VI22043: Post-harvest Actigard® application - short and long-term responses

Cover Note

Background

Actigard® (Syngenta) is used for Psa management in kiwifruit. Four foliar applications are permitted per season, with application timings for fruiting vines restricted to the pre-flowering and post-harvest period. Actigard® is a plant defence elicitor which provides protection by inducing plant defence mechanisms, which allow plants to fight infection. An active metabolic response within the plant is critical for Actigard® to be effective.

Leaf condition in kiwifruit canopies can be highly variable at fruit harvest and it is not known if this affects the vine's capacity to respond to Actigard®. In this study, the effect of applying post-harvest Actigard® was investigated by comparing the expression of plant defence genes in leaf samples selected from control (untreated) versus treated vines.

What we asked

- Are kiwifruit vines responsive to Actigard® when it is applied after fruit harvest?
- Does harvest date affect the plant's response to Actigard®?
- Do effects on plant defence in autumn persist into the following spring to protect vines through a carryover effect?

What we did

Trials were conducted in 2019 and 2021. Two Hayward and two Gold3 (Zesy002) orchards in the Waikato were selected. These included one early and one late harvest site for each cultivar, with three to four weeks separating the respective harvest dates. After fruit harvest, the vines were treated with Kocide® Opti™ (control treatment) or with a tank mix containing Kocide® Opti™ plus Actigard® (Actigard® treatment). Actigard® activity was determined by comparing the expression of eight "defence" genes which were known to be involved in plant immunity for the control vines versus the Actigard® treated vines.

What we learnt

<u>Post-harvest</u>

Both Hayward and Gold3 vines were responsive to post-harvest applications of Actigard®. Results confirmed that Actigard® induced the expression of salicylic acid (SA) defence pathway genes in kiwifruit. This supports the value of applying Actigard® or copper plus Actigard® in the post-harvest window to help vines fight a Psa infection.

Harvest date per se did not affect gene expression but leaf quality did. As leaves began to senesce and show signs of yellowing the magnitude of defence gene upregulation declined.

This shows that, like all plant defence elicitors, Actigard® requires an active plant response for success therefore it needs actively photosynthesising leaves (i.e., green) for best results. The levels of gene expression in the Actigard®-treated vines returned to baseline levels by 20 days after treatment.

Spring

The plant defence genes did not remain upregulated through to the following spring as a result of the post-harvest application of Actigard®. It is therefore likely that applying Actigard® at this timing does not have a carry-over protective effect. Spring applications are still recommended for protection in the following growing season.

Plant & Food™ Research
Rangahau Ahumāra Kai

PFR SPTS No. 22537

Postharvest Actigard® application — short- and long-term responses

Reglinski T, Vanneste J, Schipper M, Oldham J, Cornish D, Yu J, Fehlmann C, Parry F, McAlinden J, Hedderley D

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Contents

Executive summary

Postharvest Actigard® application — short- and long-term responses

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In 2020 we reported that a single postharvest application of Actigard® induced a strong upregulation of defence genes in *Actinidia chinensis* var. *deliciosa* 'Hayward', both in 'early' and 'late' harvest orchards (Reglinski et al. 2020). This report presents the results from a complementary study to confirm the 'Hayward' findings and to investigate effects of postharvest Actigard application in *Actinidia chinensis* var. *chinensis* 'Zesy002' vines. As in project VI1900, the response to Actigard was determined by comparing the expression of eight "defence marker" genes in leaves from Control vines (Kocide® Opti™) with that from vines treated with Kocide Opti and Actigard.

The results show that postharvest application of Actigard induced an upregulation of "defence marker" genes in 'Zesy002' and 'Hayward' vines. The gene expression patterns varied over time and differed between sites and cultivars. The most responsive genes to Actigard application in both cultivars were *BAD, AcDMR6, NIMIN2*, and *WRKY70i*. There was also evidence that *PR1, PR2* and *PR5* were upregulated in 'Zesy002'. The gene expression data are consistent with Actigard operating by upregulation of the salicylic acid defence pathway.

Differential gene expression patterns between 'Zesy002' at site A (early harvest) and site B (late harvest) suggested that harvest date can affect responsiveness to Actigard. At the early-harvest site gene upregulation was significant at 1 day and 7 days after Actigard treatment, whereas at the late-harvest site gene upregulation was significant only at 1 day post treatment. There was a 3-week gap between harvests at site A and site B, with canopy deterioration (leaf yellowing) becoming more evident at site B over time and leaves yielded much lower amounts of RNA. Thus leaf health was a strong driver for vine responsiveness. Frost damage at 'Hayward' site C precluded the harvest date comparison with site D in the current study. However, in project VI19001 there was no effect of harvest date in 'Hayward' vines but neither was there an obvious deterioration in leaf health. This also suggests that canopy health/integrity is the more appropriate factor to guide spray application than harvest date.

There was no evidence that the effects of postharvest Actigard application are carried over into spring. There was no difference in gene expression levels between Actigard-treated and Control vines in 'Hayward' or 'Zesy002' vines immediately before pre-flowering applications i.e. no carryover effect. Moreover, the Actigard-treated vines were not conditioned to respond more effectively to the pre-flowering Actigard application i.e. there was no priming response.

In conclusion, based on gene expression analyses, the results in this study indicate that kiwifruit vines are responsive to Actigard after fruit harvest. However, the level of defence induction by Actigard is dependent on leaf health and application is not recommended if the leaf canopy is at an advanced stage of deterioration (Kiwifruit Vine Health Technote 104289 (kvh.org.nz). Currently the determination of canopy health is subjective and further studies are recommended to develop a canopy health index as a decision tool to guide postharvest Actigard application. The absence of a carryover effect of postharvest Actigard on gene expression in spring does not negate its potential to affect *Pseudomonas syringae* pv. *actinidiae* (Psa). This remains is a significant knowledge gap with regard to the long-term effects of postharvest Actigard and further studies are recommended to investigate potential relationships between gene upregulation after harvest with Psa populations and symptom expression in spring. This would complement the current study and provide a more complete and robust assessment of the short- and long-term effects of postharvest Actigard applications.

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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) (Kiwifruit Vine Health Psa management chart 2021–22). 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 kiwifruit growing season but sprays are restricted to the pre-flowering and postharvest period on fruiting kiwifruit vines.

Actigard, like all plant defence elicitors, depends on an active plant response for efficacy and in the field this can be affected by various biotic and abiotic factors including plant developmental stage, leaf age and mineral nutrition (Steimetz et al. 2012; Walters et al. 2013; Reglinski et al. 2014). The use of Actigard on kiwifruit after fruit harvest became more widespread following a report that postharvest application reduced Psa expression on *Actinidia chinensis* var. *deliciosa* 'Hayward' vines in the following season (Brun & Max 2013). However, the effectiveness and mode of action of postharvest application in kiwifruit have not been reported in the scientific literature. In 2020, Zespri Group Limited funded a study to investigate the response of 'Hayward' and *A. chinensis* var. *chinensis* 'Zesy002' vines to a single postharvest Actigard application (Reglinski et al. 2020). The project aims were to determine if harvest date affected vine the response to Actigard, as measured by defence gene regulation, and to investigate if the response persisted into the following spring. In 'Hayward', the postharvest Actigard application induced a strong upregulation of defence genes regardless of harvest date. Defence gene expression in the Actigard-treated vines returned to control rates in spring; however, there was weak evidence that the postharvest application conditioned the vines to respond more strongly to Actigard in spring, a phenomenon known as priming (Conrath et al. 2015). Unfortunately no data were obtained for 'Zesy002' in this study because leaf samples degraded during a freezer breakdown, hence the need to repeat the orchard trials in the current study.

The aims of this study are:

- Investigate the responses of 'Hayward' and 'Zesy002' vines to postharvest Actigard application. As in project VI1900, vine response was determined by comparing the expression of eight "defence marker" genes in leaves from Control vines (Kocide® Opti™) with that from vines treated with Kocide Opti and Actigard
- Extend the investigation where appropriate to a second postharvest Actigard application after 21 days, as per label recommendation
- Determine if effects of postharvest Actigard applications persist into the following spring; this could be expressed as higher defence gene expression in new leaves before pre-flowering sprays are applied (i.e. direct carry-over) or as an enhanced response to pre-flowering Actigard application (i.e. priming/conditioning response).

1.1 Orchard sites

The trials on 'Zesy002' (sites A and B) and 'Hayward' (sites C and D) took place at four sites in the Waikato region. The kiwifruit sites were selected to be representative of early (A, C) and late (B, D) fruit harvests for each cultivar, with 3–4 weeks separating the respective harvest dates. After fruit harvest, the vines were treated with Kocide Opti (Control treatment) or with a tank mix containing Kocide Opti plus Actigard (hereon referred to as Actigard). Leaf samples were collected before and after spray application according to the schedule outlined in Figure 1. The actual dates for each site are shown in Table 1. Kocide Opti was applied at a concentration of 90 g/100 L after harvest and at 70 g/100 L for the pre-flowering application. Actigard was applied at 20 g/100 L regardless of season. Treatments were applied at a spray rate of 1000 L/ha using a pressurised handgun. There were five single-vine replicates per treatment at each site.

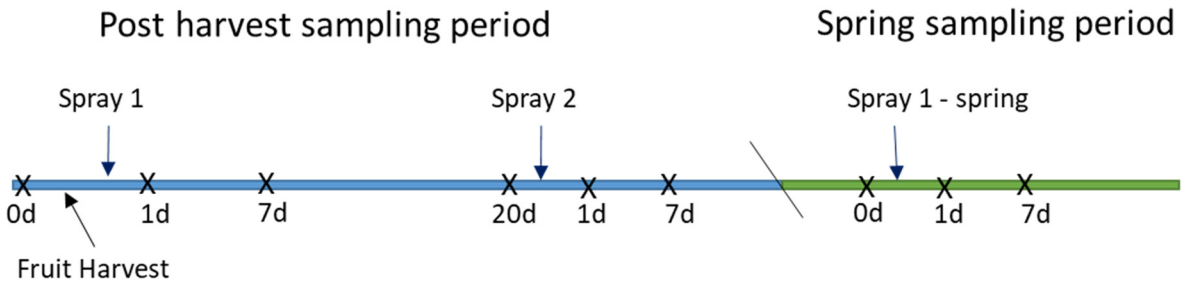

Figure 1. Proposed timing for Kocide® Opti™ and Kocide® Opti™+Actigard application and leaf tissue sampling in *Actinidia chinensis* var. *deliciosa* 'Hayward' and in *A. chinensis* var. *chinensis* 'Zesy002' vines during the postharvest period and the subsequent spring in 2021.

Table 1. Dates for fruit harvest, applications of Kocide® Opti™ and Kocide® Opti™+Actigard and leaf sampling from *Actinidia chinensis* var. *deliciosa* 'Hayward' and in *A. chinensis* var. *chinensis* 'Zesy002' vines during 2021.

		Site A	Site B	Site C	Site D
Application period	Activity	'Zesy002'	'Zesy002'	'Hayward'	'Hayward'
Postharvest	Trial Layout	13-Apr	16-Apr	13-Apr	7-May
	Pre-harvest sample	22-Apr	11-May	21-Jun	1-Jun
	Fruit harvest	28-Apr	15-May	23-Jun	2-Jun
	Application	29-Apr	17-May	24-Jun*	3-Jun
	sample 1 d	30-Apr	18-May	*	4-Jun
	sample 7 d	6-May	24-May	-	10-Jun
	sample 19/22 d	18-May	8-Jun	-	22-Jun
	Application	20-May	9-Jun	-	NA
	sample 1 d	21-May	10-Jun	-	NA
	sample 7 d	27-May	16-Jun	-	NA
Pre-flowering	Pre-spray sample	11-Oct	12-Oct	-	1-Nov
	Application	11-Oct	13-Oct	-	3-Nov
	sample 1 d	12-Oct	14-Oct	-	4-Nov
	sample 7 d	18-Oct	19-Oct	-	10-Nov

* An overnight frost destroyed the leaf canopy at site C (Appendix 2). Zespri Group Limited were notified and the decision was taken to discontinue studies at this site.

1.2 Trial design

During the postharvest period there were only two treatments, Control and Actigard. Each treatment was applied to two main plots at each site, with each plot comprising at least 10 vines (see Appendix 1 for trial layouts). The five sample vines for each treatment were randomly selected across the two main plots. In spring the postharvest main plots were split to determine if postharvest treatments affected responsiveness to Actigard in spring. Thus, half the vines treated with Actigard after harvest were again treated with Kocide Opti+Actigard in spring (Actigard–Actigard) and half were treated with Kocide Opti in spring (Actigard–Control). Similarly, the postharvest control vine plots were split and treated with either Kocide Opti or Kocide Opti+Actigard in spring. The four treatment combinations are shown in Table 2 (see Appendix 1 for trial designs).

Table 2. Treatments for postharvest and spring periods.

	Treatment	Postharvest application	Spring application
1	Control–control	Kocide® Opti™	Kocide Opti
2	Control–Actigard®	Kocide Opti	Kocide Opti+Actigard
3	Actigard–control	Kocide Opti+Actigard	Kocide Opti
4	Actigard–Actigard	Kocide Opti+Actigard	Kocide Opti+Actigard

1.3 Leaf sampling

Leaf samples were taken from each replicate by removing five leaves from within a 1.5-m radius of the main trunk. The third or fourth leaf from the base of the shoot was selected and care was taken to avoid damaged or blemished leaves as far as possible. 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 replicate 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). Leaf sampling became more problematic during the later postharvest period because of a deterioration in leaf qualify (Figure 2). No leaves were collected from site C because of severe damage caused by an overnight frost after harvest on 23–24 June 2021 (Appendix 2). At site D, leaf samples were collected up to day 19 after the first postharvest Actigard application only, by which time the leaf canopy was too thin (~10%) to justify a second application.

Figure 2. Representative leaves from site B showing changes in the quality of *Actinidia chinensis* var. *chinensis* 'Zesy002' leaves over the course of the postharvest sampling period. The leaves were collected from replicate 1 before the postharvest application (top right) and then at 1 day, 7 days and 22 days after the first application and 1 day and 7 days after the second application.

1.4 Gene expression analysis

Gene expression was determined by molecular barcoding technology using the Plexset® platform from NanoString Technologies Inc. (Seattle WA, USA). The results were analysed using the nSolver™ 4.0 software provided by NanoString. The four reference genes and eight target genes were the same as those used in project VI19001 'Autumn application of Actigard®' (Table 3).

Table 3. Reference and target genes selected for gene expression analysis by PlexSet® Nanostring. Gene expression was measured in leaves sampled from *Actinidia chinensis* var. *deliciosa* 'Hayward' and in *Actinidia chinensis* var. *chinensis* 'Zesy002' vines during the postharvest periods and subsequent spring 2021.

Gene name	Gene ID	Function
Eukaryotic small ribosomal subunit 40S	40S	Reference
Ubiquitin-conjugating enzyme	UBC	Reference
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	Reference
Protein phosphatase 2	PP2A	Reference
Pathogenesis-related protein family 1	*PR1*	Target gene
APETALA2 ethylene responsive factor 2	*AP2_ERF2*	Target gene
Glucan endo-1,3-β-glucosidase	*PR2*	Target gene
Thaumatin-like protein TG4	*PR5*	Target gene
NIM-interacting protein 2	*NIMIN2*	Target gene
Downy mildew resistance 6	*DMR6*	Target gene
WRKY transcription factor 70	*WRKY70*	Target gene
Benzyl alcohol dehydrogenase	*BAD*	Target gene

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 3. 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. Leaf tissue collected at site B from day 22 onward during the postharvest period was of poor quality (Figure 2) and yielded low amounts of RNA; gene expression data for these samples are not presented. Leaf tissue collected in spring was processed and analysed separately from the postharvest samples.

1.5 Statistical analysis

Relative expression data were log transformed for analysis. For each set of data, the relative expression for each gene was analysed using a linear model with factors for replicate, time, replicate x time, first treatment, second treatment, and the interactions between time and the two treatment factors. Where the effects were significant, the means were compared using least significant differences; comparisons were made within each time. Analysis was done using Genstat version 20 (VSNI Ltd, Hemel Hempstead, UK, 2020).

2 Results

2.1 Postharvest Actigard application activates defence gene expression in 'Zesy002' and 'Hayward' vines

Site A ('Zesy002')

Fruit were harvested on 28 April 2022 and vines were sprayed with Actigard on 29 April. At 1 day after treatment the expression of *BAD, AcDMR6, NIMIN2, and WRKY70i* was significantly greater in Actigard-treated vines than in the control vines; *AcDMR6, NIMIN2*, and *WRKY70i* were each upregulated by 3–5 fold and *BAD* by over 20-fold (Figure 3). By day 7, the expression of *BAD* in the Actigard-treated vines was not different from that in the control; however, *AcDMR6, NIMIN2*, and *WRKY70i* expression remained 5–6 fold greater, and *PR1* and *PR5* were ~3 fold greater in Actigard-treated vines than in the control. By 18 May, 19 days after the first Actigard application, there was no significant difference in gene expression between the Actigard-treated and the control vines. A second Actigard application was made on 20 May. At 1 day after treatment *BAD* and *NIMIN2* were ~10 fold greater in Actigard-treated vines than in the control, whilst *AcDMR6* and *WRKY70i* were ~3 fold and ~7 fold greater, respectively. By day 7, four genes remained upregulated in Actigard-treated vines compared with the control, namely *AcDMR6* (2 fold), *NIMIN2* (5 fold), *WRKY70i* (3 fold) and *PR2* (2 fold).

Site B ('Zesy002')

Fruit were harvested on 15 May and Actigard was applied on 17 May. At 1 day after Actigard application, five genes were expressed more strongly in Actigard-treated vines than in the control: these were *AP2_ERF2* (2 fold), *AcDMR6* (3.3 fold), *BAD* (5.6 fold), *NIMIN2* (11.6 fold) and *WRKY70i* (5.5 fold) (Figure 4). By day 7, there were no significant difference between Actigard-treated vines and the control. Leaf tissues sampled on 8 June (22 days after spray 1) and again at 1 day and 7 days after spray 2 (9 June) were of poor quality (Figure 2) and yielded low quantities of RNA. As a result, these data are not shown.

Site C ('Hayward')

Fruit were harvested on 23 June but no Actigard was applied because the canopy was destroyed by overnight frost. No further activities were performed at this site. This was the "early" harvest 'Hayward' site in project VI19001 and was therefore selected as the "early" site in the current study. The delay in fruit maturation at this site was therefore unexpected.

Site D ('Hayward')

Fruit were harvested on 2 June and Actigard was sprayed on 3 June. On 4 June, 1 day after treatment, the expression of *BAD, NIMIN2*, and *WRKY70i* was 2–4 fold greater in Actigard-treated vines than in the control (Figure 5). One week after treatment, *AcDMR6* and *WRKY70i* were over 2 fold greater in the Actigard-treated than in the control vines; however, by day 19 (22 June) there was no significant difference in gene expression between treatments. The leaf canopy was less than 10% at this time and so a second spray was not applied.

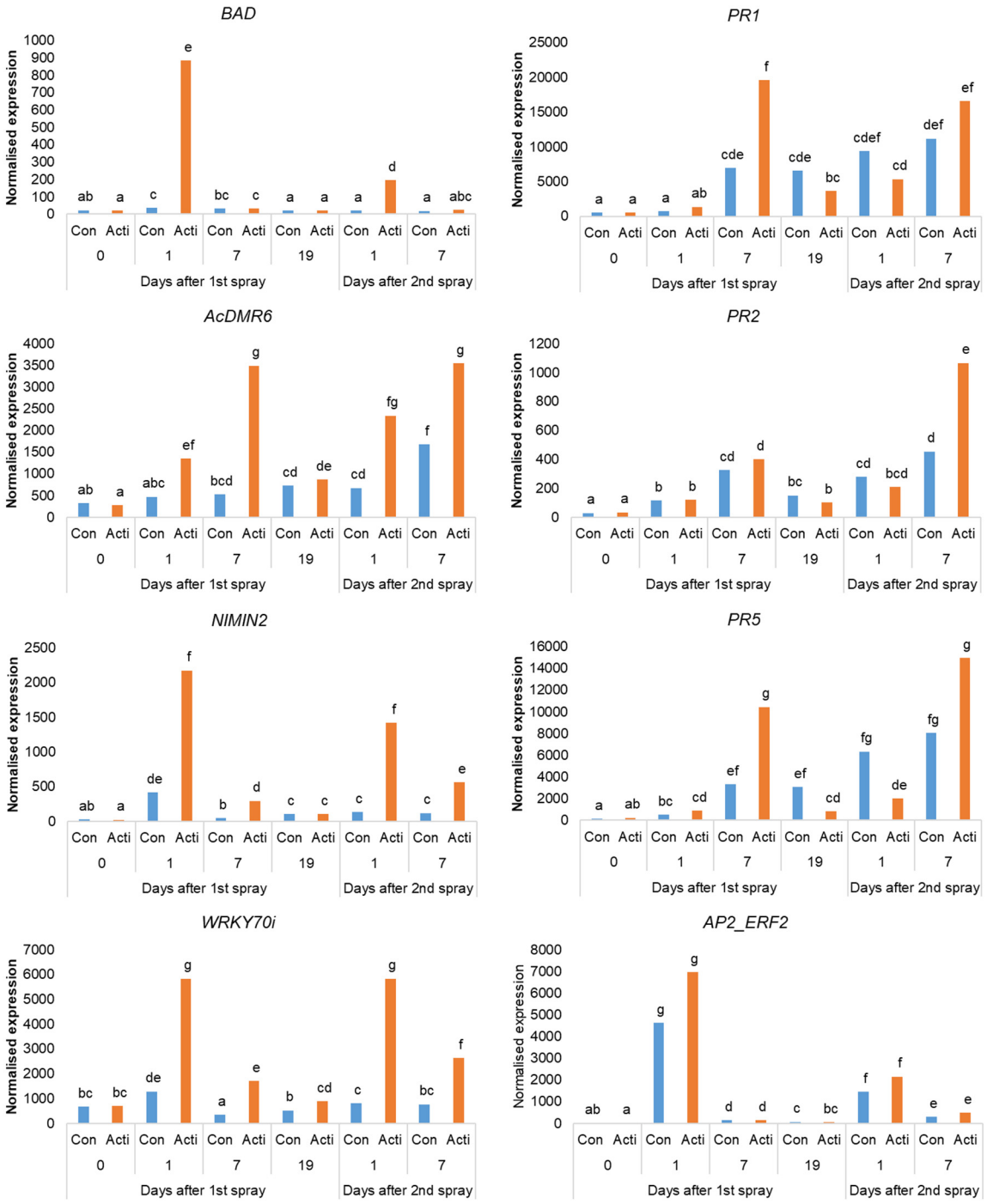

Figure 3. Effect of postharvest applications with Kocide® Opti™ (Con) or Kocide Opti+Actigard® (Acti) on gene expression in *Actinidia chinensis* var. *chinensis* 'Zesy002' vines at site A. Vines were sprayed on 29 April 2021 and leaf samples were taken 1 day before (0) and at 1 day, 7 days and 19 days after treatment. A second spray was applied on 20 May, with leaf samples taken at 1 and 7 days later. Gene expression was quantified by PlexSet® Nanostring analysis and data are presented as back-transformed means. Bars with the same letters for each gene are not significantly different at $p<0.05$ (based on $\log_2$ analysis).

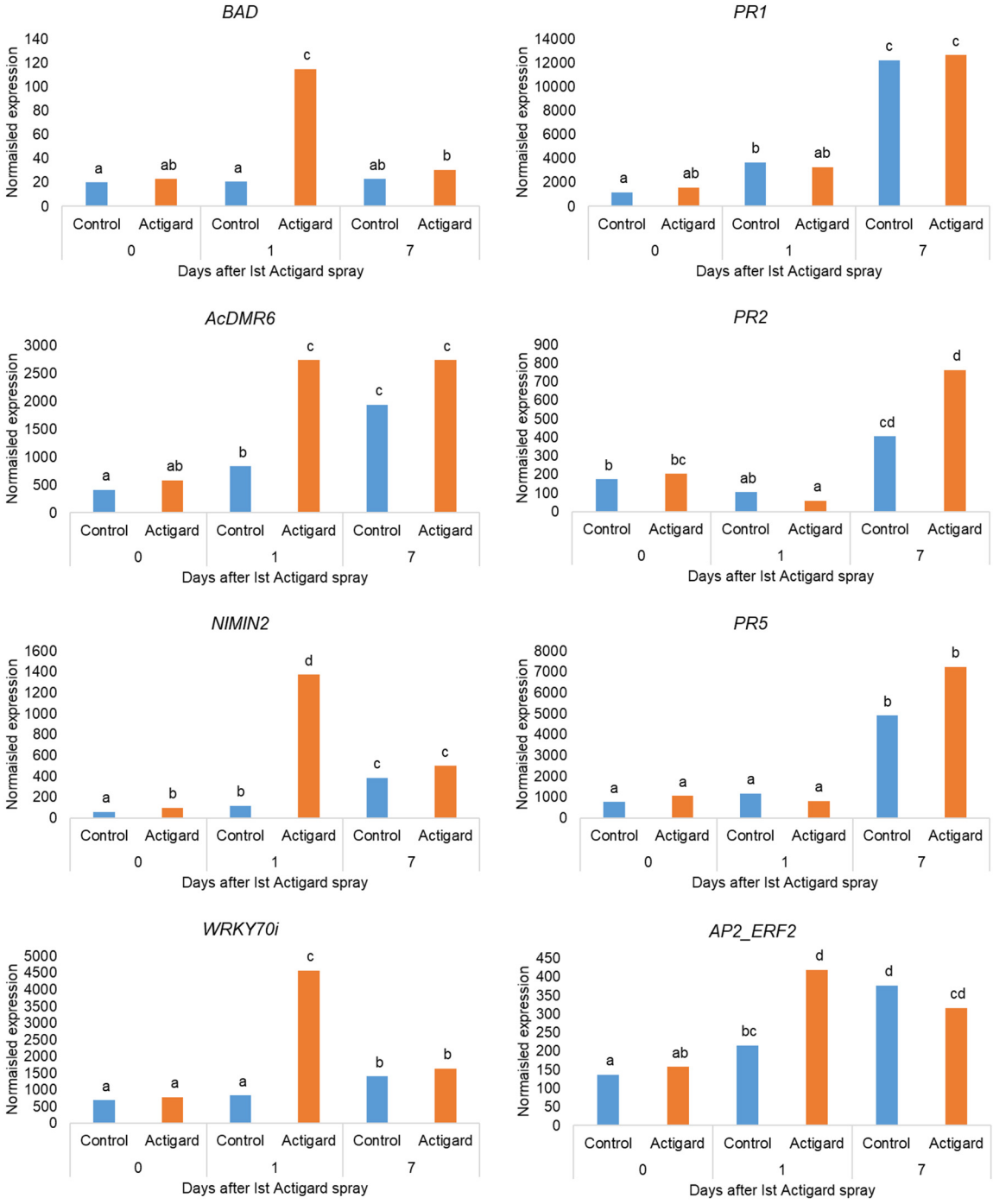

Figure 4. Effect of postharvest applications with Kocide® Opti™ (Control) or Kocide Opti+Actigard® (Actigard) on gene expression in *Actinidia chinensis* var. *chinensis* 'Zesy002' vines at site B. Vines were sprayed on 17 May 2021 and leaf samples were taken 1 day before (0) and at 1 day and 7 days after treatment. Gene expression was quantified by PlexSet® Nanostring analysis and data are presented as back-transformed means. Bars with the same letters for each gene are not significantly different at $p<0.05$ (based on $\log_2$ analysis).

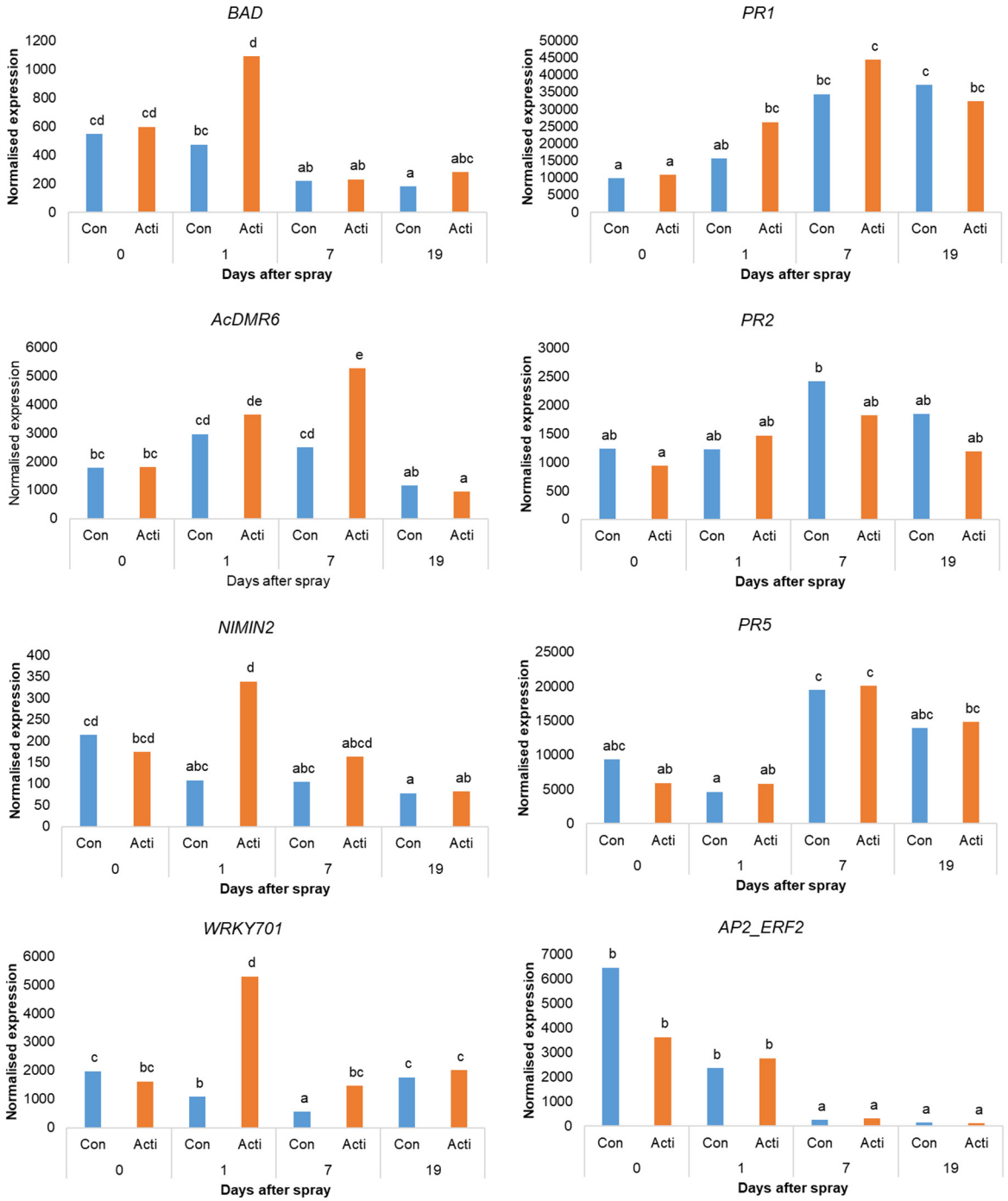

Figure 5. Effect of postharvest applications with Kocide® Opti™ (Con) or Kocide Opti+Actigard® (Acti) on gene expression in *Actinidia chinensis* var. *deliciosa* 'Hayward' vines at site D. Vines were treated on 3 June 2021 and leaf samples were taken 1 day before (0) and at 1 day, 7 days and 19 days after spray application. Gene expression was quantified by PlexSet® Nanostring analysis and data are presented as back-transformed means. Bars with the same letters for each gene are not significantly different at $p<0.05$ (based on $\log_2$ analysis).

2.2 Defence gene upregulation following postharvest Actigard application does not persist into spring

Gene expression levels before the application of pre-flowering applications did not differ significantly between vines treated with Kocide Opti at harvest (C) and those treated with Actigard (A) at any of the trial sites (Figures 6, 7 and 8). This indicates that upregulation of gene expression after postharvest Actigard application does not persist into spring.

2.3 Postharvest Actigard application does not affect responsiveness to pre-flowering applications

The phenomenon of “priming” describes an enhanced state of readiness which is induced by elicitor treatment, where defence genes express an amplified response to a subsequent stimulus such as a pathogen or second elicitor treatment (Conrath et al. 2015). In this study, evidence of priming was investigated by comparing gene expression patterns between the four postharvest/spring application combinations; vines treated with Actigard after harvest were sprayed with Actigard (AA) or Kocide Opti (AC) in spring, whilst postharvest controls (Kocide Opti) were treated with Kocide Opti (CC) or Actigard (CA) (Table 2). There was there was no statistical difference between the AA and CA treatments at 1 d or 7 d after Actigard application at any trial site (Figures 6, 7 and 8), suggesting that postharvest Actigard did not enhance responsiveness to Actigard in spring i.e. no priming response. The gene response patterns following the pre-flowering Actigard application, however, did vary between site and cultivar. At 1 d after the pre-flowering application, *BAD, AcDMR6, NIMIN2* and *WRKY70i* were significantly upregulated in Actigard-treated vines (CA and AA) compared with the controls (CC and AC) at all trial sites (Figures 6, 7 and 8). The expression levels of these genes were no longer significantly different from those of the controls by 7 d post treatment, except for *AcDMR6* and *NIMIN2* in ‘Zesy002’ at site B, which remained upregulated in Actigard-treated vines (Figure 7). The effects of Actigard on the expression of *PR1, PR2* and *PR5* , compared with controls, were greatest in ‘Zesy002’ at site B, where *PR1* was significantly upregulated at 1 d and 7 d, and *PR2* and *PR5* were upregulated at 7 d. In ‘Zesy002’ at site A, *PR1, PR2* and *PR5* tended to be more strongly expressed in Actigard-treated vines (CA and AA) than in controls (CC and AC) at day 7, but the differences were generally not statistically significant. Similarly, in ‘Hayward’ at site D, the expression of *PR1, PR2* and *PR5* tended to be greater in AA vines than in vines in other treatments at 7 d post application, but the differences were not statistically significant.

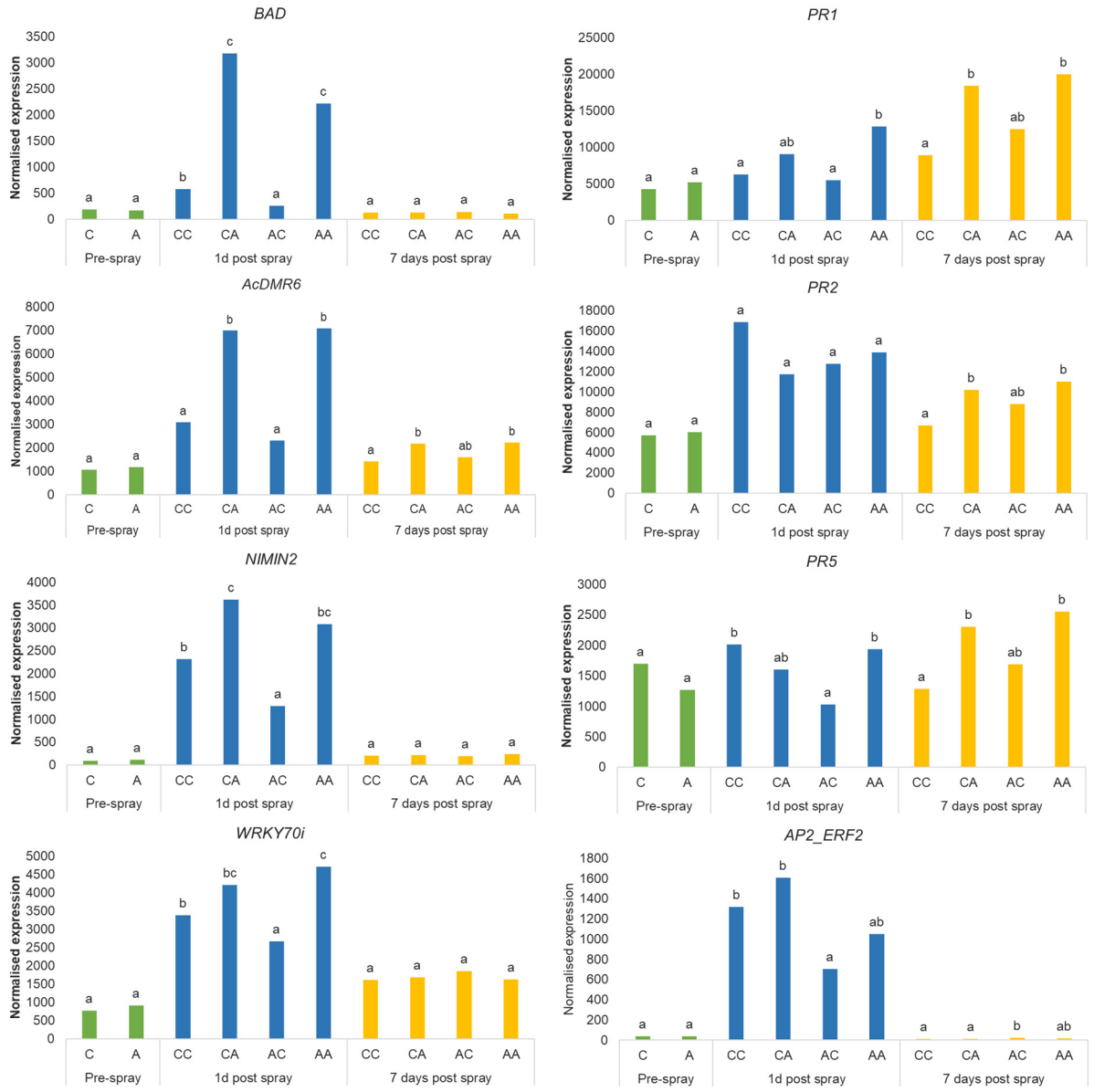

Figure 6. Gene expression in leaves of *Actinidia chinensis* var. *chinensis* 'Zesy002' vines at site A. Leaves were sampled on 11 October 2021 (pre-application), before application of Kocide® Opti™ (Control) or Kocide Opti+Actigard® (Actigard), and again at 1 day and 7 days post application. Gene expression was quantified by PlexSet® Nanostring analysis and data are presented as back-transformed means. Bars at each timepoint with the same letters are not significantly different at $p<0.05$ (based on $\log_2$ analysis). Treatment codes are: C = Control, A = Actigard, CC = Control–Control, CA = Control–Actigard, AC = Actigard–Control, AA = Actigard–Actigard, where the first letter identifies the postharvest application and the second letter the spring application.

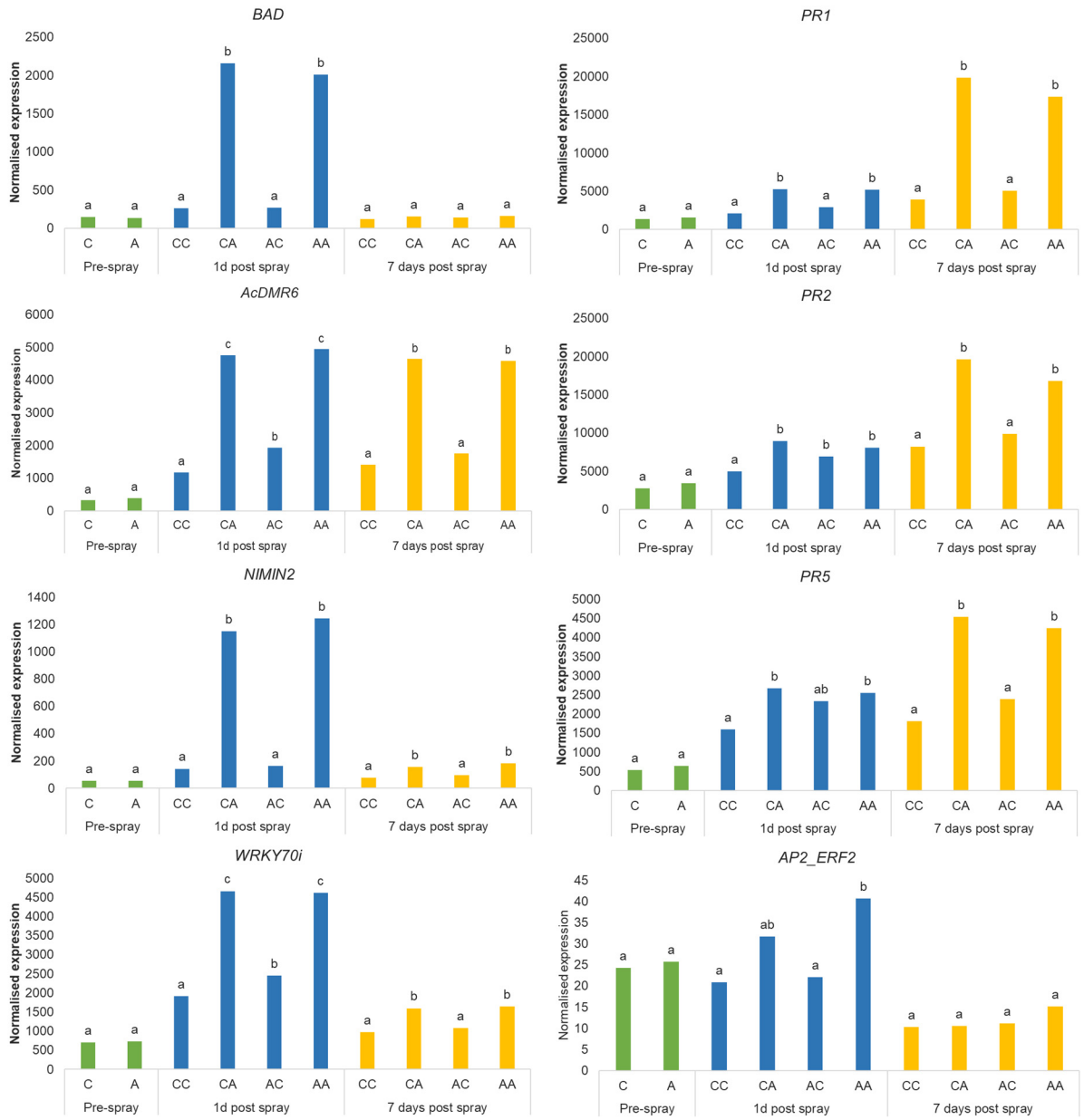

Figure 7. Gene expression in *Actinidia chinensis* var. *chinensis* 'Zesy002' vines at site B. Leaves were sampled on 12 October 2021 (pre-application), before application of Kocide® Opti™ (Control) or Kocide Opti+Actigard® (Actigard), and again at 1 day and 7 days post application. Gene expression was quantified by PlexSet® Nanostring analysis and data are presented as back-transformed means. Bars at each timepoint with the same letters are not significantly different at $p<0.05$ (based on $\log_2$ analysis). Treatment codes are: C = Control, A = Actigard, CC = Control–Control, CA = Control–Actigard, AC = Actigard–Control, AA = Actigard–Actigard, where the first letter identifies the postharvest application and the second letter the spring application.

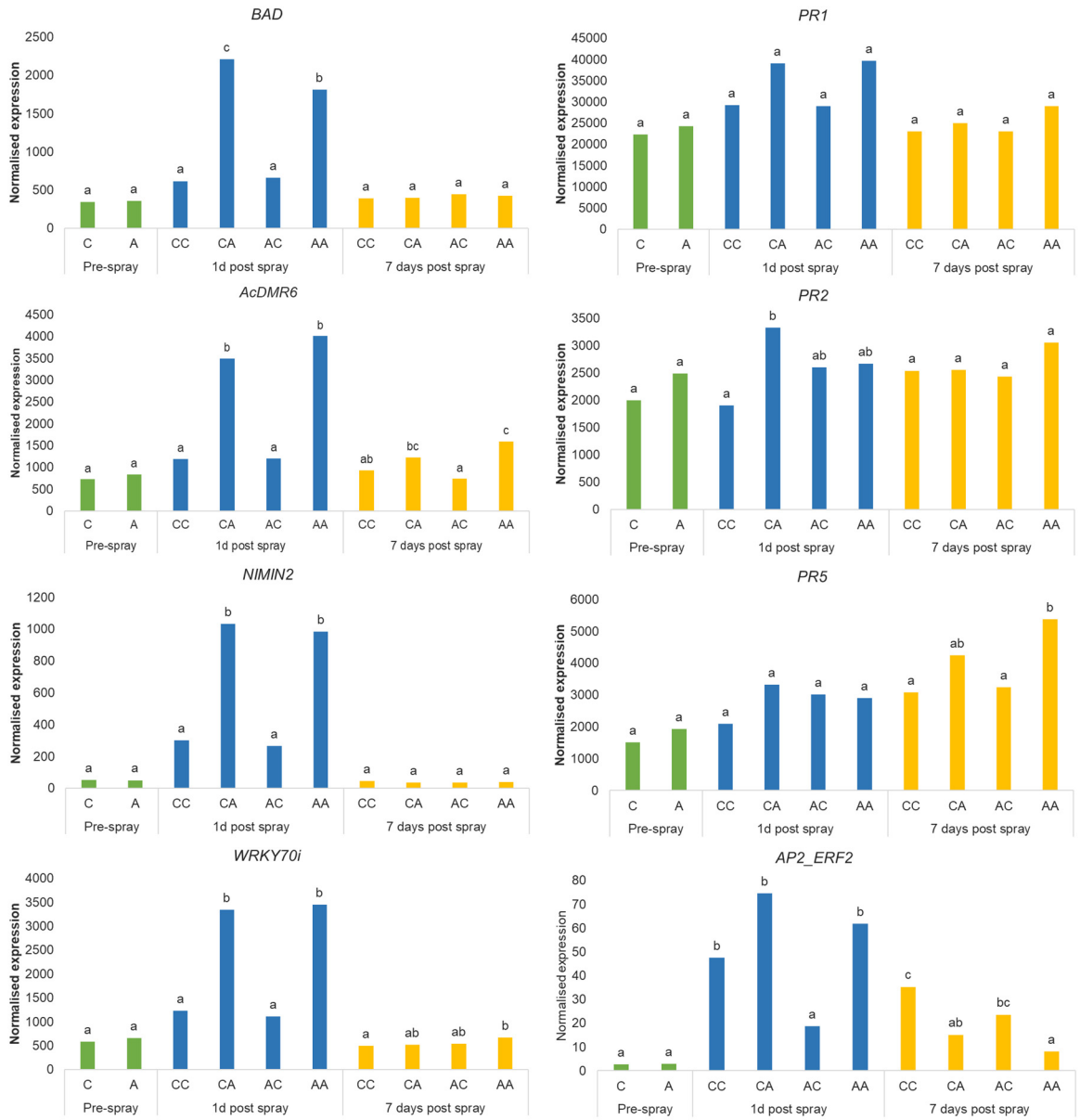

Figure 8. Gene expression in *Actinidia chinensis* var. *deliciosa* 'Hayward' vines at site D. Leaves were sampled on 1 November 2021 (pre-application), before application of Kocide® Opti™ (Control) or Kocide Opti+Actigard® (Actigard), and again at 1 day and 7 days post application. Gene expression was quantified by PlexSet® Nanostring analysis and data are presented as back-transformed means. Bars at each timepoint with the same letters are not significantly different at $p<0.05$ (based on $\log_2$ analysis). Treatment codes are: C = Control, A = Actigard, CC = Control–Control, CA = Control–Actigard, AC = Actigard–Control, AA = Actigard–Actigard, where the first letter identifies the postharvest application and the second letter the spring application.

3 Discussion

This project investigated the response of 'Zesy002' and 'Hayward' kiwifruit vines to postharvest Actigard application by comparing gene expression patterns in leaves before and after application. The data complements findings reported in Zespri project VI19001 (Reglinski et al. 2020). The main research questions in the current study were: 1) are vines responsive to Actigard after fruit harvest? 2) is the response affected by harvest date? and 3) is there a measurable carryover effect in spring?

3.1 Are vines responsive to Actigard after harvest?

Defence marker genes in both 'Zesy002' and 'Hayward' were upregulated after postharvest Actigard treatment. The specific patterns of gene expression varied between sites and cultivars but in general were greatest at 1 day and 7 days post spray and declined to control values after 3 weeks. This pattern is consistent with the transient nature of inducible resistance reported in other plant species (Walters et al. 2013). The most responsive genes to Actigard application in both cultivars were *BAD, AcDMR6, NIMIN2,* and *WRKY70i.* These genes are associated with the salicylic acid (SA) defence pathway and function by fine-tuning SA homeostasis (*BAD, AcDMR6*) (Widhalm & Dudareva 2015; Zhang et al. 2017) or regulating the transcription of pathogenesis-related (PR) proteins (*NIMIN2, WRKY70i*) (Klemme et al. 2019). There was also evidence that *PR1, PR2* and *PR5* were upregulated in 'Zesy002' at 1 week after Actigard application at site A. These data are in accord with results in project VI19001 where *DMR6, NIMIN2, WRKY70, PR1* and *PR5* were shown to be the most responsive genes to a single postharvest Actigard application in 'Hayward' vines (Reglinski et al. 2020).

3.2 Does harvest date affect the response?

In 'Zesy002', differences in gene expression patterns between site A (early) and site B (late harvest) indicated that harvest date may influence the response to Actigard. At the early-harvest orchard (site A) the expression levels of *BAD, AcDMR6, NIMIN2,* and *WRKY70i* were stronger in Actigard-treated vines than in the control at 1 d and 7 d after treatment, whereas at site B this difference was significant only at 1d post treatment. There was a 3-week gap between harvests at site A and site B, and this may indicate that harvest date, and the associated decline in canopy volume and health, can affect the duration of the induced response. Indeed site A was the only location where leaf canopy health was suitable for a second Actigard application approximately 3 weeks after the initial one (as recommended in Kiwifruit Vine Health Technote 104289 (kvh.org.nz)). At site B there was evidence of canopy deterioration (leaf yellowing) by 3 weeks after the postharvest spray and samples taken during this period yielded much lower RNA amounts. This endorses the recommendation that postharvest Actigard should be applied only if the leaf canopy condition is in good health (Kiwifruit Vine Health Technote 104289 (kvh.org.nz)). In 'Hayward', the loss of site C to frost precluded the comparison between early- and late-harvest orchards in that cultivar in the current study. However, findings in project VI19001 (Reglinski et al. 2020) suggested that harvest date did not significantly affect defence gene upregulation by Actigard in 'Hayward'.

3.3 Is there a measurable carryover effect of postharvest Actigard application in spring?

Gene expression levels in ‘Hayward’ and ‘Zesy002’ vines in spring did not differ significantly between vines treated at harvest with Kocide Opti and those treated with Kocide Opti and Actigard. This suggests that there was no carryover effect of the postharvest treatment. Moreover, the Actigard-treated vines did not express an enhanced response to the pre-flowering Actigard application, thus indicating that there was no priming effect. In project VI19001 evidence of priming was presented where postharvest Actigard enhanced responsiveness to Actigard in spring in ‘Hayward’ (Reglinski et al. 2020). However, caution was advised because the enhancement was weak (<2-fold), it affected only two genes (*PR2* and *PR5*), and it was observed in one orchard only. When taken together, the data across both studies indicate that postharvest Actigard does not condition ‘Zesy002’ or ‘Hayward’ kiwifruit vines to respond more strongly to the pre-flowering spray i.e. there is no evidence of a priming response.

In general, the most highly upregulated genes by Actigard in spring across all three sites were *BAD, AcDMR6, NIMIN2* and *WRKY70i*. The response patterns were consistent between cultivars and tended to be greatest at 1 d post treatment, hence confirming the utility of these genes as early defence response markers of Actigard-induced defence in kiwifruit. Pathogenesis-related proteins operate further downstream in the defence pathway so it was expected that upregulation of *PR1, PR2* and *PR5* would be greatest at 7 d after Actigard application. The expression patterns for the PR genes in the two ‘Zesy002’ sites were similar but the extent of upregulation tended to be greater in site B vines than at site A. In ‘Hayward’, the increases in *PR1, PR2* and *PR5* expression were generally not statistically significant and this may be a function of sampling time i.e. gene expression was not yet fully induced when the leaves were sampled. A more extensive time course is required to enable a more critical comparison of gene upregulation patterns between cultivars and orchards.

4 Conclusions and recommendations

The results from defence gene expression analysis indicate that kiwifruit vines are able to respond to Actigard after fruit harvest. However, there is no evidence that the response persists over winter and into the following spring. Whether or not harvest date affects the postharvest response is more complicated, as demonstrated by the differences in gene upregulation in 'Zesy002' at sites A and B. Overall, the data indicate that it is leaf health and not harvest date *per se* that dictates the amplitude and duration of the induced response. Biologically this makes sense, because defence activation demands actively metabolising tissue and thus vines will become less responsive to elicitors as canopy health declines. With that in mind it should be noted that the healthiest leaves were selected for analysis at each site. This was done deliberately to reduce the variability that would be introduced into the analysis. However, this means that the data in this study present a "best case scenario" and do not necessarily represent the response of the whole canopy.

Given the importance of leaf health for defence induction a more accurate measure of the relationship between whole canopy health and Actigard activity should be considered for further study. This would involve gene expression analysis of a larger selection of leaves than was practicable in the current project, including leaf types of differing health status to better reflect the condition of the canopy. Concurrent data on canopy density would be collected and the health of sampled leaves would be recorded using a SPAD (Soil Plant Analysis Development) meter to measure chlorophyll content as a proxy for metabolic capacity. There is increasing evidence of a close relationship between primary metabolism and plant defence, with the chloroplast serving as a key hub in the coordination of plant immune responses (Littlejohn et al. 2020; Kachroo et al. 2021). Together the leaf health and leaf response data could be used to develop a canopy health index as a decision tool to guide postharvest Actigard application.

Defence gene expression was used as a proxy for Actigard efficacy in this study primarily because of the difficulties with Psa assessment in the leaf canopy at this time. However, in spring, gene expression measurements alone may not be sufficient to determine if there is carryover effect of postharvest Actigard application. For example, defence gene upregulation after harvest may result in the synthesis of antimicrobial defences that protect leaf scars from new Psa infections and/or suppress existing Psa populations in the vine. No Psa measurements in spring were included in the current study because of budgetary constraints, and this information remains a significant knowledge gap with regard to longer-term effects of postharvest Actigard application. Therefore it is recommended that further studies are performed to establish if there is a correlation between the amplitude of gene upregulation after harvest with Psa populations and symptom expression in spring. This information would complement the current study and together the information would provide a more complete and robust assessment of the short- and long-term effects of postharvest Actigard applications.

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Appendix 1. Trial layouts

Site A

		Postharvest Design				
		Row1	Row2	Row3	Row4	
1	B		M		M	B
2	B		M		M	B
3	B	C1	M		M	B
4	B		M	A5	M	B
5	B		M		M	B
6	B		M		M	B
7	B	C2	M		M	B
8	B		M	A4	M	B
9	B		M		M	B
10	B		M		M	B
11	B		M	C3	M	B
12	B	A1	M		M	B
13	B		M		M	B
14	B	A2	M		M	B
15	B		M		M	B
16	B	A3	M		M	B
17	B		M	C4	M	B
18	B		M		M	B
19	B		M		M	B
20	B		M	C5	M	B
21	B		M		M	B
22			M		M	B
23						B

		Spring Design				
	Buffer	Row1	Row2	Row3	Row4	Buffer
1	B		M		M	B
2	B	CC1	M	AA5	M	B
3	B	CC2	M	AA4	M	B
4	B		M	AA3	M	B
5	B		M		M	B
6	B		M		M	B
7	B	CA1	M	AC5	M	B
8	B		M	AC4	M	B
9	B	CA3	M	AC3	M	B
10	B		M		M	B
11	B		M	CA2	M	B
12	B	AC1	M	CA4	M	B
13	B	AC2	M	CA5	M	B
14	B		M		M	B
15	B		M		M	B
16	B		M		M	B
17	B	AA1	M	CC3	M	B
18	B		M	CC4	M	B
19	B	AA2	M	CC5	M	B
20	B		M		M	B
21	B		M		M	B
22			M		M	B
23						B

Each cell is one vine

Postharvest		Spring	
Treatment	Code/rep	Treatment	Code/rep
Copper	C	Copper	CC
		Copper+ Actigard	CA
Copper +Actigard	A	Copper	AC
		Copper+ Actigard	AA

B=buffer vines, M = males

Site B

		Postharvest design				
Vine No.		Row 4	Row 3	Row 2	Row 1	
1	B	M		M	B	Shelter
2	B	M		M	B	
3	B	M		M	B	
4	B	M	C1	M	B	
5	B	M		M	B	
6	B	M	C2	M	B	
7	B	M	C3	M	B	
8	B	M		M	B	
9	B	M		M	B	
10	B	M		M	B	
11	B	M	A1	M	B	
12	B	M		M	B	
13	B	M	A2	M	B	
14	B	M	A3	M	B	
15	B	M		M	B	
16	B	M		M	B	
17	B	M		M	B	
18	B	M		M	B	
19	B	M	C4	M	B	
20	B	M		M	B	
21	B	M		M	B	
22	B	M		M	B	
23	B	M	C5	M	B	
24	B	M		M	B	
25	B	M		M	B	
26	B	M		M	B	
27	B	M		M	B	
28	B	M	A4	M	B	
29	B	M		M	B	
30	B	M		M	B	
31	B	M	A5	M	B	
32	B	M		M	B	
33	B	M		M	B	
34	B	M		M	B	

		Spring design				
Vine No.	Buffer	Row 4	Row 3	Row 2	Row 1	
1	B	M		M	B	
2	B	M	CC1	M	B	
3	B	M	CC2	M	B	
4	B	M		M	B	
5	B	M		M	B	
6	B	M	CA1	M	B	
7	B	M	CA2	M	B	
8	B	M		M	B	
9	B	M		M	B	
10	B	M	AA1	M	B	
11	B	M	AA2	M	B	
12	B	M		M	B	
13	B	M	AC1	M	B	
14	B	M	AC2	M	B	
15	B	M	AC3	M	B	
16	B	M		M	B	
17	B	M	CC3	M	B	
18	B	M	CC4	M	B	
19	B	M	CC5	M	B	
20	B	M		M	B	
21	B	M	CA3	M	B	
22	B	M	CA4	M	B	
23	B	M	CA5	M	B	
24	B	M		M	B	
25	B	M	AA3	M	B	
26	B	M	AA4	M	B	
27	B	M	AA5	M	B	
28	B	M		M	B	
29	B	M		M	B	
30	B	M	AC4	M	B	
31	B	M	AC5	M	B	
32	B	M		M	B	
33	B	M		M	B	
34	B	M		M	B	

Postharvest		Spring	
Treatment	Code/rep	Treatment	Code/rep
Copper	C	Copper	CC
		Copper+ Actigard	CA
Copper +Actigard	A	Copper	AC
		Copper+ Actigard	AA

B = buffer vines, M = males

Site D

	Postharvest design							Spring design						
Vine No.	Row 1	2	3	4	5	6	7	Row 1	2	3	4	5	6	7
1	B	M		M		M	B	B	M		M		M	B
2	B	M		M		M	B	B	M	CC1	M	AA3	M	B
3	B	M	C1	M		M	B	B	M	CC2	M	AA4	M	B
4	B	M		M	A5	M	B	B	M	CC3	M	AA5	M	B
5	B	M		M		M	B	B	M		M		M	B
6	B	M		M		M	B	B	M		M		M	B
7	B	M		M		M	B	B	M	CA1	M	AC3	M	B
8	B	M	C2	M		M	B	B	M	CA2	M		M	B
9	B	M		M	A4	M	B	B	M		M	AC4	M	B
10	B	M		M		M	B	B	M	CA3	M	AC5	M	B
11	B	M		M		M	B	B	M		M		M	B
12	B	M		M		M	B	B	M		M		M	B
13	B	M	A1	M		M	B	B	M	AC1	M	CA4	M	B
14	B	M		M	C5	M		B	M		M		M	
15	B	M	A2	M		M		B	M	AC2	M	CA5	M	
16	B	M		M	C4	M		B	M		M		M	
17	B	M	A3	M		M		B	M		M		M	
18	B	M		M	C3	M		B	M	AA1	M	CC4	M	
19	B	M		M		M		B	M	AA2	M	CC5	M	
20	B	M		M	♀	M		B	M		M		M	

Postharvest		Spring	
Treatment	Code/rep	Treatment	Code/rep
Copper	C	Copper	CC
		Copper+ Actigard	CA
Copper +Actigard	A	Copper	AC
		Copper+ Actigard	AA

B = buffer vines, M = males

Appendix 2. Frost damage at Site C after harvest on 23 June 2021

Appendix 3. Reference and target genes analysed in this project

Gene name	Gene ID	Target sequence
Eukaryotic small ribosomal subunit 40S	40S	CTACAAGCTCCTTGGTGGCCTCGCTGTTCGCAGGGCCTGCTATGGCGTTTTGAGAT TTGTTATGGAGAGCGGGGCAAAGGGATGTGAGGTGATTGTTAGT
Ubiquitin-conjugating enzyme	UBC	ATCTGAACGATTACTCACATCCACAGAATCGACCATTTCAGGAACAAAAAAATCCCC TCCAACAATTCACTGGCCTGATCGACGATCTAATTCTTCTCCG
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	ACTTTGTTGGTGACAGCAGATCGAGCATCTTTGATGCCAAGGCTGGGATTGCTTTG AACGACTTGTTCGTGAAACTGGTGTCTTGGTATGACAACGAGTG
Protein phosphatase 2	PP2A	TCCAGAATGGGCAATGCAGCACATAATTCCACAGGTATTGGACATGATTAGCAACC CACATTATCTGTACCGTATGACCATACTACACTCGATCTCTCTT
Pathogenesis-related protein family 1	*PR1*	GTTTGTGGGCACTACACTCAAATTGTGTGGAGAAACTCGGTCCGGCTCGGGTGCGC TAGGGTTCGGTGCAATAGTGGGTCTTGGTTCGTTACTTGCAACT
APETALA2 ethylene responsive factor 2	*AP2_ERF2*	TTGGCCTATGACAGGGCGGCTTTTAGTATGCGTGGGGCGAAGGCTCTCCTCAATTT TCCAGCTGAAGTAGGGGCAGAATCGTCCAAGCAAAGATTTACCC
Glucan endo-1,3-β-glucosidase	*PR2*	TGCTTGTGATTTCCCTCATAAAGAGGGCACTAGCAAAAAATAGAGTATGTACCGAGA GATTGCTCCTATGAAGACAGACAAAATATCTAATAAAGGAATA
Thaumatin-like protein TG4	*PR5*	AATATCATAAACAACTGCCCTTTCACCGTTTGGGCCGCTGCCGTTCCAGGTGGTGG CAAACGCCTTGACCGTGGCCAGAATTGGATCATCAATCCTGGTG
NIM-interacting protein 2	*NIMIN2*	AGCGGAGCGATGACGTGGAGGCCGACGCCAAGAAGGCGAGGGTAGGGGAAGATA ACGGAAAAGTGACGGAGCCGGAGGACGATGAGGTGGAGGAGTTCTT
Downy mildew resistance 6	*DMR6*	ACGCCCTCACAATTTTGCTTCAGGACCTCCAAGTCTCAGGCCTACAAGTCCTCAAG GACGGCAAGTGGATGGCCGTCAAACCCCATCCCAATGCCTTTGT
WRKY transcription factor 70	*WRKY70*	TGGAGGAAATATGGACAAAAGGAGATCCTCAATGCCAAATTTCCAAGGTGCTACTTT AGGTGCACACACAAGCCTGATCAAGGTTGCCTAGCAACAAAGC
Benzyl alcohol dehydrogenase	*BAD*	GCCGATATAGAGCTGATTCCGATGGACTATGTGAACACCGCGATGGAGCGGCTTGT GAAGGCTGACGTTAGTCCCTTGAGGCATTTTGGGCTTGACAAGC

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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