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VI19001 Autumn application of Actigard®

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Executive summary

VI19001 Autumn application of Actigard®

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Actigard® (Syngenta) is used to control *Pseudomonas syringae* pv. *actinidiae* (Psa) in kiwifruit. Four foliar applications per season are permitted with sprays restricted on fruiting vines to the pre-flowering and postharvest period. Actigard stimulates the plant defence response but little is known about the responsiveness of vines to Actigard after harvest and if the postharvest response differs between 'early' and 'late' harvest orchards. Furthermore, it is not known if there is a 'carry-over' effect of postharvest spray into the following spring. In this project the postharvest response to Actigard was investigated by comparing the expression of eight 'defence marker' genes in *Actinidia chinensis* var. *deliciosa* 'Hayward' and *Actinidia chinensis* var. *chinensis* 'Zesy002' (commonly known as Gold3) vines, before and after Actigard application. The vines were treated with Kocide Opti® ('Control' treatment) or with a tank mix containing Kocide Opti plus Actigard ('Actigard'). Changes in gene expression in Actigard-treated vines were compared with that in the control. The direct 'carry-over' effect of postharvest Actigard was investigated by comparing gene expression in the two treatment groups immediately before commencement of pre-flowering sprays in the following spring. The 'priming' response was measured in 'Hayward' by measuring the effect of pre-harvest Actigard spray on the Actigard-induced response in spring.

Results show that the early-harvest and late-harvest 'Hayward' vines were responsive to Actigard and that the gene response patterns were generally similar in the two orchards. The most highly up-regulated genes were PR1, PR5, DMR6, NIMIN2 and WRKY70 and this is consistent with Actigard operating via the salicylic acid defence pathway. No postharvest data were obtained for Gold3 because of equipment failure (freezer). In spring, pre-spray gene expression levels were not significantly different between treatments in 'Hayward' and Gold3 vines, indicating that there was no direct carry-over effect of the postharvest Actigard spray. There was, however, a weak but significant priming response of two genes (Gluc1 and PR5) to Actigard at one 'Hayward' site.

In conclusion, this study has advanced our understanding of Actigard use in kiwifruit by demonstrating that postharvest spray results in the up-regulation defence-genes in 'Hayward' vines. The evidence for a carry-over response into the following spring was not conclusive and warrants further investigation.

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1 Introduction

Actigard® (Syngenta) is a plant defence elicitor that is used for the management of bacterial canker in kiwifruit caused by the bacterial pathogen *Pseudomonas syringae* pv. *actinidiae* (Psa) (https://www.kvh.org.nz/vdb/document/99346). The active ingredient in Actigard, acibenzolar-s-methyl (ASM), is a functional mimic of salicylic acid (SA) which activates SA-responsive defence signalling cascades and induces a systemic resistance to pathogen attack (Tripathi et al. 2019). Up to four foliar applications of Actigard can be made over the season but sprays are restricted to the pre-flowering and postharvest period on fruiting vines.

Actigard efficacy depends on the activation of inducible defence mechanisms in plants. Inducible defences are considered to have evolved to be mobilised only when required, thereby minimising the metabolic costs associated with defence (Walters & Heil 2007). Moreover, the inducible component of defence declines as leaves develop greater physical resistance (Quintero & Bowers 2011; Walters et al. 2013; Barton et al. 2019) and it has been suggested that mature tissues with high constitutive resistance would not benefit significantly from further investment in induced resistance (Karban et al. 1997). This raises several important questions about the use of Actigard in kiwifruit and the relative responsiveness of the mature leaf canopy after harvest compared with that before flowering.

The main aim of this project was to investigate whether kiwifruit vines are responsive to Actigard after harvest by comparing Actigard-induced defence responses in vines immediately after harvest with that during the pre-flowering period. The responsiveness of the kiwifruit vine to Actigard is quantified by measuring the up-regulation of selected defence genes in kiwifruit leaves after spray application with Actigard. Previous studies funded by Zespri/KVH (VI1602) and Plant & Food Research (IRPsa MBIE programme) identified genes (PR1 and Gluc1) that were up-regulated following treatment with Actigard in *Actinidia chinensis* var. *deliciosa* 'Hayward' and *Actinidia chinensis* var. *chinensis* 'Zesy002' (Gold3) vines during spring. These genes encode antimicrobial proteins and are typical markers of the SA-mediated defence and SAR in plants (Ali et al. 2018). More recently, PFR-funded studies have identified other candidate genes (e.g. TLP-TG4 [PR5], NIMIN2) that were induced by Actigard in kiwifruit. Comparing the up-regulation of these 'marker genes' by Actigard, relative to controls, during the postharvest period with that during spring will enable a quantitative comparison of the vine response. Eight candidate markers are included to represent different components in the signalling cascade and so provide a more robust analysis of the defence response.

2 Methods

2.1 Orchard sites

The trials on *A. chinensis* var. *chinensis* 'Zesy002' (Gold3) and *A. chinensis* var. *delicosa* 'Hayward' were located in 4 sites within the Waikato. The kiwifruit blocks were selected to be representative of early and late fruit harvests for each cultivar with approximately three weeks separating the respective harvest dates. The vines were treated with Kocide Opti® ('Control' treatment) or with a tank mix containing Kocide Opti plus Actigard (hereon referred to as 'Actigard') according to the schedule outlined in Table 1. Kocide Opti was applied at a concentration of 90g/100L after harvest and at 70g/100L for the pre-flowering spray. Actigard was applied at 20g/100L regardless of season. Treatments were applied at a spray rate of 1000 L/ha. There were five single-vine replicates per treatment at each site.

At sites A–C, the same vines were used for the Actigard and the control treatments after harvest and in spring, i.e. the treatment sequence was Actigard-Actigard versus Control-Control. However, at site D the entire trial area was accidentally sprayed with Actigard on 22 October 2019, hence losing the Control treatment. After consultation with Zespri, it was agreed to proceed with sampling at site D and to investigate whether the postharvest treatments affected vine responsiveness to Actigard in spring, i.e. Actigard-Actigard versus Control-Actigard.

Table 1. Schedule of activities at the orchard trial sites

		Postharvest 2019***					Pre-flowering 2019			
Cultivar	Site	Leaf sample	Harvest	Spray applied	1/2d sample	6/7d sample	Leaf sample	Spray applied	2d sample	6/7d sample
Gold3 'early'	A	17 Apr	18 Apr	18 Apr	20 Apr	24 Apr	7 Oct	9 Oct	11 Oct	16 Oct
Gold3 'late'	B	6 May	8 May	9 May	11 May	15 May	14 Oct	16 Oct	18 Oct	23 Oct
'Hayward' 'early'	C	3 May	6 May	8 May	10 May	14 May	29 Oct	30 Oct	1 Nov	6 Nov
'Hayward' 'late'	D	22 May	23 May	24 May	25 May	30 May	*	22 Oct	24 Oct	29 Oct

A, B, C, D.
*no sample collected because grower did not notify of intention to spray.
**The entire trial at site C sprayed with Kocide Opti® plus Actigard® on 22 October 2019.
*** All postharvest Gold3 samples, with shaded background, were lost because of freezer failure.

2.2 Leaf sampling

Leaf samples were taken from each rep by removing six leaves from within a 1.5m radius of the main trunk. Care was taken to select unblemished leaves of similar size and from similar locations on the shoot. The selection of late harvest samples was more problematic because of deteriorating leaf quality (Figure 1). Immediately after sampling, six discs (18 mm diam.) per leaf were cut from unblemished areas of the leaf using a cork borer and then pooled by rep in a plastic vial before immersion in liquid nitrogen. The frozen samples were stored at -70°C until RNA extraction and analysis (see Section 2.3). A freezer failure at Ruakura resulted in the loss of all Gold3 postharvest samples.

a

'Zesy002'/Gold3 – early harvest

b

'Zesy002'/Gold3 - late harvest

c

'Hayward' early harvest

d

'Hayward' late harvest

Figure 1. Representative leaves from the four trial sites collected before fruit harvest.

2.3 Gene expression analysis

Gene expression was determined by molecular barcoding technology using the Plexset® platform from NanoString Technologies Inc. (Seattle WA, USA) with a total of 12 genes; the results were analysed using the nSolver™ 4.0 software provided by NanoString. Four of the 12 genes, eukaryotic small ribosomal subunit 40S (40S), ubiquitin-conjugating enzyme (UBC1), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and protein phosphatase 2 (PP2A), were used as reference genes to normalise the data. They were chosen because their expression was found stable in previous experiments with kiwifruit (Wurms et al. 2011). The other eight genes were selected because in previous experiments on kiwifruit they were found to be overexpressed in response to treatment with ASM (Cellini et al. 2014; Wurms et al. 2017, Michelotti et al. 2018; de Jong et al. 2019). They are: pathogenesis-related protein family 1 (PR1), APETALA2 ethylene responsive factor 2 (AP2_ERF2), Glucan endo-1,3-β-glucosidase (Gluc1), NIM-interacting protein 2 (NIMIN2), Downy mildew resistance 6 (DMR6), WRKY transcription factor 70 (WRKY70), and benzaldehyde dehydrogenase (BAD), thaumatin-like protein TG4 (PR5).

For each gene a capture probe and a reporter probe, each binding to an adjacent 50 bp DNA sequence specific for the gene being analysed, was synthesised by Integrated DNA Technologies Private Limited (IDT, Singapore). The 100 bp target sequences of the 12 genes used in this study are presented in Appendix 2.

Total RNA was prepared from about 100 mg of kiwifruit tissue ground by mortar and pestle in liquid nitrogen, using the Spectrum Plant Total RNA Kit (Sigma-Aldrich) following the supplier's recommendations. Sample purity and RNA concentrations were determined using a Nanophotometer® (Implen, CA, USA). RNA samples were sent at -80°C to the Grafton Clinical Genomics of the School of Medical Science, Auckland University for processing.

The RNA counts of genes of interest were normalised against the counts of the reference genes before the statistical analysis.

2.4 Statistical analysis

Relative expression data were log transformed for analysis. For each set of data ('Hayward'/Gold3 x Spring/Autumn x Early/Late), relative expression for each gene was analysed using a linear model with factors for replicate and the treatment x time combination. Where the treatment x time effect was significant, the means were compared using least significant differences. Analysis was done using the stats and predictmeans packages in R (R: A Language and Environment for Statistical Computing, R Core Team, R Foundation for Statistical Computing, Vienna, Austria, 2019).

3 Results

3.1 Effects of postharvest Actigard spray on gene expression

The control treatment (Kocide Opti) and the Actigard treatment (Actigard+Kocide Opti) each induced a change in the expression of the candidate genes in 'Hayward' vines at site C 'early-harvest' (Figure 2) and site D 'late-harvest' (Figure 3). In most cases gene up-regulation was significantly greater in the Actigard-treated vines than in the controls, however, the response patterns varied by orchard and by sample time point. At the 'early harvest' orchard (site C), the expression of PR1, DMR6, NIMIN2 and WRKY70 was significantly greater in Actigard-treated vines than in the control at both post-treatment time points. AP2-ERF2 and Gluc1 were induced to a similar degree in the control and the Actigard vines whereas BAD was not affected. At the 'late-harvest' orchard (site D), DMR6, NIMIN2 and WRKY70 were significantly more strongly expressed in Actigard-treated vines than in the controls at both post-treatment time points. PR1, PR5 and Gluc1 were greater in the Actigard treatment than in the control only at 6 d post treatment. BAD was up-regulated by Actigard at 1 d post spray, relative to the pre-spray level, but was down-regulated in the control. AP2ERF2 was strongly down-regulated in both the control and Actigard-treated vines.

The Gold3 samples degraded because of a freezer failure and were not analysed.

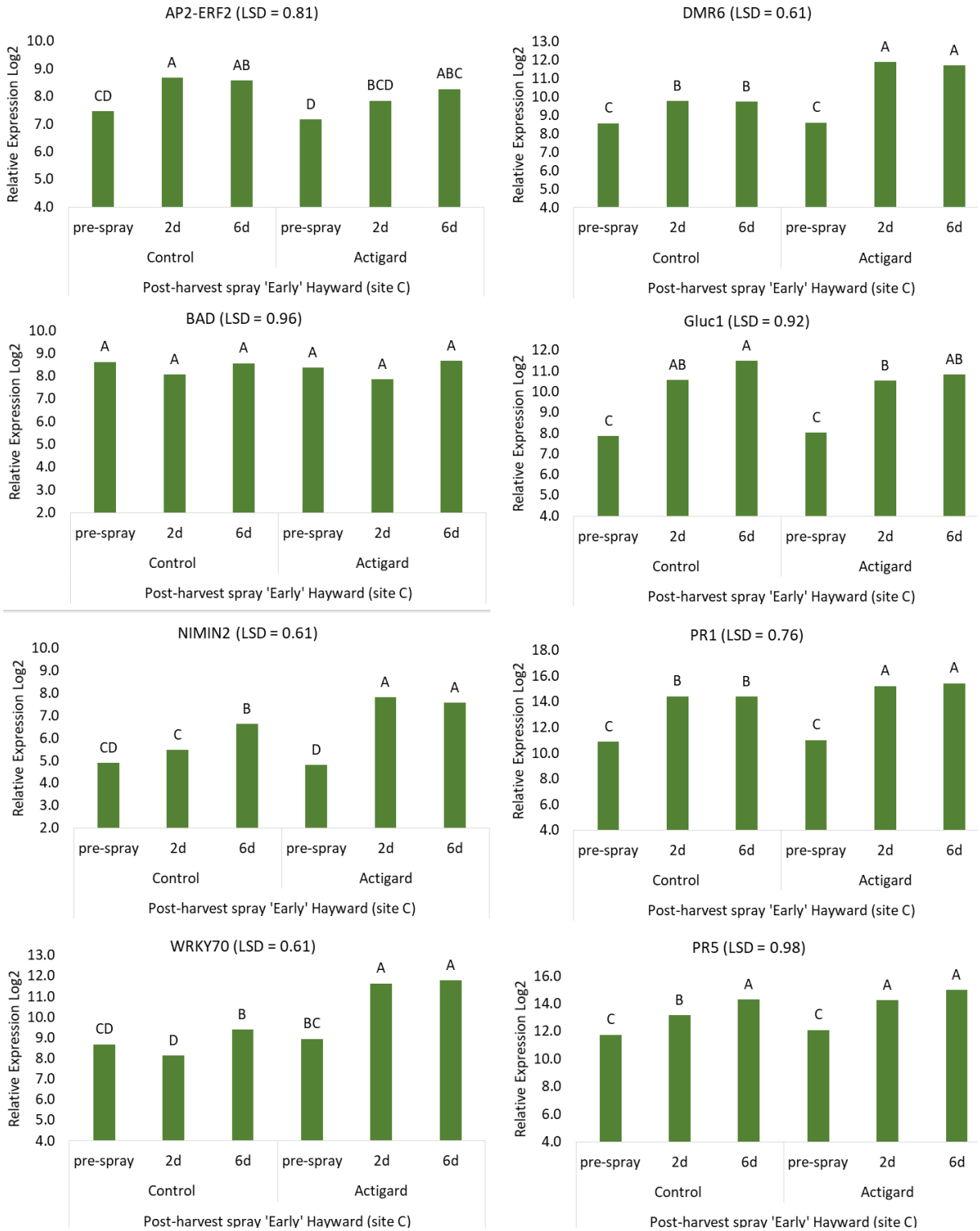

Figure 2. Effect of postharvest sprays on gene expression in *Actinidia chinensis* var. *deliciosa* 'Hayward' vines at site C. Fruit was harvested on 6 May 2019 and vines were treated with Kocide Opti® ('Control') or Kocide Opti+Actigard® ('Actigard') on 8 May. Leaf samples were taken before harvest (pre-spray) and at 2 days and 6 days after spray application. Gene expression was quantified using nanostring and data are presented as log2 means. Least Significant Difference (LSD) values are in parenthesis. Bars with the same letters are not significantly different at $p<0.05$.

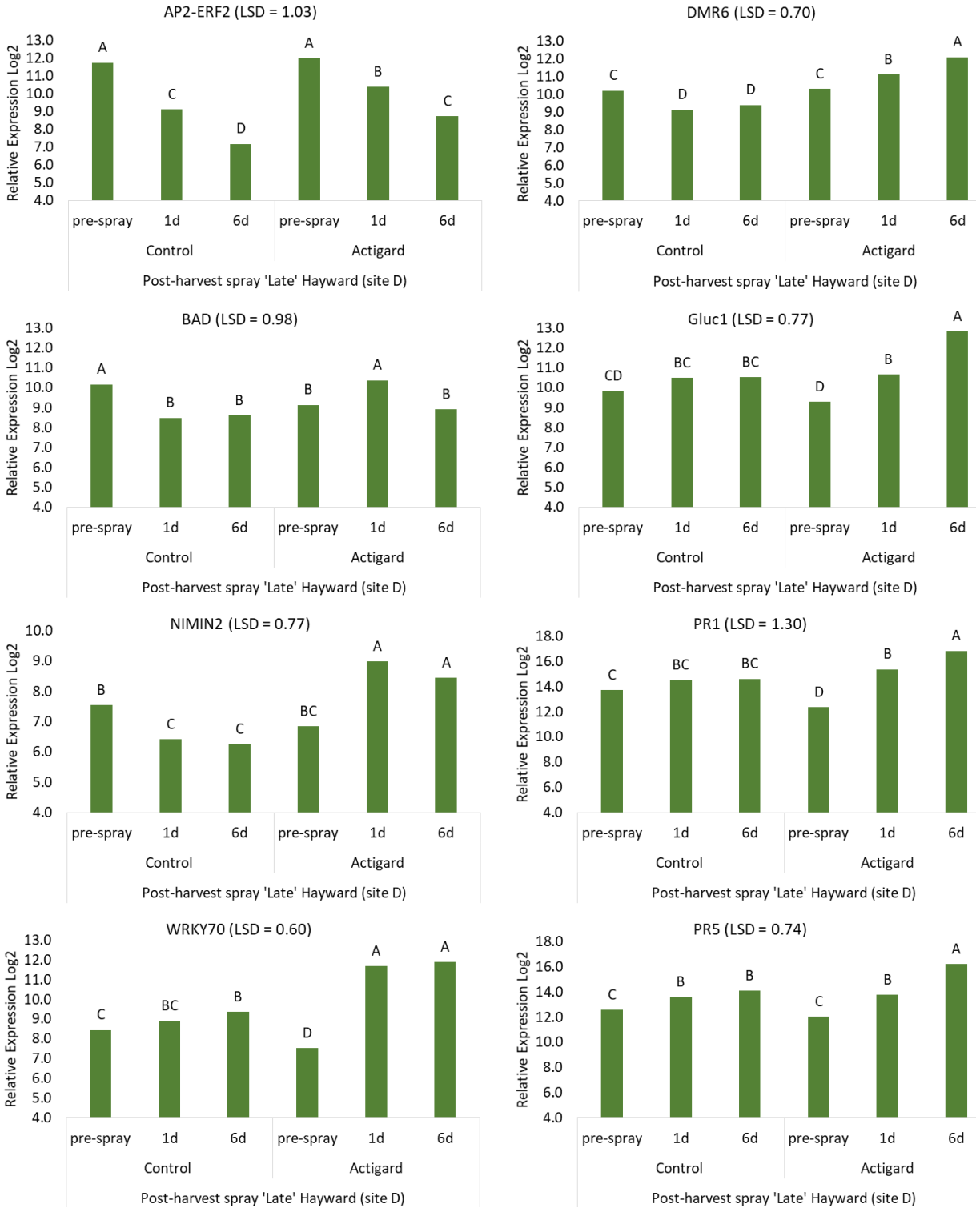

Figure 3. Effect of postharvest sprays on gene expression in *Actinidia chinensis* var. *deliciosa* 'Hayward' vines at site D. Fruit was harvested on 23 May 2019 and vines were treated with Kocide Opti® ('Control') or Kocide Opti+Actigard® ('Actigard') on 24 May. Leaf samples were taken before harvest (pre-spray) and at 1 days and 6 days after spray application. Gene expression was quantified using nanostring and data are presented as log2 means. Least Significant Difference (LSD) values are in parenthesis. Bars with the same letters are not significantly different at $p<0.05$.

3.2 Gene expression in spring

3.2.1 'Hayward'

Gene expression was measured before the pre-flowering spray (pre-spray) to determine if the response to the autumn Actigard spray persisted into spring. At site C, there was no significant difference in the pre-spray gene expression levels between Actigard and control vines except for APR-ERF2 and NIMIN2, which were lower in the Actigard vines (Figure 4). After pre-flowering sprays were applied, the expression of all genes, except AP2-ERF2 and BAD, increased significantly in Actigard-treated vines compared with the control at 2 days and/or 7 days post treatment.

At site D, the grower accidentally sprayed the whole trial area with Actigard, thus eliminating the opportunity to compare the Actigard and Control vines as originally intended. Instead, samples were collected at 2 and 7 days post treatment to investigate whether the postharvest treatments (Control and Actigard) affected vine responsiveness to the pre-flowering Actigard spray. In essence the new question was did the postharvest Actigard spray 'condition' or 'prime' the vines to respond more effectively to Actigard in spring? Two genes, Gluc1 at 2d and 7d, and PR5 at 7d, were expressed at significantly greater levels in the Actigard-Actigard vines compared with the Control-Actigard, suggesting that these genes may have been primed by the postharvest spray (Table 2). None of the other genes were affected.

Table 2. Gene expression in *Actinidia chinensis* var. *deliciosa* 'Hayward' vines at site D. The postharvest Control and Actigard®-treated vines were treated with Kocide Opti®+Actigard ('Actigard') on 22 October 2019. Leaf samples were taken at 2 days and 7 days after spray application. Gene expression was quantified using nanostring and data are presented as log2 means. Values in rows with the same letters are not significantly different at $p<0.05$. Bold text identifies where significant differences were recorded.

Gene transcript	2d post spray		7d post spray	
	Control-Actigard	Actigard-Actigard	Control-Actigard	Actigard-Actigard
AP2-ERF2	4.2a	4.2a	4.5a	4.5a
DMR6	12.7a	12.8a	11.8b	12.3ab
BAD	9.5a	9.6a	8.2b	7.9b
Gluc1	**10.4b**	**11.2a**	**10.4b**	**11.4a**
NIMIN2	9.3a	9.3a	7.6b	7.6b
PR1	16.3a	16.0ab	15.3c	15.6bc
PR5	11.7ab	12.1a	**11.3b**	**12.3a**
WRKY70	11.9a	11.8a	11.0b	10.7b

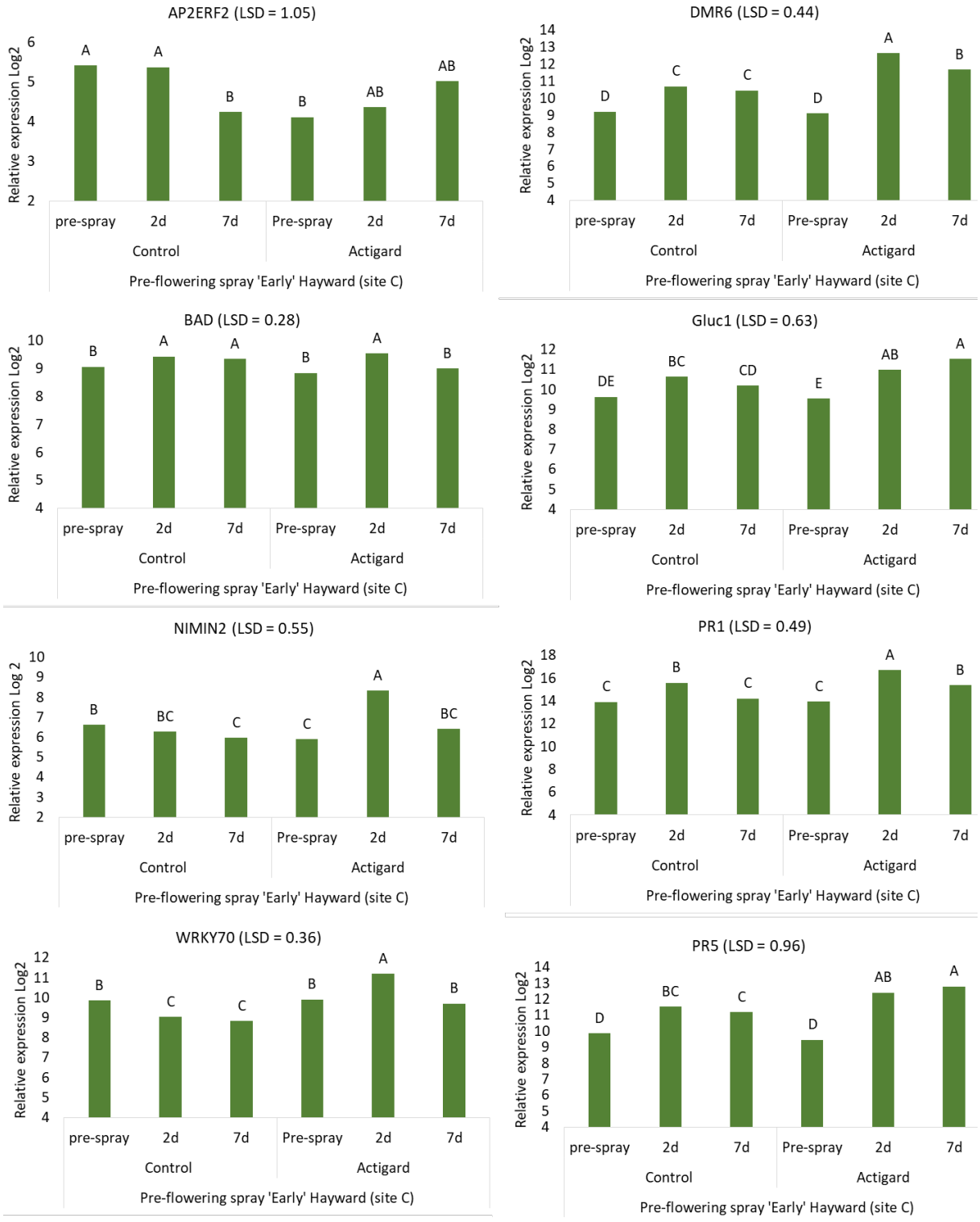

Figure 4. Effect of pre-flowering sprays on gene expression in *Actinidia chinensis* var. *deliciosa* 'Hayward' vines at site C. Vines were treated with Kocide Opti® ('Control') or Kocide Opti+Actigard® ('Actigard') on 30 October 2019. Leaf samples were taken one day before (pre-spray) and at 2 days and 7 days after spray application. Gene expression was quantified using nanostring and data are presented as log2 means. Least Significant Difference (LSD) values are in parenthesis. Bars with the same letters are not significantly different at $p<0.05$.

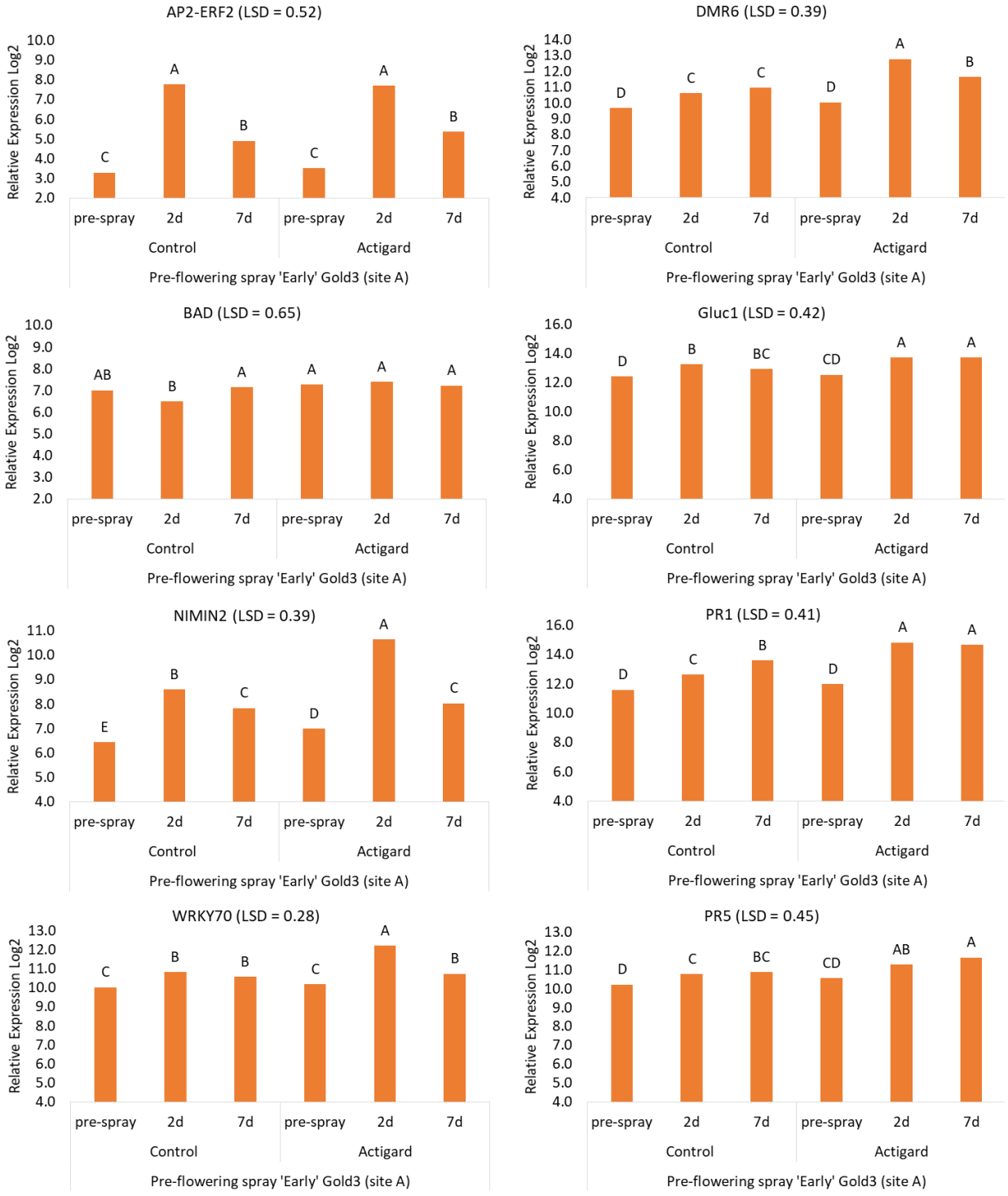

Figure 5. Effect of pre-flowering sprays on gene expression in *Actinidia chinensis* var. *chinensis* 'Zesy002' (Gold3) vines at site A. Vines were treated with Kocide Opti® ('Control') or Kocide Opti+Actigard® ('Actigard') on 9 October 2019. Leaf samples were taken two days before (pre-spray) and at 2 days and 7 days after spray application. Gene expression was quantified using nanostring and data are presented as log2 means. Least Significant Difference (LSD) values are in parenthesis. Bars with the same letters are not significantly different at $p<0.05$.

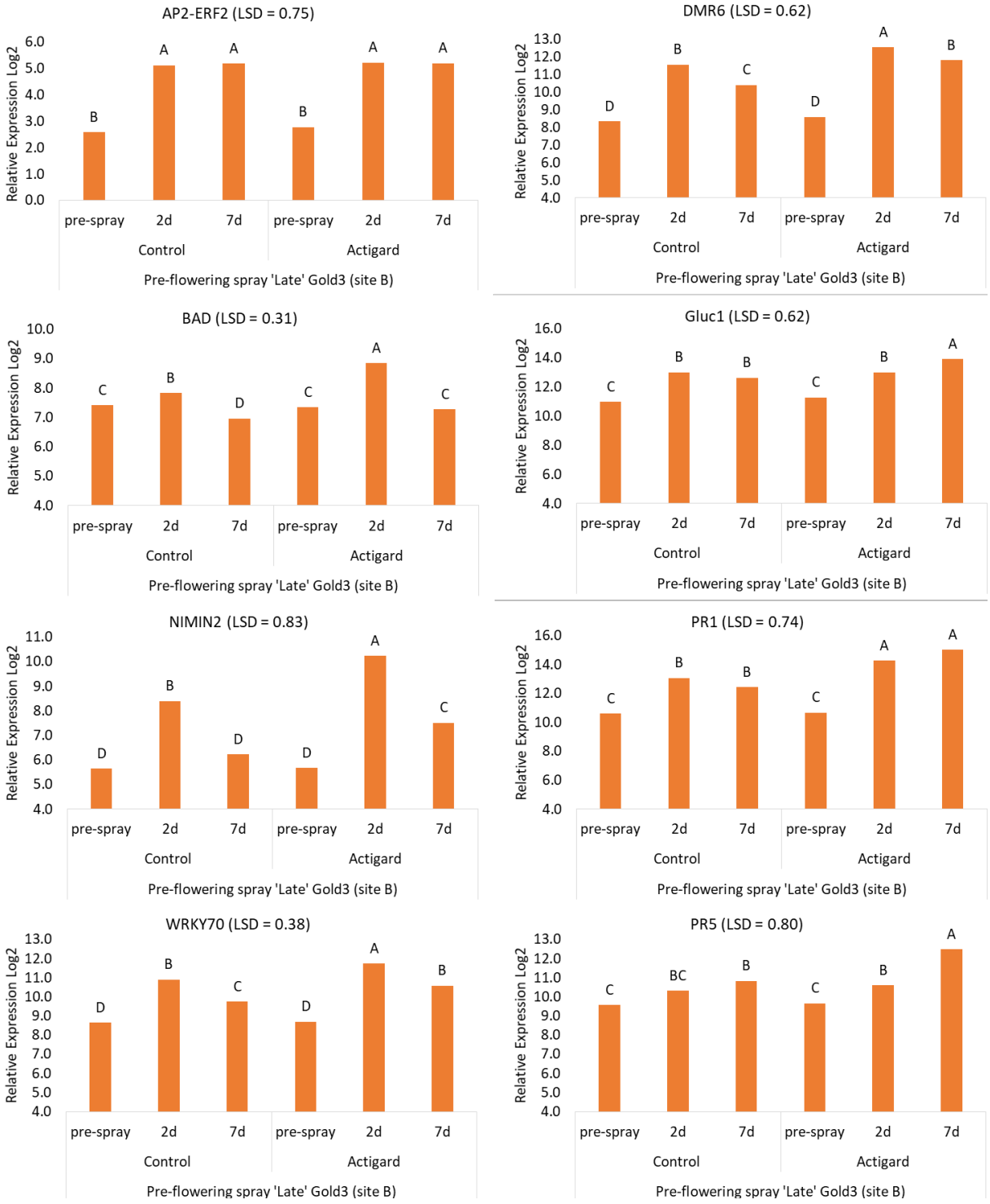

Figure 6. Effect of pre-flowering sprays on gene expression in *Actinidia chinensis* var. *chinensis* 'Zesy002' (Gold3) vines at site B. Vines were treated with Kocide Opti® ('Control') or Kocide Opti+Actigard® ('Actigard') on 16 October 2019. Leaf samples were taken two days before (pre-spray) and at 2 days and 7 days after spray application. Gene expression was quantified using nanostring and data are presented as log2 means. Least Significant Difference (LSD) values are in parenthesis. Bars with the same letters are not significantly different at $p<0.05$.

3.2.2 Gold 3

Only one gene, NIMIN2, was significantly greater in the Actigard vines than in the control before the pre-flowering spray was applied at site A (Figure 5). However, this was not repeated at site B where there were no significant differences in the pre-spray gene expression levels between Actigard and control vines (Figure 6). Taken together, these data suggest that there is no carry-over effect from the postharvest Actigard treatment. After the pre-flowering sprays, the expression of PR1, PR5, Gluc1, DMR6, NIMIN2 and WRKY70 were significantly greater in Actigard-treated vines than in the control at 2 days and/or 7 days post treatment. The expression of BAD was not affected by either treatment at site A, whereas at site B BAD increased significantly at 2 d after Actigard. APR-ERF2 increased after treatment at both sites but there was no significant difference between Actigard and the control.

4 Discussion

Postharvest application of Actigard has become widely accepted mostly based on observations from a field experiment on 'Hayward' vines in France in 2012 (Brun & Max 2013). However, the mode of action of postharvest application has not been reported. In this project the response of kiwifruit vines to postharvest Actigard application was determined by comparing gene expression patterns in leaves before and after spray application. The primary questions as outlined in the contract were; 1) does harvest date affect vine responsiveness to Actigard? and 2) is there a measurable carry-over effect in spring?

Does harvest date affect Actigard response?

Vines were responsive to Actigard after harvest. The defence 'marker genes' in 'Hayward' vines were up-regulated following Actigard spray in both the 'early-harvest' (early May) and 'late-harvest' (late May) orchards. The gene expression profiles for six of the eight genes exhibited subtle differences between sites but in general the data suggest that the harvest date and the treatment date did not affect vine responsiveness to Actigard. The most responsive genes, DMR6, NIMIN2, WRKY70, PR1 and PR5, are typically associated with the salicylic acid signalling pathway and this is consistent with Actigard operating as a functional mimic of salicylic acid. Two of the genes (AP2-ERF2 and BAD) either showed no response or a weak response to Actigard. No postharvest data were obtained for Gold3 vines because freezer failure resulted in sample breakdown.

Is there a measurable carry-over effect of postharvest Actigard spray in spring?

The carry-over effect of postharvest Actigard was examined in two ways: firstly, by determining if gene expression in spring, before further treatment, was greater in Actigard vines than in the control, and secondly by investigating if the postharvest Actigard spray affected vine responsiveness to Actigard in spring, the phenomenon of priming. The former 'direct response' was investigated in 'Hayward' vines at site C and in Gold3 at sites A and B whilst the latter 'priming response' was studied only in 'Hayward' vines at site D.

Direct effect: In most cases, there was no significant difference in 'pre-spray' gene expression levels between the Actigard and the control vines. The only exceptions were in Gold3 at site A, where the expression of NIMIN2 was greater in Actigard than the control, and in 'Hayward' at site C, where the expression of AP2-ERF2 and NIMIN2 were lower in Actigard than the control. Taken together there is no strong evidence of a direct carry-over effect from the postharvest Actigard spray. After spray applications in spring, all genes except AP2-ERF2 and BAD, were more strongly induced in Actigard-treated vines than with the control in both 'Hayward' and Gold3.

Priming response: There was evidence of priming in 'Hayward' vines at site D where the postharvest Actigard conditioned a stronger induction of Gluc1 and PR5 by Actigard in spring. However, caution is advised because the priming response was relatively weak (< 2-fold) and no other genes were affected.

Other observations

Gene expression was shown to increase in the control treatment (Kocide Opti) although generally to a lesser degree than in the Actigard treatment (Actigard plus copper tank mix). Copper has been reported to induce PR proteins in pepper (Chmielowska et al. 2010), *Arabidopsis* (Liu et al. 2018), and peach (Goto et al. 2019). However, copper is known to induce oxidative stress (Drakiewicz et al. 2004;

Liu et al. 2018) and it is possible that gene up-regulation in the control kiwifruit vines is associated with the stress response rather than defence elicitation *per se*. Nevertheless, the elevation of gene expression in the control somewhat complicates this study and it would be of interest to compare the response of vines to Actigard, with Actigard plus copper and copper only.

5 Conclusions and recommendations

In conclusion, this study has shown that kiwifruit vines respond to postharvest Actigard application by up-regulating the expression of defence-related genes. In 'Hayward' the gene expression profiles were generally similar in the 'early' and 'late' harvest orchards, suggesting that treatment date did not impact the Actigard response. There was no strong evidence to suggest that postharvest Actigard spray affected gene expression in spring either directly or indirectly (priming). This study has advanced our understanding of Actigard use in kiwifruit but there remain some important knowledge gaps as listed below:

- How does Gold3 respond to postharvest Actigard spray? – this was part of the original study but could not be completed because of the freezer failure.
- Are vines equally responsive to a second Actigard application at 21d after the first postharvest spray? – this is currently recommended in the spray guide with a cautionary note on leaf health.
- Do leaves of different age in the canopy vary in their response to Actigard? – care was taken with leaf selection in the current study but does that give a true reflection of the vine response?
- Does the gene expression response to Actigard alone differ with that of the Actigard plus copper mix used in this study? i.e. additive or antagonistic effects?
- During spring, how well does gene upregulation correlate with suppression of Psa?

6 Acknowledgements

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Appendix 1. Trial layouts

Site A ('Early' Gold3) Block 7

Row						
1	2	3	4	5	6	7
B	M	B	M			
B	M	A5	M			
B	M	C5	M	B		
B	M	A4	M	B		
B	M	C4	M	B	M	
B	M	A3	M	C2	M	
B	M	C3	M	A1	M	B
B	M	A2	M	C1	M	B
B	M	B	M	B	M	B
B	M	B	M	B	M	B

A1-A5 = Actigard®+Kocide Opti®, C1-5 = Kocide Opti, M= males, B= buffer (sprayed with Kocide Opti only)

Figure A1. 'Early' 'Zesy002'/Gold3 block at Site A one day before harvest 17 May 2019.

Site B ('Late' Gold3) Block 1

Block 1	Gold (2 vines/Bay)			
	1	2	3	
	M	B	M	
	M	C1	M	
	M	C2	M	
	M	A1	M	
	M	A2	M	
	M	C3	M	
	M	C4	M	
	M	A3	M	
	M	C5	M	
	M	A4	M	
	M	A5	M	
	M	B	M	

A1-A5 = Actigard®+Kocide Opti®, C1-5 = Kocide Opti, M= males, B= buffer (sprayed with Kocide Opti only).

Figure A2. 'Late' 'Zesy002'/Gold3 block at Site B one day before harvest 6 May 2019.

Site C ‘Early’ Hayward, Block 6

Row				
1	2	3	4	5
M	B	M	B	M
M	A5	M	C5	M
M	A4	M	C4	M
M	A3	M	C3	M
M	A2	M	C2	M
M	A1	M	C1	M
M	B	M	B	M
M	B	M	B	
M	B	M	B	
M	B	M	B	
M	B	M	B	

A1-A5 = Actigard®+Kocide Opti®, C1-5 = Kocide Opti, M= males, B= buffer (sprayed with Kocide Opti only).

Figure A3. ‘Early’ ‘Hayward’ block at Site A one day before harvest 3 May 2019.

Site D ‘Late’ ‘Hayward’, Block 1

Row						
1	2	3	4	5	6	7
B	M	B	M	B	M	B
B	M	B	M	B	M	B
B	M	B	M	B	M	B
B	M	C5	M	A5	M	B
B	M	C4	M	A4	M	B
B	M	C3	M	A3	M	B
B	M	C2	M	A2	M	B
B	M	C1	M	A1	M	B
B	M	B	M	B	M	B
B	M	B	M	B	M	B
B	M	B	M	B	M	B
B	M	B	M	B	M	B

A1-A5 = Actigard®+Kocide Opti®, C1-5 = Kocide Opti, M= males, B= buffer (sprayed with Kocide Opti only).

Figure A4. ‘Late’ ‘Hayward’ block at Site D one day before harvest 22 May 2019.

Appendix 2. Reference and target genes analysed in this project

Gene name	Gene ID	Target sequence
Eukaryotic small ribosomal subunit 40S	40S	CTACAAGCTCCTTGGTGGCCTCGCTGTTCGCAGGGCCTGCTATGGCGTTTTG AGATTTGTTATGGAGAGCGGGGCAAAGGGATGTGAGGTGATTGTTAGT
Ubiquitin-conjugating enzyme	UBC	ATCTGAACGATTACTCACATCCACAGAATCGACCATTTCAGGAACAAAAAAAT CCCCTCCAACAATTCACTGGCCTGATCGACGATCTAATTCTTCTCCG
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	ACTTTGTTGGTGACAGCAGATCGAGCATCTTTGATGCCAAGGCTGGGATTGC TTTGAACGACTTGTTCGTGAAACTGGTGTCTTGGTATGACAACGAGTG
Protein phosphatase 2	PP2A	TCCAGAATGGGCAATGCAGCACATAATTCCACAGGTATTGGACATGATTAGCA ACCCACATTATCTGTACCGTATGACCATACTACACTCGATCTCTCTT
Pathogenesis-related protein family 1	PR1	GTTTGTGGGCACTACACTCAAATTGTGTGGAGAAACTCGGTCCGGCTCGGGT GCGCTAGGGTTCGGTGCAATAGTGGGTCTTGGTTCGTTACTTGCAACT
APETALA2 ethylene responsive factor 2	AP2_ERF2	TTGGCCTATGACAGGGCGGCTTTTAGTATGCGTGGGGCGAAGGCTCTCCTCA ATTTTCCAGCTGAAGTAGGGGCAGAATCGTCCAAGCAAAGATTTACCC
Glucan endo-1,3-β-glucosidase	Gluc1	TGCTTGTGATTTCCCTCATAAAGAGGGCACTAGCAAAAAATAGAGTATGTACC GAGAGATTGCTCCTATGAAGACAGACAAAATATCTAATAAAGGAATA
Thaumatin-like protein TG4	PR5	AATATCATAAACAACTGCCCTTTCACCGTTTGGGCCGCTGCCGTTCCAGGTG GTGGCAAACGCCTTGACCGTGGCCAGAATTGGATCATCAATCCTGGTG
NIM-interacting protein 2	(NIMIN2)	AGCGGAGCGATGACGTGGAGGCCGACGCCAAGAAGGCGAGGGTAGGGGAA GATAACGGAAAAGTGACGGAGCCGGAGGACGATGAGGTGGAGGAGTTCTT
Downy mildew resistance 6	DMR6	ACGCCCTCACAATTTTGCTTCAGGACCTCCAAGTCTCAGGCCTACAAGTCCTC AAGGACGGCAAGTGGATGGCCGTCAAACCCCATCCCAATGCCTTTGT
WRKY transcription factor 70	WRKY70	TGGAGGAAATATGGACAAAAGGAGATCCTCAATGCCAAATTTCCAAGGTGCTA CTTTAGGTGCACACACAAGCCTGATCAAGGTTGCCTAGCAACAAAGC
Benzaldehyde dehydrogenase	BAD	GCCGATATAGAGCTGATTCCGATGGACTATGTGAACACCGCGATGGAGCGGC TTGTGAAGGCTGACGTTAGTCCCTTGAGGCATTTTGGGCTTGACAAGC

The target sequence is a 100 bp DNA sequence to which the capture probe and the reporter probe hybridise to form a target-probe complex which is purified, immobilised on a special support and counted using an automated fluorescence microscope. The 40S, UBC, GAPDH and PP2A genes were used as reference genes,

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