), 2. 10 µL L^{-1} ethylene before curing (EB), 3. both 10 µL L^{-1} ethylene and 1 µL L^{-1}1-MCP before curing (EMB), 4. 1 µL L^{-1} 1-MCP after curing (MA), 5. 10 µL L^{-1} ethylene after curing (EA), 6. both 10 µL L^{-1} ethylene and 1 µL L^{-1} 1-MCP after curing (EMA) and 7. control (no treatment). Treatments were applied in water-sealed air tight polypropylene chambers (88 cm x 59 cm x 59 cm) which housed two 8 x 8 cm electric fans (Nidec Beta SL, Nidec, Japan) to circulate the gases during treatments. Onions were treated in the chambers for 24 h at 20°C and the control bulbs held at 20°C in air. In the treatment boxes, levels of CO_2 did not rise above 0.30%. The 1-MCP was applied by adding 1.8 g SmartFresh

(0.14 %, Rohm and Haas, PA) to a 50 mL conical flask and sealed with Nescofilm (Bando Chemical Ind. Ltd., Kobe, Japan). To release 1 µL L^{-1} 1-MCP gas, 20 mL warm (50°C) water was injected into the conical flask through the Nescofilm using a needle and syringe prior to transfer to the chamber (Chope et al., 2007a). Ethylene treatment (10 µL L^{-1}) was administered by injecting 3.25 mL ethylene (100 % ethylene; SIP Analytical Ltd., Kent, UK) directly into the chamber via a tapped tube (polyvinyl chloride) followed by repeated full withdrawal-injection displacements to flush the ethylene into the chamber.

Pre-storage treated onions

After curing, onions were transported to Cranfield University within 2.5 h. Diseased or damaged onions were removed and the remaining onions randomly placed in individual plastic stackable crates and stored in air for 29 weeks at 0-1°C in the dark (Figure 1). At each sampling time, four onions per treatment, curing temperature and replicate (n = 168) were selected randomly, taken after harvest (day 0), immediately after curing (6 weeks) then at intervals during cold storage (17, 25 and 35 weeks after harvest) (n = 840).

Pre-storage and storage treated onions

After curing, a subset of the treated onions was transported at ambient temperature (6h ± 1 h) to the Research Institute of Vegetable Crops (Skierniewice, Poland) for continuous air or ethylene treatment (Figure 1). Control onions and onions treated with EB, MB, EMB, EA and EMA cured at either 20 or 28°C for six weeks were placed in individual plastic trays and stored in air or 10 µL L^{-1} ethylene for a further 29 weeks at 0-1°C in the dark. Six onions per pre-storage treatment, post-curing treatment and curing temperature (n = 144) were selected at random at the end of storage (35 weeks after harvest).

Sample preparation

Onions stored in the UK were removed from storage a day prior to sample preparation for gas analysis. Each bulb was then halved and visible sprout growth recorded in mm and expressed as a % of the bulb height (Chope et al., 2007b). Two longitudinal wedges were cut and snap-frozen in liquid nitrogen and each then stored at -40°C for biochemical analysis and -80°C for RNA extraction. Frozen tissue for

biochemical analysis was lyophilised using an Alpha 1-4 Christ LDC-1 freeze-dryer and pump (Edwards Super Modulo, Sussex, UK) and powdered using a pestle (Chope et al., 2007b). Sprout and root growth and disease incidence were measured in the onions sent to Poland for continuous ethylene treatment. Onions were snap-frozen in liquid nitrogen in Poland and returned to the UK on dry ice for microarray analysis.

Physiological measurements

Respiration rate

Respiration rate was measured immediately before and after curing and at each time point throughout cold storage. Four onions were placed in 3 L jars with air-tight lids and septum. The jars were sealed for 4 h at room temperature and gas samples removed with repeated full withdrawal-injection displacements using a 30 mL plastic syringe (Chope et al., 2007a). Gas samples were analysed using gas chromatography (GC model 8340, DP800 integrator, Carlos Erba Instruments, Herts., UK) coupled with hot wire detection (Chope et al., 2007a; Terry et al., 2007a). The GC was calibrated using 10.06 % CO_2 (10 % CO_2, 2 % O_2, 88 % N_2; Certified Standard from BOC). The four onions were weighed and respiration rate expressed in mmoles kg^{-1} h^{-1}.

Biochemical measurements

High pressure liquid chromatography (HPLC) was used to quantify the concentration of sugars and fructans. All chemicals for these assays were purchased from Sigma (Dorset, UK) unless otherwise stated.

Extraction and quantification of sugars

Fructose, glucose, sucrose and fructans were extracted according to Downes and Terry (2010). Onion powder (150 mg) was extracted using 2.25 mL HPLC grade water for 10 min at 75°C to extract the fructans. To the slurry, 3.75 mL MeOH was added to give a final 62.5% MeOH solution and extracted for 15 min at 55°C. The mixture was then passed through a 0.2 µm Millex-GV syringe driven filter (Millipore Corporation, MA, USA). The extract was then stored at -40°C until further use. Glucose, fructose and sucrose were quantified according to Chope et al. (2007a). Fructans were quantified according to Downes and Terry, (2010). Extracts were thawed and loaded into a HPLC system with a P680 pump and ASI-100 Automated

Sampling Injector. The extract (10 µL) was injected into a Prevail Carbohydrate ES column of 250 mm x 4.6 mm diameter, 5 µm particle size (Alltech, UK; Part no. 35101) with a Prevail Carbohydrate ES guard cartridge of 7.5 mm x 4.6 mm diameter (Alltech, UK; Part no. 96435). The mobile phase consisted of HPLC grade water (A) and EtOH (B). The gradient involved a linear increase/decrease of solvent B; 85-65%, 9 min; 65-85%, 3 min; 85% 8 min at a flow rate of 0.5 mL min^{-1} and column temperature was set at 40°C. An evaporative light scattering detector (ELSD 2420, Waters, MA) connected to the system via a UCI-50 universal chromatography interface detected the eluted carbohydrates. Carbohydrate concentrations were calculated against calibration standards; fructose, glucose, sucrose, 1-kestose and nystose ranging from 5 – 0.05 mg mL^{-1}.

Microarray analysis

RNA extraction

Six samples were chosen for microarray analysis; four samples were taken before curing immediately after treatment with ethylene or 1-MCP or ethylene and 1-MCP in combination for 24 h at 20°C. The other two samples were taken after 29 weeks cold storage (1°C) in continuous air or continuous ethylene. There were three biological replicates of each of the six treatments making 18 samples in total. Total RNA was isolated according to Chang et al. (1993) with modifications.

Total RNA was extracted from frozen, ground onion tissue (100 mg) homogenised in 1 mL extraction buffer (2% (w/v) CTAB (cetyl trimethylammonium bromide), 0.8 M NaCl, 20 mM Na_2EDTA, 0.2 M boric acid, adjusted to pH 7.6 with TRIZMA base, β-mercaptoethanol added to 1% (v/v) just prior to use) using a pestle and mortar. The mixture was transferred to a 2 mL microtube and incubated at 65ºC for 10 min, then allowed to return to room temperature. Chloroform (1 mL) was added and mixed before being centrifuged at 13,000 rpm for 5 min at room temperature. The aqueous phase was removed to a clean tube and an equal volume of precipitation buffer (0.5% (w/v) CTAB, 50 mM Na_2EDTA, 50 mM MES (2-(N-morpholino) ethanesulfonic acid), adjusted to pH 5.8 with NaOH, and filtered through a 0.2 µM sterile filter), mixed and incubated on ice for 30 min. Samples were centrifuged at 13,000 rpm for 20 min at 4ºC and the supernatant removed. The pellet was resuspended in SSTE (1.0 M NaCl, 0.5% SDS, 10 mM TrisHCl (pH 8.0), 1 mM

Na_2EDTA (pH 8.0)) and briefly incubated at 37ºC, before being allowed to return to room temperature. Chloroform (1 mL) was added and mixed, before being centrifuged at 13,000 rpm for 5 min at room temperature. The aqueous phase was removed to a clean tube and an equal volume of isopropanol added and incubated on ice for 20 min. Samples were centrifuged at 13,000 rpm for 20 min at 4ºC and the supernatant removed. The pellet containing total nucleic acid was washed with 1 mL 70% (v/v) ethanol, then left to air dry and finally resuspended in 50 µL DEPC-treated water. Then, 30 µL 8 M lithium chloride solution was added and the samples incubated on ice overnight to selectively precipitate RNA. Samples were centrifuged for at 13,000 rpm for 30 min at 4ºC, the supernatant was removed, the pellet washed with 0.5 mL 70% ethanol and resuspended in 15 µL RNase-free water. Sample purity and integrity were verified using the RNA 6000 Nano Assay on the Agilent 2100 BioAnalyzer (Agilent Technologies Inc., Santa Clara, CA, USA) and then treated with Baseline Zero DNase (Epicentre, Madison, WI, USA) according to the supplier's instructions.

Microarray

A total of 13,310 onion nucleotide sequences were available for the construction of a 60-mer oligonucleotide custom *Allium cepa* microarray. The majority were obtained from public databases; 13,154 from the Onion Gene Index (http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=onion) and 102 from GenBank (http://www.ncbi.nlm.nih.gov/genbank/), with the remaining 54 sequenced directly from onion bulb tissue. Microarrays were designed using Agilent Technologies e-array microarray design platform (https://earray.chem.agilent.com/earray/). The design process ensures probes are designed to unique sequences within all sequences submitted for the design process, avoiding redundancy in representation of sequences by probes. Initially, a prototype chip was designed in a 4 x 44K format where 60-mer oligonucleotide probes for ESTs and singletons were designed to both sense and anti-sense. Test hybridizations of RNA from a range of onion tissues (root, shoot, bulb and leaf) were used to orientate these probes, thus reducing the number of probes, so the final format was 8 x 15K, consisting of eight independent arrays of 15K probes, on a single glass slide. Each array consisted of 15,736 60-mer oligonucleotide probes in total, representing 536 internal control probes and 15,200 probes representing 13,310 unique

onion sequences. In order to further our analyses of onion gene expression, the annotation for individual probes was populated with annotations from the closely related, fully sequenced, genome of rice (*Oryza sativa*). Translated blastx alignments were made between onion sequences downloaded from the Onion Gene Index (Release 2.0; http://compbio.dfci.harvard.edu/tgi/plant.html) and rice cDNA sequences from the Rice Genome Annotation project (Version 6.1; http://rice.plantbiology.msu.edu/index.shtml). The tblastx alignments were performed with an E-value cut-off of 0.01 (Altschul et al., 1997). Annotations, including descriptions and Gene Onotology assignments were then cross-referenced from rice sequences with significant homology to onion sequences, allowing GO analysis and more informative descriptions on the putative role of onion genes.

Agilent One Color Quick Amp Labelling Kit (Agilent Technologies Inc.) was used to amplify and label target RNA with Cyanine 3-CTP to generate complementary RNA (cRNA) according to the manufacturer's instructions. Purification of the labelled cRNA was performed using RNeasy mini spin columns (Qiagen, Hilden, Germany) and quantified using a NanoDrop ND-1000 UV-VIS spectrometer. Agilent One Color RNA Spike-In Kit (Agilent Technologies Inc.) was used as a positive control for monitoring sample amplification, labelling and microarray processing. The cRNA was fragmented and hybridized to an onion oligonucleotide microarray, representing 13,310 unique onion sequences, using the Agilent Gene Expression Hybridisation Kit, and then washed with Gene Expression Wash Buffers 1 and 2, according to the manufacturer's instructions (Agilent Technologies Inc.).

The microarray slides were scanned using an Agilent G2565BA Microarray scanner with Agilent Scan Control version A8.4.1 at a resolution of 5 µm, using the extended dynamic range option. Signal values for individual probes were extracted using Agilent Feature Extraction version 10.5.1.1 software (Agilent Technologies Inc.). All microarray data have been submitted to the online database Gene Expression Omnibus for public access and long term storage (accession number GSE27132).

Quantitative real-time PCR validation

To validate the microarray results, transcript levels of five differentially expressed transcripts identified in the microarray data were confirmed using real time quantitative PCR (Figure 6,Table 6). cDNA was synthesised using the

633 ThermoScript™ RT-PCR System for First-Strand cDNA Synthesis kit (Invitrogen,
634 Cat. No. 11146-024) from total RNA samples (1 µg) using a combination of random
635 hexamers and oligo dT primers (20:80 mix, respectively). Gene specific primers were
636 designed using Primer 3 and PrimerSelect (Lasergene) software. Transcript
637 abundance detected by an ABI Prism 7900HT sequence detection system (Applied
638 Biosystems) controlled by SBS 2.1 software (Applied Biosystems) using a SensiMix
639 SYBR Green qPCR MasterMix (Bioline, London, UK). The qPCR was performed in
640 384 well plates using the "Standard Curve" method (Wong and Medrano, 2005) for
641 mRNA quantification with normalization to the endogenous control gene, tumour
642 protein (TC4554 CUST_716_P1403527117; F TCCGACTACAGGAACAACCAG, R
643 AAACTCCTCTGCCTTCTCAGC). The control gene was selected from six genes
644 evaluated for stability within our samples using the geNorm software package
645 (Vandesompele et al., 2002). Quantitative PCR conditions, efficiency calculations and
646 data normalizations were as described previously (Hammond et al., 2006).
647
648
649 **Statistical analysis**
650 Statistical analyses were conducted using Genstat for Windows Version
651 10.1.0.147 (VSN International Ltd., Herts., UK). Analysis of variance was used to
652 identify the main effects of cultivar, treatment and time, and the interactions between
653 these factors to a probability of $P < 0.05$ unless otherwise stated. The first sampling
654 time (day 0; before curing) consisted of three treatments and the outturns thereafter
655 consisted of five treatments. This imbalance was resolved by considering the first
656 time point as a common baseline to which the remaining time points could be
657 compared. Least significant differences (LSD; $P = 0.05$) were calculated from each
658 analysis. Microarray data analysis was performed using Genespring GX11 (Agilent).
659 There were three replicates for each treatment (control, ethylene before curing, 1-MCP
660 before curing, ethylene and 1-MCP before curing, continuous storage in air and
661 continuous storage in ethylene) totalling 18 samples. The continuous treated samples
662 ($n = 6$) were analysed separately to those treated before curing ($n = 12$). Raw
663 expression data were subject to quantile normalization, and then baseline
664 normalization was applied to individual probes, by dividing probe signal values by the
665 median probes signal of control samples. Significantly differentially expressed
666 transcripts were selected using a one-way ANOVA (GeneSpring GX) with a

667 Benjamini-Hochberg corrected *p*-value <0.05 and a fold-change cut-off > 2.
668 Significantly differentially expressed transcripts were then grouped using the K-means
669 clustering algorithm in GeneSpring GX.
670
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Figure legends

Figure 1 Schematic diagram of experimental plan.

Figure 2 Respiration rate (mmol CO_2 kg^{-1} h^{-1}) of 'Sherpa' onions treated with either ethylene before curing (EB), 1-MCP before curing (MB), ethylene and 1-MCP before curing (EMB), ethylene after curing (EA), 1-MCP after curing (MA), ethylene and 1-MCP after curing (EMA) or no treatment (control) for 24 h at 20°C (n = 12); LSD bars (P = 0.05) are shown.

Figure 3 Fructose, glucose, sucrose and total fructans (DP3-DP8; degrees of polymerisation) of 'Sherpa' onions treated with either ethylene before curing (EB), 1-MCP before curing (MB), ethylene and 1-MCP before curing (EMB), ethylene after curing (EA), 1-MCP after curing (MA), ethylene and 1-MCP after curing (EMA) or no treatment (control) for 24 h at 20°C (n = 12); LSD bars (P = 0.05) are shown.

Figure 4 K-means cluster analysis of altered onion gene expression sampled following treatment before curing for 24 h at 20°C with ethylene (EB), 1-MCP (MB), ethylene and 1-MCP (EMB) or untreated (control).

Figure 5 Functional classification of (A) 272 probes up-regulated by continuous ethylene treatment, (B) 302 probes down-regulated by continuous ethylene treatment sampled after 29 weeks storage.

Figure 6 Correlation between the gene expressions of five genes quantified using the onion microarray and qPCR. The expression of the five genes was quantified for each onion treatment (r = 0.68, P < 0.001).

Table 1 Sprout length (% of the bulb height) of 'Sherpa' onions measured 25 and 35 weeks after harvest (six weeks curing then transferred to cold storage) treated before or after curing with 10 µL L^{-1} ethylene and/or 1 µL L^{-1} 1-MCP for 24 h at 20°C (*n* = 12).

Treatment	Sprout length (% of bulb height)			
	25 weeks		35 weeks	
	20°C	28°C	20°C	28°C
Control	51.3	39.2	55.4	58.9
EB	47.3	30.8	68.0	58.8
MB	39.8	6.1	58.0	42.9
EMB	24.8	13.9	60.7	53.5
EA	46.0	19.1	51.6	54.3
MA	40.1	22.0	62.5	56.1
EMA	15.3	7.9	61.6	64.0

Treatments: EB, ethylene before curing; MB, 1-MCP before curing; EMB, ethylene and 1-MCP before curing; EA, ethylene after curing EMA, ethylene and 1-MCP after curing. LSD (*P* = 0.05) = 22.86.

Table 2 Sprout length (% of the bulb height) of 'Sherpa' measured 35 weeks after harvest treated before or after curing with 10 µL L^{-1} ethylene and/or 1 µL L^{-1} 1-MCP for 24 h at 20°C then transferred to air or continuous ethylene storage at 0-1°C (n = 6).

Pre-storage	Sprout length (% of bulb height)			
	Air		Ethylene	
	20°C	28°C	20°C	28°C
Control	63.1	56.2	49.3	38.9
EB	68.3	61.0	33.2	51.3
MB	64.1	61.0	41.9	49.7
EMB	63.4	60.1	28.8	37.4
EA	63.0	51.6	36.0	40.6
EMA	37.6	62.8	64.2	43.9

Treatments: EB, ethylene before curing; MB, 1-MCP before curing; EMB, ethylene and 1-MCP before curing; EA, ethylene after curing EMA, ethylene and 1-MCP after curing. LSD (P = 0.05) = 14.78.

Table 3 Root incidence (% of bulbs with roots) of 'Sherpa' onions measured 35 weeks after harvest (six weeks curing then transferred to cold storage) treated before or after curing with 10 µL L^{-1} ethylene and/or 1 µL L^{-1} 1-MCP for 24 h at 20°C then transferred to air or continuous ethylene storage at 0-1°C (*n* = 6).

Pre-storage	Root incidence (% of bulbs with roots)			
	Air		Ethylene	
	20°C	28°C	20°C	28°C
Control	83.3	50.0	0.0	16.7
EB	33.3	83.3	0.0	16.7
MB	83.3	66.7	0.0	83.3
EMB	66.7	83.3	0.0	0.0
EA	50.0	83.3	0.0	33.3
EMA	0.0	66.7	33.3	33.3

Treatments: EB, ethylene before curing; MB, 1-MCP before curing; EMB, ethylene and 1-MCP before curing; EA, ethylene after curing EMA, ethylene and 1-MCP after curing. LSD (*P* = 0.05) = 40.30.

Table 4 Functional classification of onion probes differentially expressed when treated before curing for 24 h at 20°C with ethylene, 1-MCP, ethylene and 1-MCP or untreated (control).

Functional category	Cluster									
	0	1	2	3	4	5	6	7	8	Row Totals
Housekeeping	11	20	40	17	49	37	21	14	6	215
Stress and defence	3	10	6	4	5	8	6	9	3	54
Chaperones	0	3	3	2	5	0	2	0	0	15
Photosynthesis	0	1	4	1	5	3	2	1	0	17
Cell wall metabolism	0	3	2	1	4	3	2	3	0	18
Secondary metabolism	3	8	7	1	4	3	3	4	2	35
Cell death	1	0	2	0	2	1	0	0	0	6
Peptidase / Kinase	8	11	29	10	13	27	6	19	3	126
Transport	4	10	24	3	9	20	3	15	2	90
Signalling	3	0	4	1	8	6	2	4	0	28
Metabolism	8	22	29	4	20	23	8	13	2	129
PGR	1	6	7	0	5	2	2	5	0	29
Cell cycle	1	1	2	0	5	3	0	1	1	13
Transcription factor	9	11	34	2	15	15	6	14	1	107
Unclassified	19	44	75	19	49	64	21	34	2	327
Phosphatase	0	2	3	1	2	4	3	4	0	19
Row Totals	71	152	271	66	200	219	87	140	22	1228

Table 5 The 30 most highly up and down-regulated onion probes compared with controls after treatment with ethylene before curing (EB), 1-MCP before curing (MB) or ethylene and 1-MCP before curing (EMB).

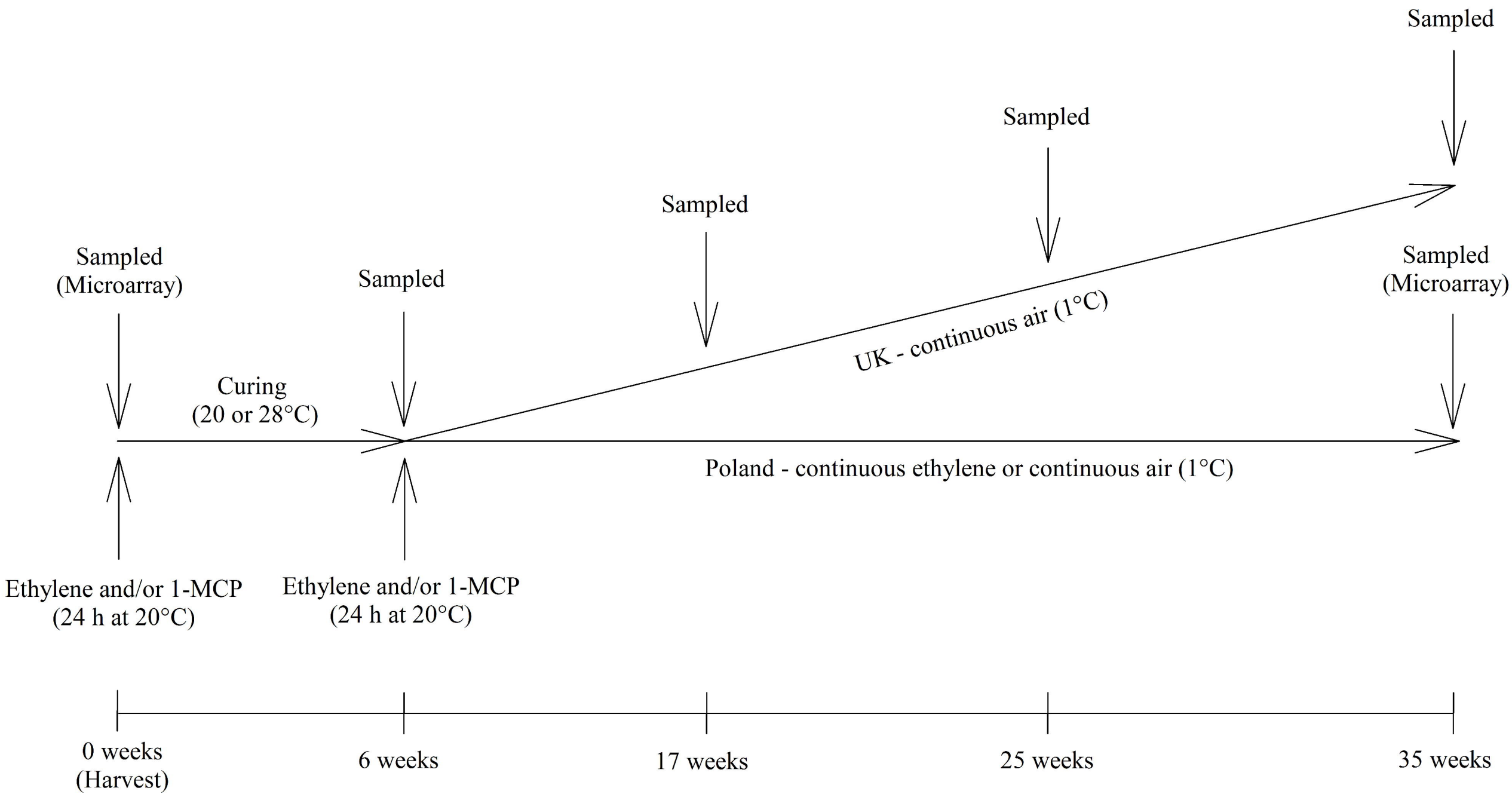
Probe	Tentative annotation	Fold change[a]	Regulation	Onion Sequence ID	Treatment
CUST_792_PI403527117	integral membrane protein	51.1	up	TC4630	EB
CUST_2054_PI403527117	3-hydroxy-3-methylglutaryl-coenzyme A reductase	48.1	up	TC5892	EMB
CUST_390_PI403527117	transferase family protein	42.0	up	TC4228	MB
CUST_5592_PI403527117	WD domain, G-beta repeat domain containing protein	33.9	up	CF447771	MB
CUST_3008_PI403527117	retrotransposon protein	28.9	up	TC6846	MB
CUST_2287_PI403527117	LTPL121 - Protease inhibitor/seed storage/LTP family protein precursor	26.4	down	TC6125	EB
CUST_10068_PI403527117	1-aminocyclopropane-1-carboxylate oxidase homolog 4	24.7	up	CF438875	EMB
CUST_7201_PI403527117	starch synthase	23.8	down	CF437167	EMB
CUST_160_PI403527117	per1-like family protein	22.6	up	TC3998	EMB
CUST_3247_PI403527117	CHIT5 - Chitinase family protein precursor	21.8	up	TC7085	EMB
CUST_4826_PI403527117	protein kinase family protein	21.0	up	CF438357	MB
CUST_7052_PI403527117	S-formylglutathione hydrolase	18.4	up	CF448815	MB
CUST_160_PI403527117	per1-like family protein	18.2	up	TC3998	EB
CUST_11478_PI403527117	stress responsive protein	18.1	up	BI095628	EMB
CUST_10708_PI403527117	amino acid transporter	17.9	up	CF440190	EB
CUST_10973_PI403527117	monocopper oxidase	17.7	down	BE205651	EB
CUST_1451_PI403527117	dihydrodipicolinate synthase, chloroplast precursor	17.1	up	TC5289	MB
CUST_36_PI403527117	peroxidase precursor	16.6	up	TC3874	MB
CUST_10095_PI403527117	Ser/Thr protein phosphatase family protein	16.3	up	CF440115	MB

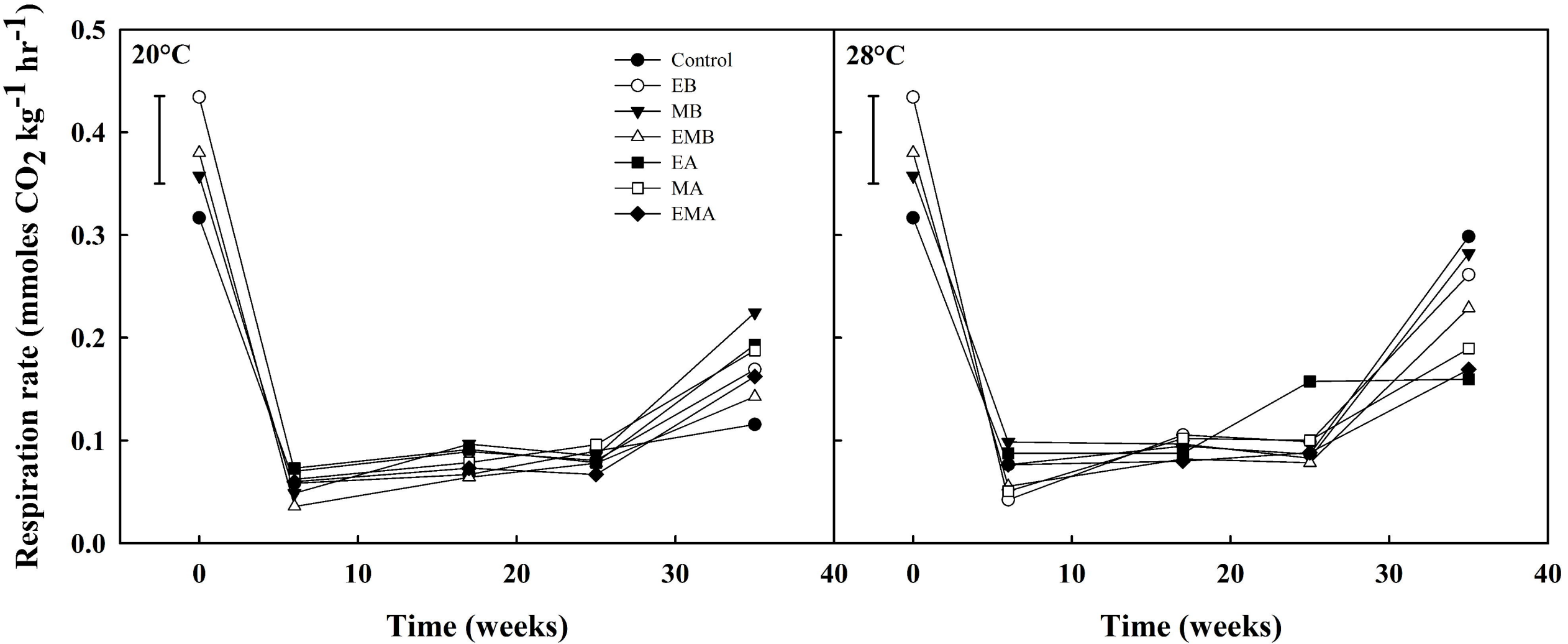
CUST_600_PI403527117	EF hand family protein	15.9	up	TC4438	EMB
CUST_6021_PI403527117	OsWRKY48 - Superfamily of TFs with WRKY and zinc finger domains	15.3	down	CF439568	EMB
CUST_6801_PI403527117	zinc finger family protein	15.3	down	CF435756	EB
CUST_9681_PI403527117	alpha-soluble NSF attachment protein	15.1	up	BQ580069	EMB
CUST_792_PI403527117	integral membrane protein DUF6 containing protein	15.0	up	TC4630	EMB
CUST_11354_PI403527117	mitochondrial carrier protein	14.8	up	CF441173	MB
CUST_2054_PI403527117	3-hydroxy-3-methylglutaryl-coenzyme A reductase	14.5	up	TC5892	EB
CUST_160_PI403527117	per1-like family protein	14.4	up	TC3998	MB
CUST_10095_PI403527117	Ser/Thr protein phosphatase family protein	12.7	up	CF440115	EB
CUST_4897_PI403527117	aldehyde dehydrogenase	12.6	up	CF442148	EB
CUST_10269_PI403527117	myristoyl-acyl carrier protein thioesterase, chloroplast precursor	12.6	up	CF445478	EB

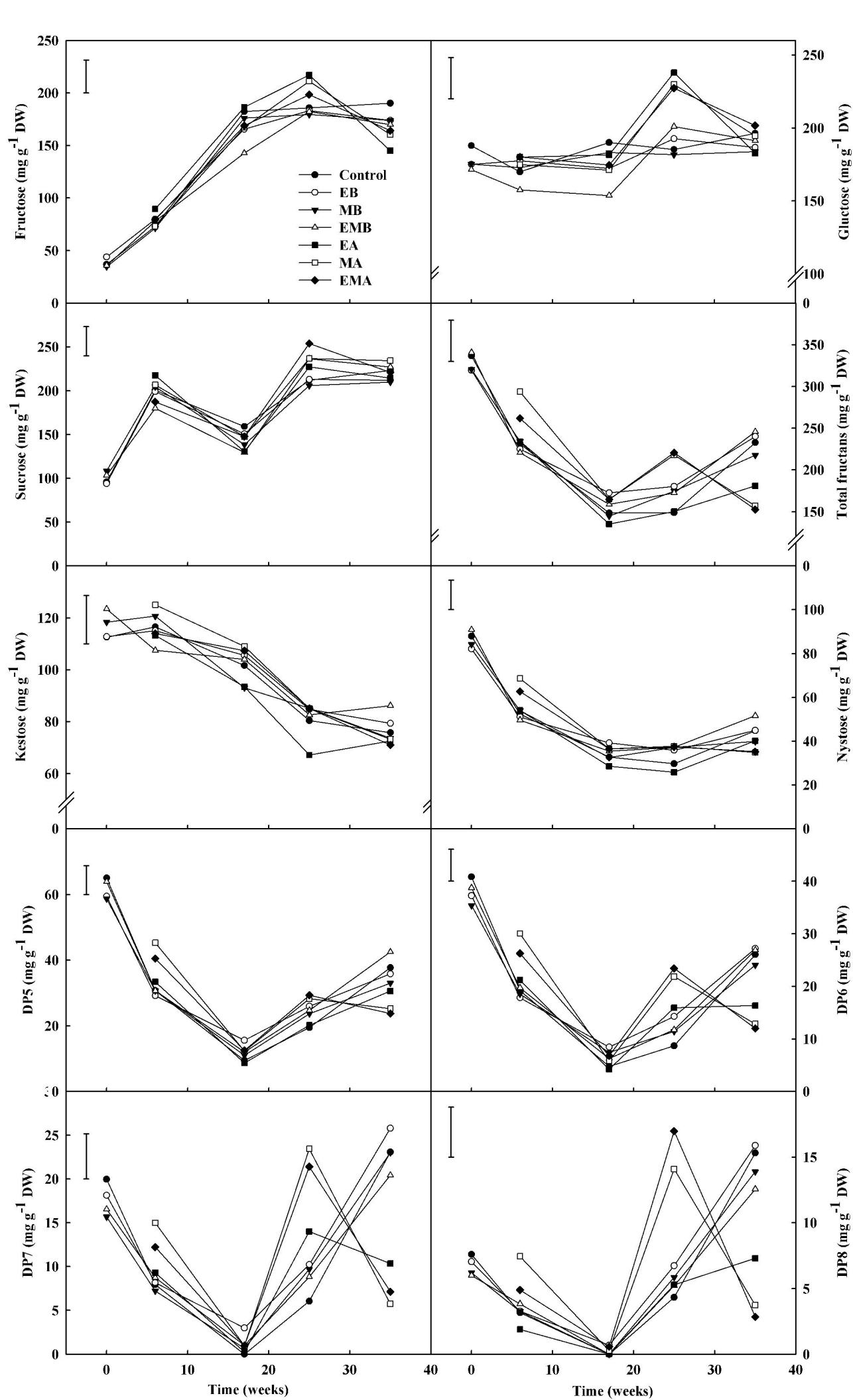
[a] Fold change compared with expression of control, calculated as 2^x, where x = absolute value of (normalised treatment / normalised control)

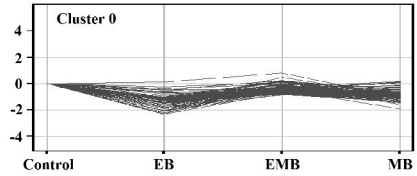
Table 6 Primers used for qPCR analysis

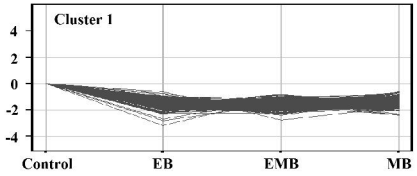
Probe	Gene	Forward Sequence	Reverse Sequence
3995_P1403527117	ABTB1-Armadillo repeat	TTGGCTCTTGCTCATCTTTG	ACCATCTTGCTGTTGCTTTG
10973_P1403527117	Monocopper oxidase	GATCGGAGAATTGGGAAAGAC	TTAGCTCGGCCACACAGAAG
2287_P1403527117	LTPL121-Protease inhibitor / seed storage	CTGCACTCCTTGCCCTAAAC	CTCCCAGCTTCAGTGTATCG
1126_P1403527117	RNA polymerase	AAGTGGCGGTGGTCTGATAG	AGGCAGCAACAAAGATGGTAAG
2252_PI403527117	Starch synthase	ATGTTCGGGTTCTTTGTTCAG	GCCTCTTCTTCACTTACTTTCCAG

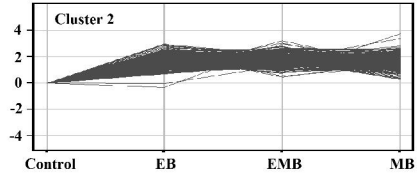

Sampled
Sampled
Sampled
Sampled
Sampled
(Microarray)
Sampled
(Microarray)
UK - continuous air (1°C)
Curing
(20 or 28°C)
Poland - continuous ethylene or continuous air (1°C)
Ethylene and/or 1-MCP
(24 h at 20°C)
Ethylene and/or 1-MCP
(24 h at 20°C)
0 weeks
(Harvest)
6 weeks
17 weeks
25 weeks
35 weeks

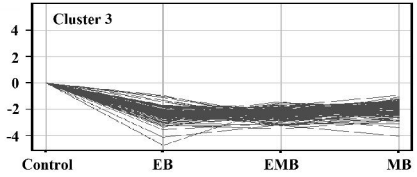

20°C
28°C
Control
EB
MB
EMB
EA
MA
EMA
Respiration rate (mmoles CO2 kg-1 hr-1)
0.0
0.1
0.2
0.3
0.4
0.5
0
10
20
30
40
Time (weeks)

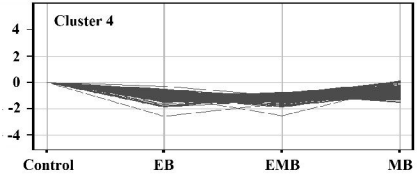

Fructose (mg g-1 DW)
Glucose (mg g-1 DW)
Sucrose (mg g-1 DW)
Total fructans (mg g-1 DW)
Kestose (mg g-1 DW)
Nystose (mg g-1 DW)
DP5 (mg g-1 DW)
DP6 (mg g-1 DW)
DP7 (mg g-1 DW)
DP8 (mg g-1 DW)
Control
EB
MB
EMB
EA
MA
EMA
Time (weeks)
Time (weeks)

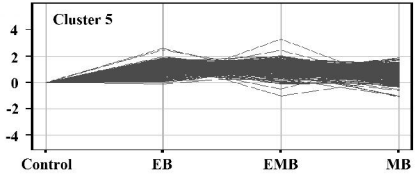

Cluster 0
4
2
0
-2
-4
Control
EB
EMB
MB

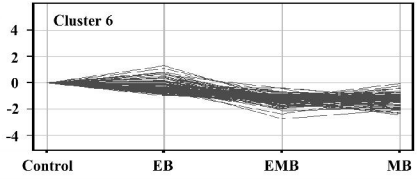

Cluster 1
4
2
0
-2
-4
Control
EB
EMB
MB

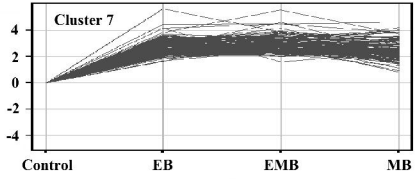

Cluster 2
4
2
0
-2
-4
Control
EB
EMB
MB

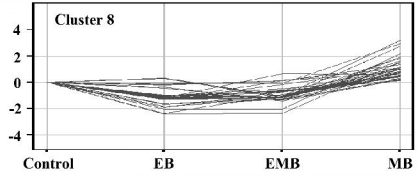

Cluster 3
4
2
0
-2
-4
Control
EB
EMB
MB

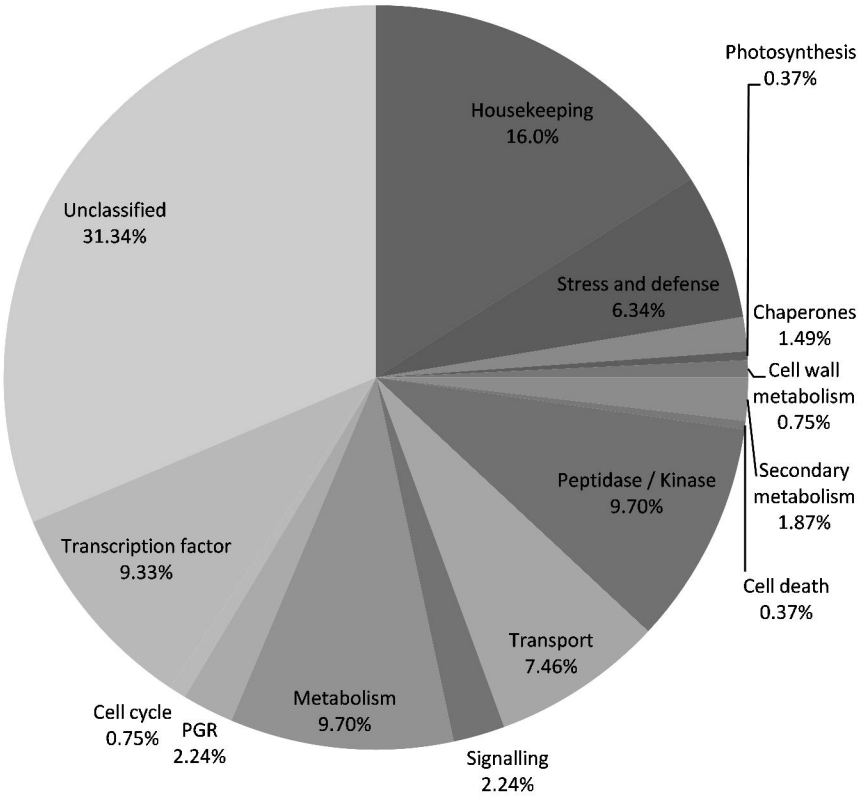

Cluster 4
4
2
0
-2
-4
Control
EB
EMB
MB


Cluster 5
4
2
0
-2
-4
Control
EB
EMB
MB

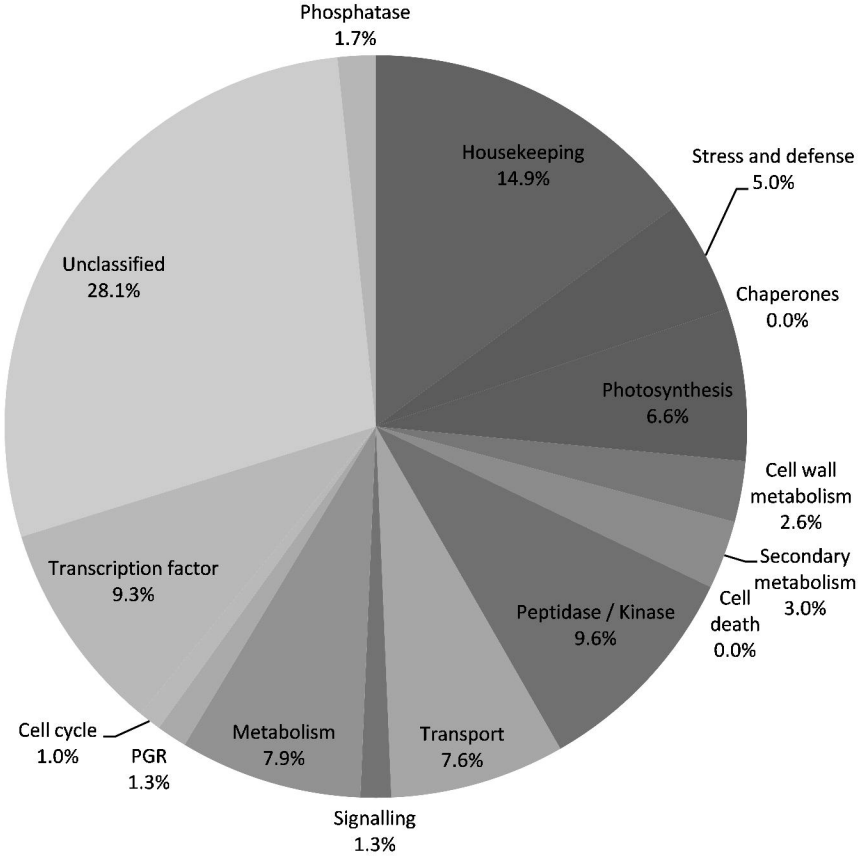

Cluster 6
4
2
0
-2
-4
Control
EB
EMB
MB


Cluster 7
4
2
0
-2
-4
Control
EB
EMB
MB

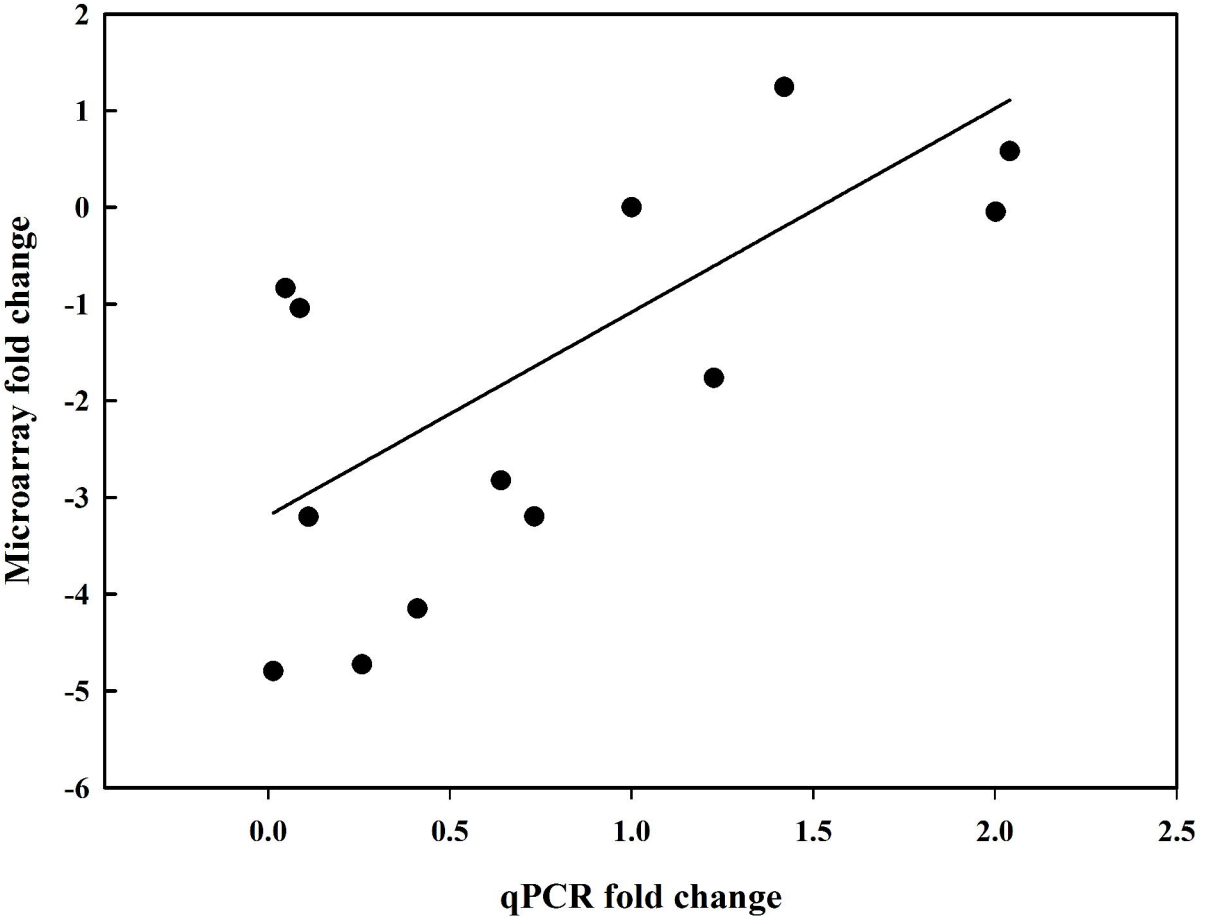

Cluster 8
4
2
0
-2
-4
Control
EB
EMB
MB


Housekeeping
16.0%
Stress and defense
6.34%
Chaperones
1.49%
Cell wall
metabolism
0.75%
Secondary
metabolism
1.87%
Cell death
0.37%
Photosynthesis
0.37%
Peptidase / Kinase
9.70%
Transport
7.46%
Signalling
2.24%
Metabolism
9.70%
PGR
2.24%
Cell cycle
0.75%
Transcription factor
9.33%
Unclassified
31.34%

(A)


Phosphatase
1.7%
Housekeeping
14.9%
Stress and defense
5.0%
Chaperones
0.0%
Photosynthesis
6.6%
Cell wall
metabolism
2.6%
Secondary
metabolism
3.0%
Cell
death
0.0%
Peptidase / Kinase
9.6%
Transport
7.6%
Signalling
1.3%
Metabolism
7.9%
PGR
1.3%
Cell cycle
1.0%
Transcription factor
9.3%
Unclassified
28.1%

(B)


Microarray fold change
qPCR fold change
2
1
0
-1
-2
-3
-4
-5
-6
0.0
0.5
1.0
1.5
2.0
2.5