Studentship Project: Annual Progress Report October/2020 to September/2021

Student Name:	**Raisa Osama**	**AHDB Project Number:**	**CP 185**
Project Title:	**Role of auxin in Phytophthora root rot disease development in soft fruit**		
Lead Partner:	**The James Hutton Institute, Dundee**		
Supervisor:	**Dr Eleanor Gilroy, Dr Craig Simpson, Prof Grant Murray**		
Start Date:	**October 2019**	**End Date:**	**March 2023**

1. Project aims and objectives

Our research question is "What role might auxin play in plants challenged with *Phytophthora* species and how can this knowledge be applied to combat Raspberry root rot (PRR) disease and develop novel strategies for disease control". The overall aims of the project are:

1. Investigate the effect of auxin, activators and inhibitors on *Phytophthora rubi* isolates *in vitro*
2. Investigate the role of auxin on raspberry root rot disease development
3. Functional analysis of ABP19a on auxin signalling and disease resistance

2. Key messages emerging from the project

1. TIBA or 2,3,5-Triiodobenzoic Acid inhibits mycelial growth of nine different isolates of *Phytophthora rubi* and causes deformation and aggregation of sporangia and hyphae
2. The root-based application of TIBA on hydroponically grown raspberries have shown to promote root growth when pathogen is present.
3. Genes related to auxin signalling and root growth were upregulated in infected and TIBA treated roots.
4. ABP19a, a germin-like protein is highly upregulated in resistant raspberry cultivar Latham after infection.
5. Host immune response is compromised in NbABP19 silenced *N. benthamiana* plants

3. Summary of results from the reporting year

TIBA consistently inhibits mycelial growth across all isolates

To investigate the effect of auxin-related chemicals on mycelial growth of *P. rubi*, nine different isolates from a range of locations and years were previously screened with the following chemicals: auxin IAA (Indole-3-Acetic acid), a potent synthetic auxin mimic NAA (1-Napthaleneacetic Acid), the auxin herbicide, 2,4-D (2,4-dichlorophenoxyacetic acid), the auxin transport inhibitors TIBA and NPA. Although different chemicals had varying effect on *P. rubi* growth, we previously reported that TIBA consistently showed suppression of growth across all isolates with greater inhibition as the concentration of the chemical increased (Fig 1A). To understand whether this suppression was due to the perturbance of polar auxin transport, NPA, another polar auxin transport inhibitor was tested. Interestingly, NPA showed no significant suppression (Fig 1A). TIBA is a Benzoic acid (BA) derivative. BA and their derivatives such as salicylic acid are inhibitory to *Phytophthora* species (Williams et al., 2003). To test this hypothesis, the same isolates of *P. rubi* were screened with BA (Fig 1A), salicylic acid (2-hydroxybenzoic acid) and gallic acid (3,4,5-Trihydroxybenzoic acid) (Fig 1B). BA appeared to suppress *P. rubi* growth only at 100ppm leading to total inhibition of some isolates (Fig 1A). On the other hand, salicylic acid had a similar pattern of increased inhibition with higher dose leading to around <50% inhibition at 10ppm while gallic acid showed a promotional effect (Fig 1B). Salicylic acid is a signalling compound, which mediates defence responses against pathogen in different plant species and can induce CD at high concentrations (Edgar et al., 2006). Gallic acid is another derivative of BA that can be found in bark, leaf, wood, fruit, root and seeds of different plants. The promotional effect of gallic acid depending on the isolate has been previously reported (Jarvis, 1961). Therefore, the finding that TIBA has a suppressive effect could be partially explained by it being a BA derivative. However, the pattern of inhibition by TIBA was different from BA. TIBA showed progressively more inhibition with increase in concentration. Whereas BA showed different level of inhibition only at higher concentration. It has been reported that TIBA strongly inhibited endoreduplication in spinach and Arabidopsis which was thought to be due to the stabilization of actin filaments (Amijima et al., 2014). It may be that TIBA is also interfering with the actin cytoskeleton of *P. rubi*.

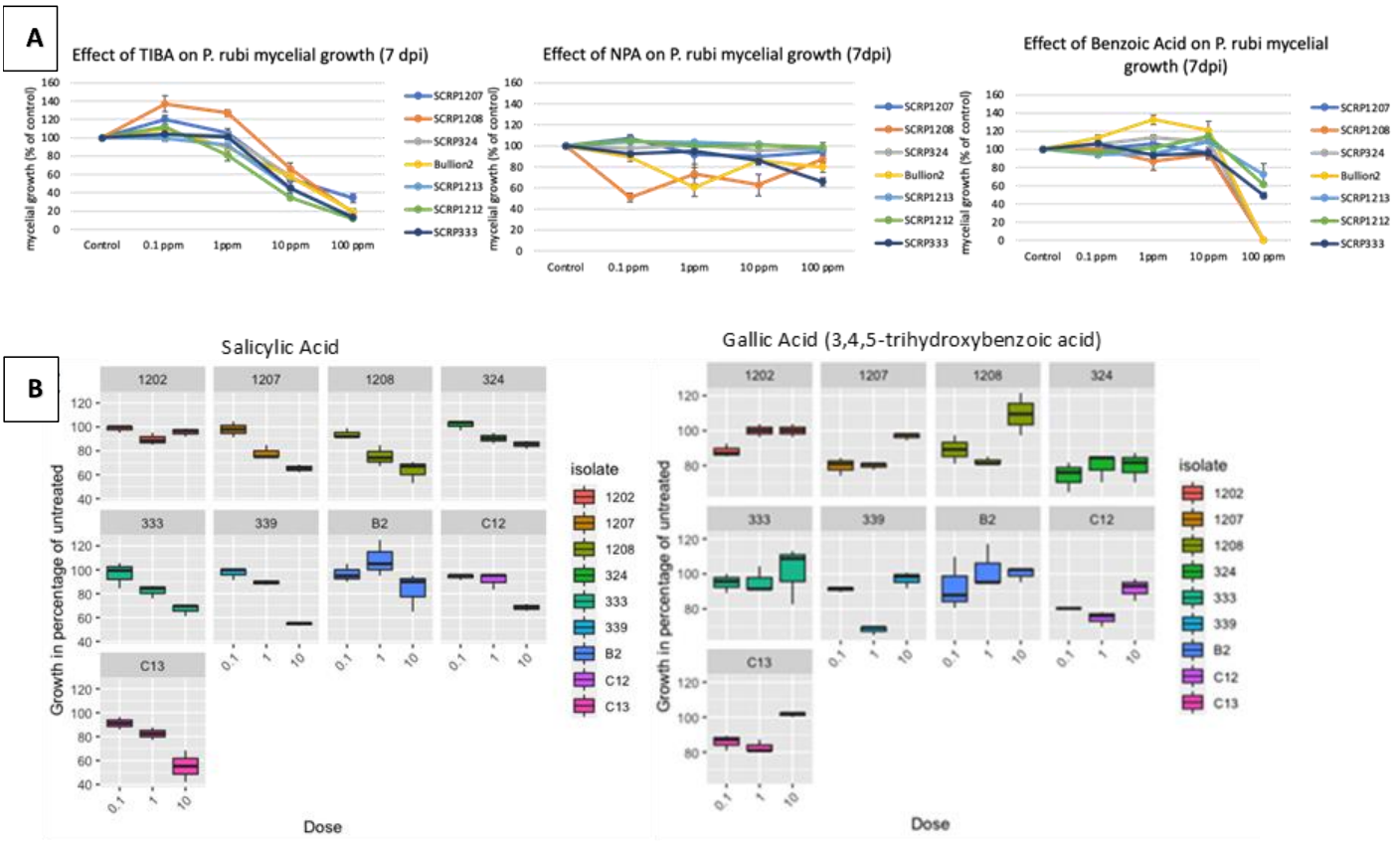

Figure 1: The effect of different chemicals on the growth of *P. rubi* isolates. The average diameter of mycelial growth was measured (mm) at 7dpi and calculated in % compared to the control. Here, 100% = the same as the control, less than 100% implies inhibition and over 100% promote growth compared to control. **A**: Line graph showing the pattern of inhibition of growth by TIBA, NPA and BA **B**: Effect of salicylic acid and gallic acid on mycelial growth.

TIBA causes deformation and aggregation of sporangia and hyphae

To investigate whether TIBA has lethal effect on sporangia development, *P. rubi* inoculum containing sporangia and zoospores were treated with 1ppm, 10ppm and 100ppm of TIBA and observed under the microscope. When compared to the control, the cytoplasm of TIBA treated hyphae and sporangia were unevenly distributed and appeared in aggregation (Fig 2). TIBA is used as growth regulator in some countries

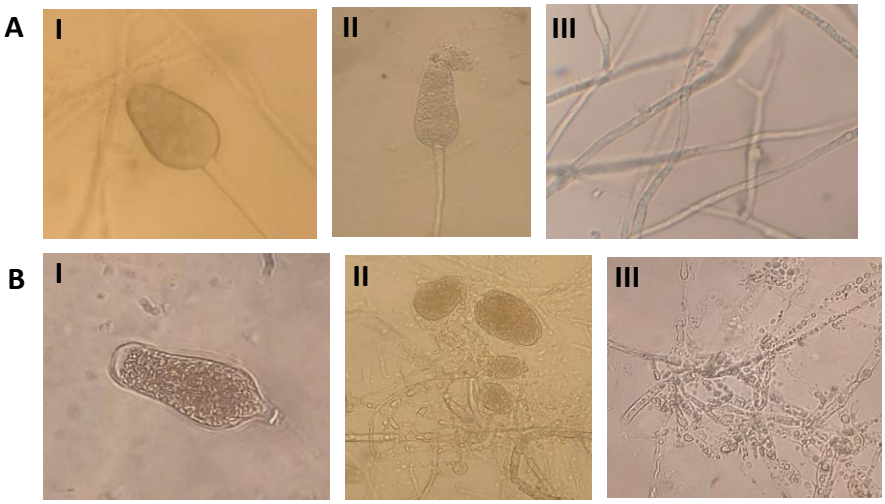

Figure 2: Effect of TIBA on *P. rubi* sporangia and hyphae A: Control I. Healthy sporangium with evenly distributed cytoplasm II. Mature sporangium releasing zoospores III. Healthy hyphae **B**: TIBA treated *P. rubi* I. Deformed sporangium with uneven distribution of cytoplasm II. Deformed aggregated mycelium and sporangia III. Hyphae are deformed and fragmented.

to supress growth and promote fruiting (Harms & Oplinger, 1988), but the use of TIBA to control disease has not been reported. However, the commercial use of TIBA is not approved in the UK (http://sitem.herts.ac.uk/aeru/ppdb/en/Reports/2941.htm). Therefore, specifying the target of TIBA on *P. rubi* growth can lead to the use of other approved chemicals with similar target to control root rot in raspberries.

TIBA promotes root growth when P. rubi is present

Next, the effect of TIBA on disease development was further analysed by challenging the raspberry susceptible cultivars, Glen Dee and Glen Moy, with root-based application of 1 and 10ppm TIBA in soil-free media. At the same time plants were infected with *P. rubi*. After seven days, root length, surface area and root volume were measured using the WinRhizo image analysis. It was observed that, the root surface area and root volume of G. Dee increased from 0ppm to 10ppm but only in infected samples. For the non-infected samples there was a decline in all parameters at 10ppm (Fig 3A). On the other hand, in G. Moy, root length increased, and root volume decreased in infected samples when compared to non-infected samples (Fig 3B).

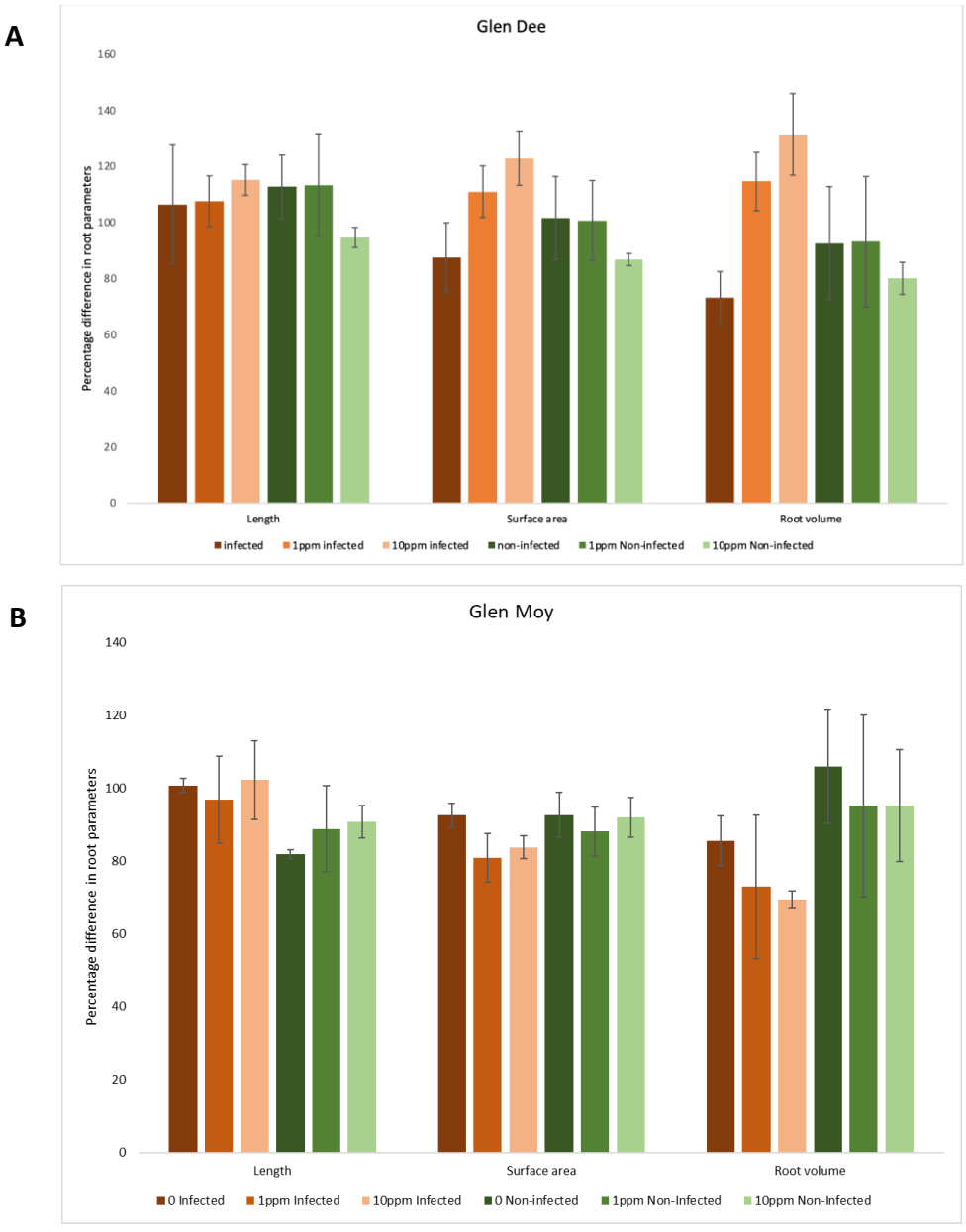

Figure 3: Root architecture analysis of susceptible cultivars after TIBA treatment and infection. Percentage difference in root length, surface area and root volume of **A:** Glen Dee and **B:** Glen Moy measured with WinRhizo software after 7 days of infection and treatment with TIBA.

TIBA is an auxin transport inhibitor, so TIBA alone inhibits root growth (Fujita & Syono, 1996). However, TIBA and pathogen have promotional effect on root growth, which can be seen in Figure 3A. The oomycete cell walls consist of 80-90% of glucans of which the major constituent is β-1,3 glucans (Tokunaga & Bartnicki-Garcia, 1971). It has been published that yeast cell wall also contain 30-45% β-1, 3 glucans (Klis et al., 2006) and a plant activator prepared from yeast cell wall extract (YCWE) induces disease resistance against fungal and bacterial pathogen (Narusaka et al., 2015). The application of YCWE has shown to promote plant height at later growth stage (Hamasaki et al., 2014). In the same study it has been mentioned that YCWE promoted root spread of Arabidopsis and wheat seedlings (data not published). Taken together it can be assumed that the application TIBA leads to the degradation of *P. rubi* cell walls releasing polymers of glucose, which may provide nutrition for root growth but also induce immune response by acting as a PAMP.

TIBA and infection shows upregulation of auxin and root growth related genes in G. Dee roots

Finally, RNA-seq analysis was performed with the root tip samples collected from the susceptible raspberry cultivar, Glen Dee. Since TIBA and pathogen showed a synergistic effect on root growth, the focus was to investigate differentially expressed (DE) genes in infected root samples where T0=infected only, T1=infected+1ppm TIBA, T10=infcetde+10ppm TIBA. In total, 1,777 out of 21,216 expressed genes were classified as DE with P-value cut-off at 0.05 and log2fold change cut-off at 1. The number of upregulated and downregulated genes in different treatment are presented in Figure 4. From the heat map, it can be seen that, cluster five and six are highly upregulated in T10. Detailed examination of these genes led to the identification of genes involved in auxin signalling and response and root growth and morphogenesis.

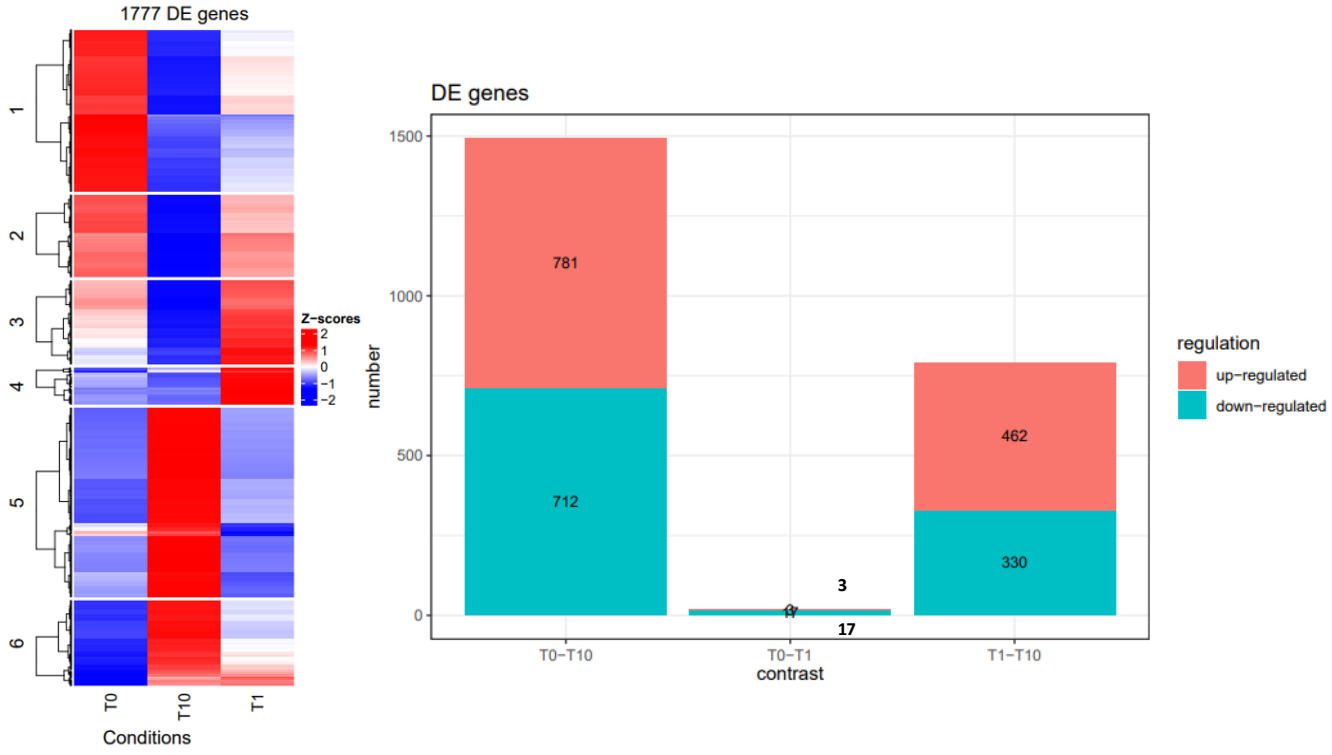

Figure 4: Expression and number of differentially expressed (DE) genes. Cluster 5 and 6 are highly expressed in T10. In T0-T10 samples, 781 and 712 genes were upregulated and downregulated respectively. Only 3 out of 20 genes were upregulated in T0-T1 samples. Comparing T1 to T10, identified 462 upregulated and 330 downregulated genes.

Genes related to auxin signalling and response include auxin responsive protein IAA-like, auxin induced protein AUX-like, auxin efflux carrier component 2-like (PIN2), auxin transporter like protein (LAX2), auxin response factor 3 (ARF3), ABP19a, and indole-3-acetic acid-amido synthetase GH3.1. For all of these except LAX2 and ARF3, expression was upregulated from T0 to T10 (Fig 5). Auxin plays a very important role in remodelling of root structure that aid in adaptation to stress. Several genes coding for auxin induced protein and auxin responsive protein were significantly upregulated in T10 samples. Auxin gradients required for root cell elongation, differentiation, and patterning, are regulated by membrane localised proteins such as PIN2 and ABC transporters, which are both highly induced in T10 samples. Another important auxin gene GH3 is also upregulated in T10. GH3 is thought to be involved in the regulation of expansin gene that loosens the cell wall and activates SA and JA-mediated disease resistant pathways (Ding et al., 2008).

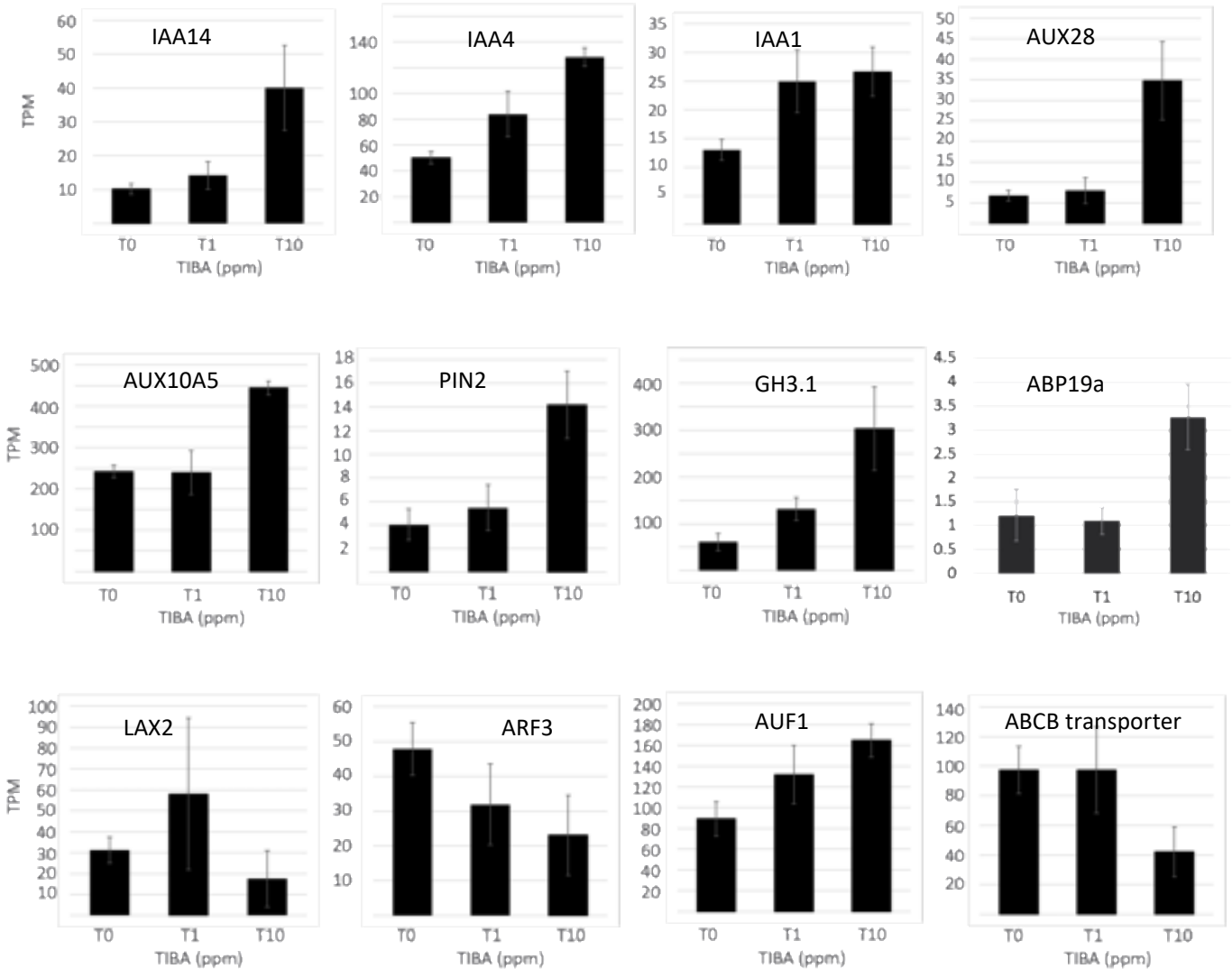

Figure 5: The expression of different auxin-related genes. Expression values are in transcripts per million (TPM). The genes were identified using the online tool, 3D RNA-seq App (Guo et al., 2020).

Interestingly, two genes were downregulated in T10, LAX2 and ARF3 (Fig 5). It is known that ARF3 is a negative regulator of auxin signalling leading to inhibition of primary root growth and activation of lateral root development (Yoon et al., 2014). This finding supports the physiological data, where the surface area of Glen Dee roots increased significantly from T0 to T10 but not the root length (Fig 3). Although LAX2 is a

member of the auxin influx transporter, its role in auxin-regulated root development is limited as LAX2 mutants do not show any root growth related defects (Péret et al., 2012)

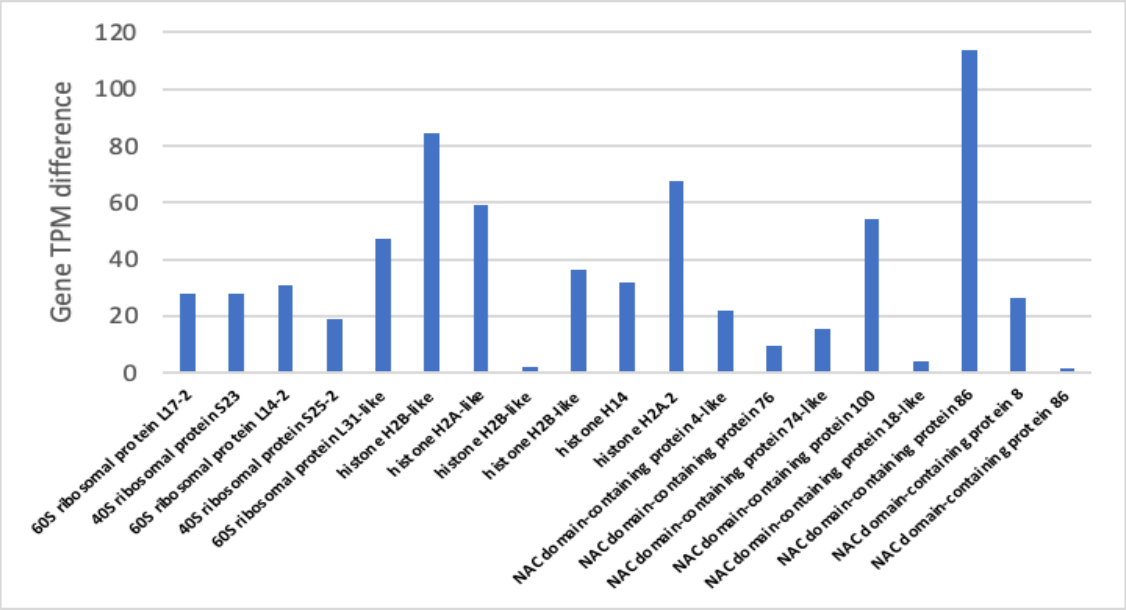

Figure 6: Overexpression of genes associated with root growth. Upregulated genes encoding ribosomal protein, histone protein and NAC-domain proteins. The bar graphs show the TPM difference between the T0 and T10 samples

Additionally, several DEGs associated with root growth and morphogenesis were identified (Fig 6). Ribosomal proteins play a crucial role in root development and silencing of genes encoding ribosomal protein can lead to inhibition of root growth (Popescu & Tumer, 2004; Van Minnebruggen et al., 2010). Five ribosomal proteins were identified from cluster five and six. For each gene there was a twofold increase in gene expression from T0 to T10. Histone modifications are also known to be important for root growth and development (Takatsuka & Umeda, 2015; Yi et al., 2002). Six genes encoding histone protein were also upregulated in T10. Moreover, eight genes encoding NAC-domain containing protein were all upregulated in T10. NAC are transcriptional regulators in plants that have been reported to regulate biotic and abiotic stress in plants (Nuruzzaman et al., 2013). One of the genes with >1000 TPM expression value included the ethylene-response transcription factor ERF071 (data not shown). This gene was induced by twofold from T0 to T10. ERF071 is involved in root development through the regulation of root meristem cell division. Silencing and overexpression of the ERF73 exhibited reduced and increased primary root elongation respectively (Seok et al., 2020; Yang et al., 2011). The upregulation of these genes suggests their role in root growth process and support the root growth phenotype observed in T10 samples.

ABP19 is a germin-like protein that may have potential role in resistance

We have previously reported that a putative germin-like protein, ABP19 is significantly upregulated in infected resistant cultivar Latham. This gene is closely located to Rub118b, the genetic marker for root rot resistance (Graham et al., 2011). We identified nine copies of ABP19 closely located to each other. RT-qPCR was conducted to confirm the upregulation of *ABP19* in Latham. Among all the nine copies, only one copy (*ABP19.5*) showed consistent expression across all samples and confirmed the upregulation in the resistant

cultivar (Fig 7A). To know whether *ABP19.5* had tissue specific expression RT-qPCR was conducted on uninfected leaf, primary root and lateral roots of both cultivars. *ABP19.5* was expressed in all tissues tested (Fig 7B).

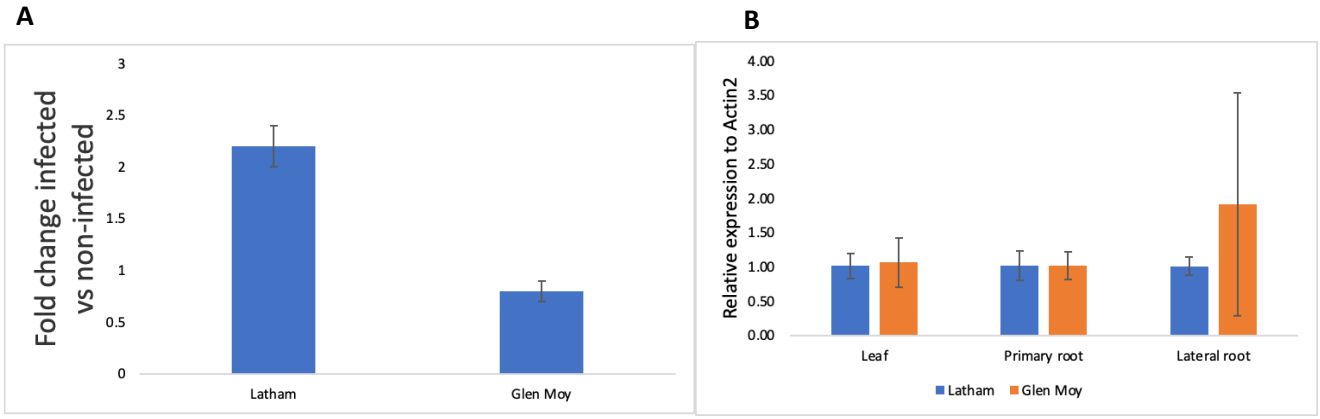

Figure 7: Expression of ABP19.5 in Moy and Latham plants. A. qRT-PCR confirming the upregulation of *ABP19.5* in infected Latham roots. **B.** Expression of *ABP19.5* in different tissues. Total RNA was extracted, and first-strand cDNA was synthesized for expression analysis. The expression level was normalized against the expression of the housekeeping gene *Actin2*.

There is increasing evidence for the involvement of germins (GER) and germin-like proteins (GLP) in host resistance. A number of studies have shown that these proteins are upregulated or activated during pathogen infection or when chemicals associated with resistance such as H_2O_2, salicylic acid or ethylene are applied (Lou & Baldwin, 2006; Zimmermann et al., 2006). Overexpression and knockdown of endogenous GER and GLP studies have also supported the involvement of these proteins in host defence. Moreover, the transient overexpression of many GERs and GLPs in transgenic plants have provided with increased resistance against diseases (Ilyas et al., 2016).

Host immune response is compromised in NbABP19 silenced N. benthamiana plants

Four homologs of *ABP19* genes were identified in the model plant *N. benthamiana*. To further understand the importance of ABP19 in disease resistance, cell death (CD) assays were conducted on *NbABP19* silenced *N. benthamiana* plants with well-established immune response triggering proteins. Virus-induced gene silencing (VIGS) was used to knock down the expression of *NbABP19-like* genes in such a way that in the first construct, TRV:ABP19AB, two *ABP19* genes with sequence similarity, A and B were silenced and in the second construct, TRV:ABP19CD, C and D genes were silenced. In a third construct, TRV:ABP19A-D, all four *ABP19* genes were silenced.

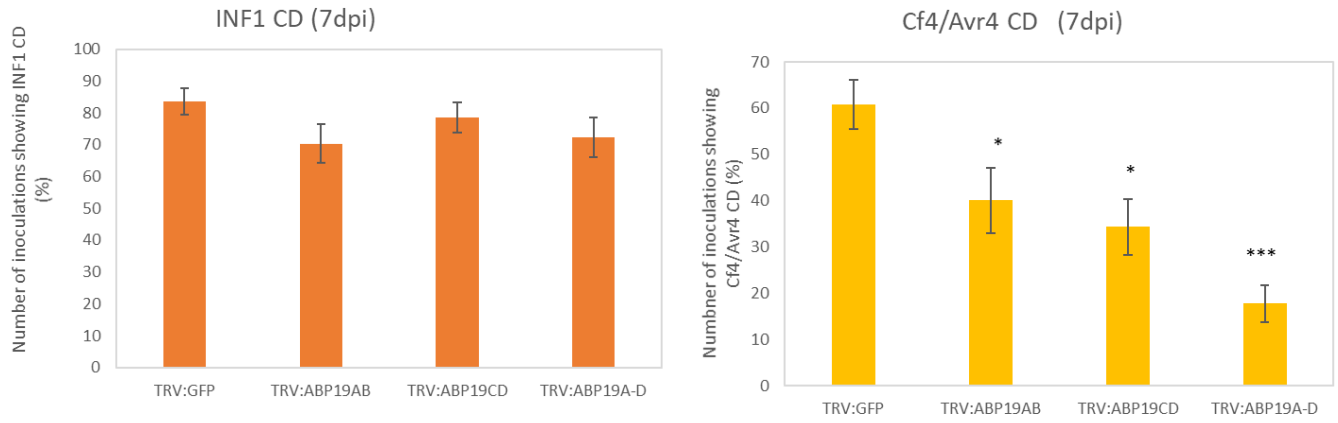

Figure 8. **Role of ABP19 in two separate immune triggers that lead to cell death.** Cf4/Avr4 and INF1 CD in *NbABP19* silenced plants. The graph depicts the average percentage of infiltrated sites that develop CD at 7 dpi. Error bars are standard errors and the graph represents the combined data from six biological replicates. Significant differences to the GFP control by Holm-Sidak method one-way ANOVA are indicated by the asterisks. * P=<0.05 and *** P= <0.001

INF1 is a *Phytophthora infestans* elicitor protein that triggers Pathogen triggered immunity (PTI) in *N. benthamiana* plants. Avr4 on the other hand is an apoplastic virulence protein secreted by tomato fungal pathogen *Cladosporium fulvum* which triggers CD response when co-infiltrated with its cognate host receptor Cf4. INF1 and Cf4/Avr4 triggers immune responses that are controlled by separate MAP kinase signalling cascades that converge downstream to promote the same immune responses through activities of Salicylic acid induced protein kinase (SIPK) and Wound induced protein kinase (WIPK) (Ishihama et al., 2011; Zhang & Liu, 2001). Although there was no difference in INF1 CDs, the hypersensitive response to Avr4/Cf4 was significantly lower in *ABP19* silenced plants (Fig 8), implying that ABP19 may be involved in immune response triggered by Cf4/Avr4 protein but has no effect on the INF1- mediated CD. The host receptors for both INF1 and Avr4/Cf4 proteins are located at the plasma membrane whereas the receptors for RXLR effector proteins are in the host cells. Hence, *P. infestans* RXLR effector proteins, Avr2 and Avr3a were also used for CD assays to test whether silencing of *ABP19* had any effect on effector triggered immunity (ETI). After two replicates of this assay, there was no suppression of R3a/Avr3a but a reduction in R2/Avr2 CD was observed (Fig 9). More replicates of this assay is needed, to see if the reduction is statistically significant.

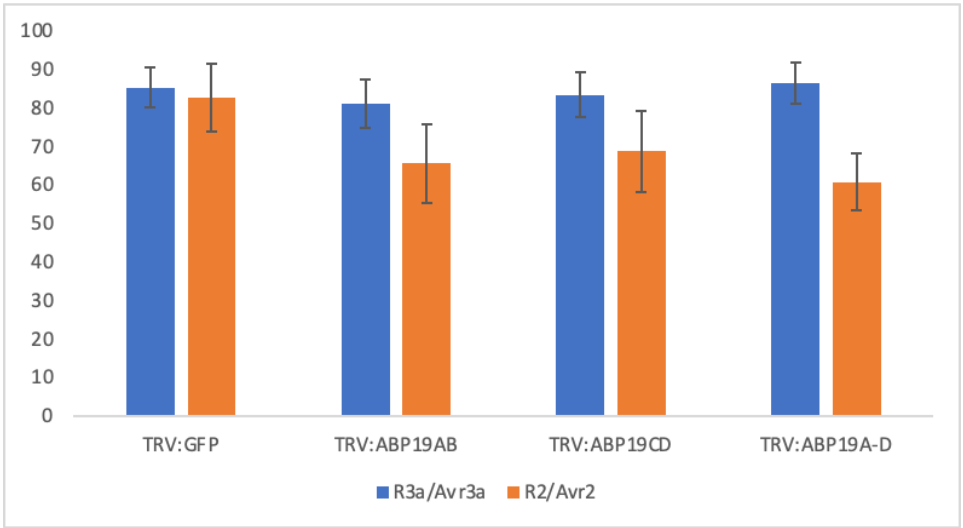

Figure 9. **Role of ABP19 in response to cell death triggered by effector proteins.** R2/Avr2 and R3a/Avr3a CD in NbABP19 silenced plants. The graph depicts the average percentage of infiltrated sites that develop CD at 7 dpi. Error bars are standard errors, and the graph represents the combined data from two biological replicates.

4. Key issues to be addressed in the next year

ABP19-related experiments

- Subcellular localization of ABP19in benthamiana leaves
- Expression of RiABP19 in Arabidopsis/ benthamiana roots
- purification of ABP19 protein
- SOD assay with purified protein
- Anti-fungal activity assay/screening of *P. rubi* isolates with purified protein
- Root staining assay to determine ROS burst in resistant vs susceptible roots
- Followed by checking whether ROS burst and ABP19 expression occurs at the same cellular localization
- Followed by using the same assay to check whether ABP19 is induced by auxin/H_2O_2/TIBA
- Try to clone and analyse ABP19.10 that we found recently

Cell death related experiment:

- AvrPto shares the same signalling cascade (i.e. MAP3Kε signalling cascade) as Avr4. CD assays will be conducted on ABP19 silenced benthamiana plants with AvrPto proteins
- Check if ABP19 activity increases the trigger response by Avr4 and AvrPto by overexpressing ABP19
- Y2 hybridization assay to see if ABP19 interacts with components of the MAP3Kε signalling pathway

Other experiments:

- Check if *P. rubi* enters roots after treating with TIBA and Zn complex with microscopy using tdt333
- Transcriptomic analysis of resistant cultivar Latham infected with P. rubi
- Do more replicates of the phenotypic experiments to establish significant difference.
- qRT-PCR to confirm upregulation of auxin related genes in raspberry roots

5. Outputs relating to the project

(events, press articles, conference posters or presentations, scientific papers):

Output	**Detail**
Oral presentation	The Hutton Postgraduate Student Event 2021
Infographic poster	AHDB Crops PhD Conference 2021
Poster	University of Warwick Postgraduate Symposium 2021
Poster	ISRR Dundee Medal Lecture in Root Research and Workshop 2021
Infographic poster	BSPP Infographics Student Competition 2020
3-Minute Thesis presentation	SCOTIA agriculture club competition 2021

6. Partners (if applicable)

Scientific partners	
Industry partners	
Government sponsor	

References:

Edgar, C., McGrath, K., Dombrecht, B., Manners, J., Maclean, D., Schenk, P., & Kazan, K. (2006). Salicylic acid mediates resistance to the vascular wilt pathogen Fusarium oxysporum in the model host Arabidopsis thaliana. *Australasian Plant Pathology, 35*(6), 581-591.

Fujita, H., & Syono, K. (1996). Genetic analysis of the effects of polar auxin transport inhibitors on root growth in Arabidopsis thaliana. *Plant Cell Physiol, 37*(8), 1094-1101. doi:10.1093/oxfordjournals.pcp.a029059

Graham, J., Hackett, C., Smith, K., Woodhead, M., MacKenzie, K., Tierney, I., . . . Jennings, N. (2011). Towards an understanding of the nature of resistance to Phytophthora root rot in red raspberry. *Theoretical and applied genetics, 123*(4), 585-601.

Guo, W., Tzioutziou, N. A., Stephen, G., Milne, I., Calixto, C. P., Waugh, R., . . . Zhang, R. (2020). 3D RNA-seq: A powerful and flexible tool for rapid and accurate differential expression and alternative splicing analysis of RNA-seq data for biologists. *RNA biology*, 1-14.

Hamasaki, T., Kitagawa, T., & Yasuhara, T. (2014). *Efficacy of yeast cell wall extract, a byproduct of beer brewing, in tomato (Solanum lycopersicum) culture.* Paper presented at the 2nd International Conference on Environment, Energy and Biotechnology.

Harms, C. L., & Oplinger, E. S. (1988). Plant growth regulators: their use in crop production.

Ishihama, N., Yamada, R., Yoshioka, M., Katou, S., & Yoshioka, H. (2011). Phosphorylation of the Nicotiana benthamiana WRKY8 transcription factor by MAPK functions in the defense response. *The Plant Cell, 23*(3), 1153-1170.

Klis, F. M., Boorsma, A., & De Groot, P. W. J. (2006). Cell wall construction in Saccharomyces cerevisiae. *Yeast, 23*(3), 185-202. doi:https://doi.org/10.1002/yea.1349

Narusaka, M., Minami, T., Iwabuchi, C., Hamasaki, T., Takasaki, S., Kawamura, K., & Narusaka, Y. (2015). Yeast cell wall extract induces disease resistance against bacterial and fungal pathogens in Arabidopsis thaliana and Brassica crop. *PLoS One, 10*(1), e0115864.

Nuruzzaman, M., Sharoni, A. M., & Kikuchi, S. (2013). Roles of NAC transcription factors in the regulation of biotic and abiotic stress responses in plants. *Frontiers in Microbiology, 4*(248). doi:10.3389/fmicb.2013.00248

Popescu, S. C., & Tumer, N. E. (2004). Silencing of ribosomal protein L3 genes in N. tabacum reveals coordinate expression and significant alterations in plant growth, development and ribosome biogenesis. *The Plant Journal, 39*(1), 29-44. doi:https://doi.org/10.1111/j.1365-313X.2004.02109.x

Seok, H.-Y., Ha, J., Lee, S.-Y., Bae, H., & Moon, Y.-H. (2020). Two alternative splicing variants of AtERF73/HRE1, HRE1α and HRE1β, have differential transactivation activities in Arabidopsis. *International journal of molecular sciences, 21*(19), 6984.

Takatsuka, H., & Umeda, M. (2015). Epigenetic Control of Cell Division and Cell Differentiation in the Root Apex. *Front Plant Sci, 6*, 1178. doi:10.3389/fpls.2015.01178

Tokunaga, J., & Bartnicki-Garcia, S. (1971). Structure and differentiation of the cell wall of Phytophthora palmivora: cysts, hyphae and sporangia. *Archiv für Mikrobiologie, 79*(4), 293-310.

Van Minnebruggen, A., Neyt, P., Groeve, S. D., Coussens, G., Rosa Ponce, M., Luis Micol, J., & Van Lijsebettens, M. (2010). The ang3 mutation identified the ribosomal protein gene RPL5B with a role in cell expansion during organ growth. *Physiologia Plantarum, 138*(1), 91-101. doi:https://doi.org/10.1111/j.1399-3054.2009.01301.x

Williams, M., Senaratna, T., Dixon, K., & Sivasithamparam, K. (2003). Benzoic acid induces tolerance to biotic stress caused by Phytophthora cinnamomi in Banksia attenuata. *Plant Growth Regulation, 41*(1), 89-91. doi:Doi 10.1023/A:1027355604096

Yang, C.-Y., Hsu, F.-C., Li, J.-P., Wang, N.-N., & Shih, M.-C. (2011). The AP2/ERF transcription factor AtERF73/HRE1 modulates ethylene responses during hypoxia in Arabidopsis. *Plant Physiology, 156*(1), 202-212.

Yi, H., Mysore, K. S., & Gelvin, S. B. (2002). Expression of the Arabidopsis histone H2A-1 gene correlates with susceptibility to Agrobacterium transformation. *The Plant Journal, 32*(3), 285-298. doi:https://doi.org/10.1046/j.1365-313X.2002.01425.x

Zhang, S., & Liu, Y. (2001). Activation of Salicylic Acid–Induced Protein Kinase, a Mitogen-Activated Protein Kinase, Induces Multiple Defense Responses in Tobacco. *The Plant Cell, 13*(8), 1877-1889. doi:10.1105/tpc.010044