Bioengineering inducible gene expression in leafy brassicas to address post-harvest-specific requirements

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Abstract

Food loss and waste has a significant negative impact on the sustainability of the global food system. It is estimated that ~13% of harvested fresh horticultural crops are never consumed owing to deterioration of plant health and quality in the post-harvest period. Harvested leafy crops are living tissues which can remain edible for periods of one week to six months, depending on the variety; this is astonishing when we consider that they are unable to uptake nutrients or water, and are subject to the stress of the harvest and storage processes. Improvements to the health and quality of post-harvest leafy crops could increase their storage life, and consequently reduce food loss. The aim of this study is to identify changes in transcription and immunity caused by harvest, and bioengineer a harvest-inducible genetic system to bolster identified weaknesses in post-harvest health.

Post-harvest improvements to crop health have mostly focused on storage conditions and abiotic stress. However, disease can contribute significantly to food loss and waste, and the changes to immunity of leafy crops after harvest remain obscure. Accordingly, in Chapter 3, I examine post-harvest pattern-triggered immunity (PTI) and selected immune signalling pathways in an Arabidopsis model harvest system. Here I show that harvest suppresses pathways involved in resistance to biotrophic pathogens, and by contrast harvest enhances pathways providing defence against necrotrophic pathogens. Therefore, this study identifies that harvest has a significant impact on the immune system, and presents attenuated immune pathways as potential targets for post-harvest enhancement.

The multiple stresses of harvest and storage can lead to physiological changes in harvested leafy crops, including accelerated tissue senescence. In Chapter 4 I explore the progression of post-harvest transcriptional changes in leafy brassicas, comparing Arabidopsis with a long-storage leafy crop, cabbage (*Brassica oleracea* var. *capitata*), and existing datasets in short-storage brassicas broccoli (*Brassica oleracea* var. *italica*) and salad rocket (*Eruca sativa*). I demonstrate that there is commonality of post-harvest abiotic responses in short-storage brassicas and the model system, which are not seen in the cabbage dataset. This study shows that the post-harvest needs of leafy crops are likely to be markedly different depending on the length of shelf-life.

The post-harvest shift in the transcriptome and immune system of harvested leafy brassicas underlines the requirement for post-harvest-specific interventions, which would avoid negative impacts on the health or yield of the plant while it is growing on soil. To this end, in Chapter 5 I identify likely regulatory control of harvest-inducible genes, which show low expression on soil, and high expression post-harvest. Promoter sequences from these harvest-inducible genes are used to drive reporter gene expression in a harvest-responsive manner.

Overall, our findings indicate that the changes in the immune system and transcriptome of harvested leafy brassicas necessitate different interventions from soil-growing plants. As such, I provide proof-of-concept of a harvest-inducible system that could form the future basis of bioengineering strategies to improve health and quality of harvested leafy brassicas, and thereby reduce food loss and waste.

Lay summary

Food waste and loss have a negative impact on the sustainability of the global food system, yet ~13% of harvested crop plants are never eaten. Harvested vegetable crops become inedible when they are damaged by disease, or their quality deteriorates; many leafy vegetables are particularly likely to be wasted owing to their short shelf-life. Harvested leafy crops are living plant tissues that can still respond to their environment, and still have the cellular immune components needed to respond to pathogen attack. This study explores the post-harvest changes in the immune system that take place in leafy plants, using the model brassica plant Arabidopsis, and also examines the gene expression changes over the course of storage in Arabidopsis and closely related brassica crops cabbage, broccoli, and rocket. My results show that harvest dampens the plant's ability to respond to pathogens that feed from living plant cells; by contrast, harvest accentuates the defences of leaf tissues against pathogens that kill host plant cells to feed from their contents. Brassica crops with a shorter shelf-life shared strong responses to the stress of harvest, and their gene expression changes were markedly different from those of cabbage, which has a much longer shelf-life. Through this analysis of gene expression and immunity, I identified targets that would benefit from enhancement in order to improve post-harvest health and quality. I then designed and provided proof-of-concept in Arabidopsis of a genetic method of switching on these traits as soon as the leafy crop is harvested. This study lays the groundwork for genetically-engineering improved post-harvest plant health and quality without negatively affecting the crops as they grow on soil.

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Declaration

I declare that this thesis has been composed solely by myself and that it has not been submitted, in whole or in part, in any previous application for a degree. Except where stated otherwise by reference or acknowledgment, the work presented is entirely my own.

Lindsay Williams

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KEY ABBREVIATIONS AND ACRONYMS

ABA	abscisic acid
ABRE	abscisic acid response element
ANOVA	analysis of variance
CI	confidence interval
CRE	cis-regulatory element
DEG	differentially expressed gene
dph	days post-harvest
EH	early harvest (harvested before infection)
ET	ethylene
FC	fold change
FDR	false discovery rate
GFP	green fluorescence protein
GO BP	Gene ontology biological process
hph	hours post harvest
hpi	hours post-inoculation/infiltration
hps	hours post-spray
JA	jasmonic acid
JAZ1-GS	JAZ1-tagged with protein G/ streptavidin-binding peptide
KEGG	Kyoto encyclopedia of genes and genomes
LH	late harvest (infected before harvest)
MeJA	methyl-jasmonate

PAGE	Parametric Analysis of Gene Set Enrichment
PAMP	pathogen-associated molecular pattern
PMSF	phenylmethylsulfonyl fluoride
PRR	pattern recognition receptor
Psm	*Pseudomonas syringae* pv. *maculicola*
PTI	pattern-triggered immunity
PTM	post-translational modification
SA	salicylic acid
SE	standard error
siRNA	small interfering RNA
SSC	smoothing spline clustering
tasiRNA	trans-acting small interfering RNA
TF	transcription factor
TLCK	tosyl-L-lysine chloromethyl ketone
TPCK	tosyl-L-phenylalanine chloromethyl ketone
TSS	transcription start site
WT	wild type

Chapter 1: Introduction

1.1 The global importance of post-harvest health

Improving the health of harvested crops is crucial for sustainable food security. Although disconnected from their source of nutrition, harvested plants remain living organisms and tissues. The processes of harvest and storage exert multiple stresses on plants; understanding how these affect plant health throughout storage is crucial to prevent spoilage, which reduces shelf-life and leads to food loss. The seasonality of agriculture means that most crops can only be harvested in a limited window of time, after which post-harvest storage or import from a region with a different growing season is required to make that food available throughout the year. As the world population becomes more urbanised and trade in food commodities has been globalised, storage and transportation of fresh food has become increasingly important. With 9% of the >8 billion world population undernourished (FAO et al., 2023), ensuring that edible, nutritious food reaches as many people as possible has never been more important. Post-harvest deterioration of plant health leads to ~13% of crops being discarded (FAO, 2021), which wastes not only the potential nutrition provided by that food, but also the plethora of intensive agricultural inputs required to grow and harvest that crop, such as fresh water, fertilisers and fossil fuels. Accordingly, the wasted inputs from discarded food are estimated to contribute to 6% of global greenhouse emissions (Ritchie, 2020; Poore and Nemecek, 2018). And yet there is insufficient attention given to the post-harvest period of crop life: e.g., of the

~47 billion USD spent on agricultural research globally (Beintema et al., 2020), it is estimated that less than 5% of US funding and 15% of Australian funding is spent on post-harvest-related research (Kader, 2003; Goletti and Wolff, 1999). Improving post-harvest health of crops has the potential to have a significant positive impact on both human health and climate change.

1.2 Shifting priorities in post-harvest research

Optimisation of post-harvest quality is certainly not a modern phenomenon: the earliest known grain silos were built ~10,500 years ago (Kuijt and Finlayson, 2009), and there is evidence of ice houses in China from 2000 BC for cooling food (Muller, 1991). However, consumers' expectation that all fresh fruit and vegetables will be available year-round is a very recent development reliant on advances in food storage and long-distance transportation (Calvin et al., 2004). Development of cooling technologies and controlled atmosphere storage has increased the global trade of many vegetables. For example, controlled atmosphere storage can increase the shelf-life of Chinese cabbage (*Brassica rapa* spp. *pekinensis*) by 129 days (Fang and Wakisaka, 2021). However, the cold chain is energy intensive. It is estimated that food refrigeration accounts for 1% of global CO_2 emissions and 3.2% of the UK's total greenhouse gas emissions (Ravishankar et al., 2020; Foster et al., 2023). While it is unlikely that refrigeration will be superseded as a strategy to maintain post-harvest health, alternative parallel and future strategies should strive to be carbon neutral. In the same way that crop breeding as a whole is focussing increasingly on low input agriculture (Fess et al., 2011), so post-harvest trait development should prioritise

minimal energy and resource expenditure. One way to achieve this is through development of crop varieties specifically designed for improved post-harvest quality and shelf-life without compromising on-soil yields.

Strategies to improve post-harvest health depend on understanding the physiology of the stored crop, which can be markedly different from that of the soil-growing plant. Modern academic research on post-harvest physiology can be considered to have commenced in the 1920s (Laties, 1995) with studies of the role of ethylene (ET) in respiration of stored apples (Kidd and West, 1925; Blackman and Parija, 1928; Gane, 1934). Subsequently, much of twentieth century post-harvest research continued to focus on high-value fruit, the role of the ripening-associated hormone ET, and storage conditions (Dewey, 1979; Kader, 2003). However, the physiological changes in aerial organ crops that are in active vegetative growth at the point of harvest, such as rocket and broccoli, have been less well characterised. These actively growing crops (hereafter referred to as 'leafy vegetables' or 'leafy crops') have the potential to provide fascinating insights into the impact of harvest on the plant, as direct comparisons can be drawn with their on-soil counterparts, which are at the same developmental stage. What is more, the relatively short shelf-life of many leafy vegetables means that even small gains in post-harvest longevity or quality can significantly increase the proportion of these crops being consumed.

1.3 Post-harvest leafy vegetables

As a result of root detachment, harvest exerts multiple abiotic stresses on leafy vegetable crops, including wounding, nutrient starvation and dehydration. Consequently, harvested leaves enter premature senescence: reducing energy-expensive activities, such as growth and photosynthesis, and breaking down chlorophyll, which leads to the yellowing that consumers associate with inedibility (Spadafora et al., 2019). However, like other living tissues, their metabolism, transcription and protein translation remain intact (albeit likely somewhat reduced), allowing harvested tissues to respond to their environment (Zhang et al., 2023; Goodspeed et al., 2013).

1.3.1 Post-harvest leafy brassica crops

Leafy brassica crops are particularly fascinating for post-harvest studies, owing to their astonishing phenotypic range, even within individual species. Within the single species of *Brassica oleracea*, the range of storage life varies from 15 days to 6 months (Figure 1.1A). This is the result of domestication and breeding programmes that have selected for distinctive traits, such as a proliferation of immature inflorescences in broccoli (*Brassica oleracea* var. *italica* L.) or the formation of a compact, protective core of leaves (leaf heading) in cabbages (*Brassica oleracea* var. *capitata* L.) (Cheng et al., 2016).

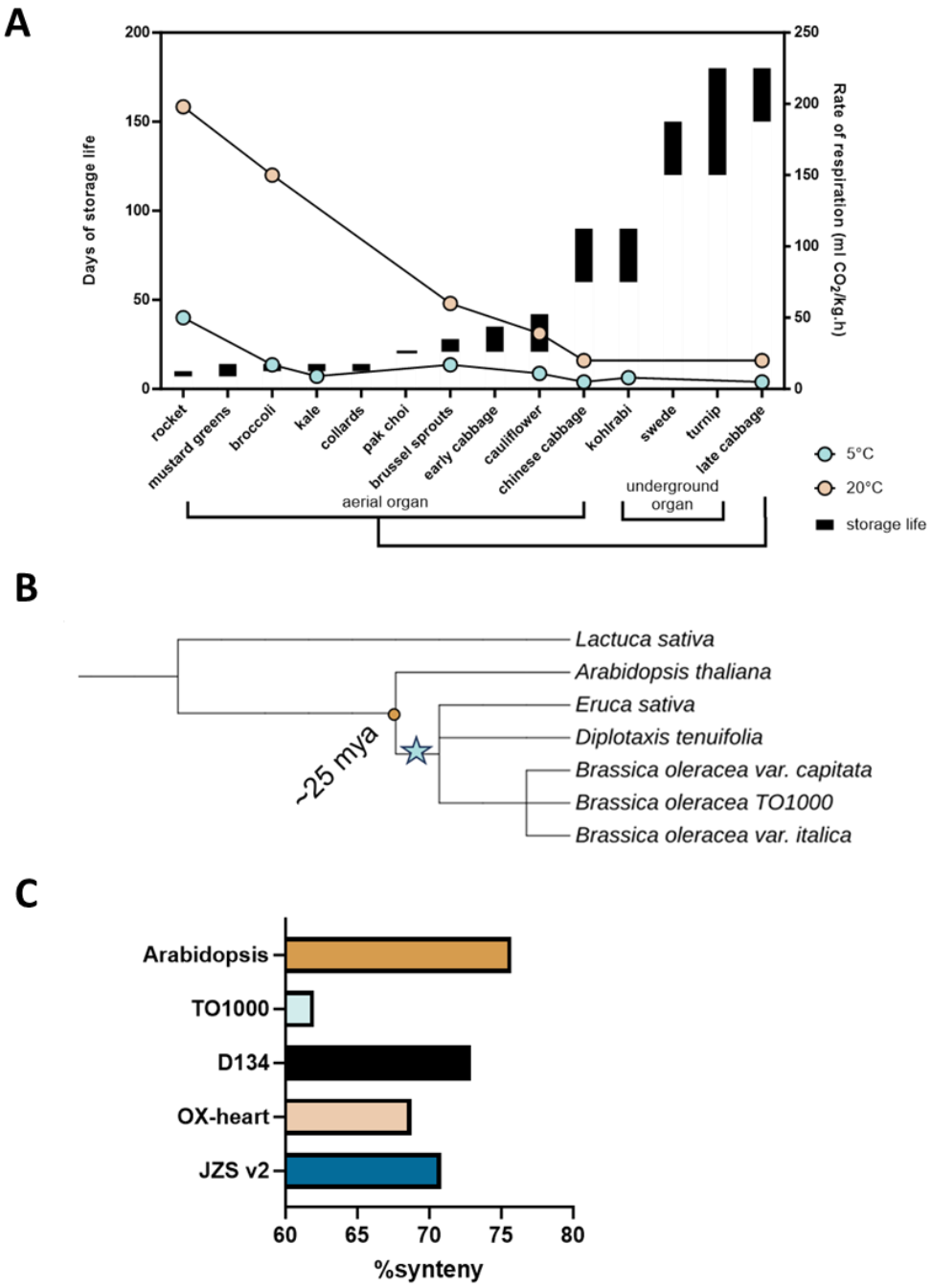


Figure 1.1 Leafy brassica crop variation in genomics and phenomics **(A)** Range of post-harvest storage life of brassica crops (black bar showing min-max) compared to their rate of post-harvest respiration at 5°C and 20°C (2 days post-harvest, where known). [data from postharvest.ucdavis.edu; Wang et al., 2022; Albornoz and Cantwell, 2016; and Escalona et al., 2006] **(B)** Phylogeny of brassica species used in this study based on taxonomy data in NCBI; blue star indicates whole-genome triplication event; branch length is not representative of relatedness (created in phylot.biobyte.de, visualised in iTOL). **(C)** The estimated levels of synteny between different leafy brassica datasets used in this study (data from Ji et al., 2023). See also Table 1.1.

Brassicas in which the floral organs or rosette leaves are the high-value crop, such as broccoli or rocket, have high respiration post-harvest, and consequently a shorter shelf-life compared to crops that are over-wintering storage organs, such as cabbage and turnips (Saltveit, 2016) (Figure 1.1A).

Leafy brassicas are particularly valued nutritionally for their fibre, vitamins and more recently for phytochemicals with additional antioxidant properties, such as glucosinolates, vitamin C and flavonoids (Sami et al., 2013). As such, post-harvest studies into leafy brassicas have mainly focussed on the profile of desirable nutrients over the course of storage (Zhao et al., 2020; Bell et al., 2020; Dewhirst et al., 2017; Spadafora et al., 2016), or the impact of storage conditions on senescence and accompanying discolouration (Ahlawat and Liu, 2021; Mastrandrea et al., 2016; Luo et al., 2019). Risk of infection is especially relevant to leafy vegetables, because their high water content and surface area make them particularly susceptible to tissue damage, enabling opportunistic pathogens to infect the plants in storage (Kader and Saltveit, 2003).

1.3.2 Genetic research in leafy brassica crops

In the last decade, research into leafy brassica crops has been facilitated by the construction of reference genomes (Table 1.1). However, molecular studies in brassicas are limited by the relative difficulty of making transgenic lines and lengthy generation time. Fortunately, the model plant *Arabidopsis thaliana* is a close relative of key brassica crops (Figure 1.1B), has a well-characterised genome, a short generation time, and is amenable to genetic transformation.

Binomial	Cultivar/ line	Chromosomes (ploidy)	Common name	Reference
Arabidopsis thaliana (L.) Heynh.	Col-0 ecotype (TAIR10.1)	5 (2n)	Thale cress	The Arabidopsis Genome Initiative, 2000
Brassica oleracea L. var. *capitata*	OX-heart_923	9 (2n)	Pointed cabbage	Guo et al., 2021
	Cap02-12 aka JZS v1	9 (2n)	Heading cabbage	Liu et al., 2014
	D134			Lv et al., 2020
	Cap02-12 aka JZS v2			Cai et al., 2020
Brassica oleracea L. var. *alboglabra*	T01000 DH3	9 (2n)	Chinese kale	Parkin et al., 2014
Brassica oleracea L. var. *italica*	HDEM	9 (2n)	Broccoli	Belser et al., 2018
Diplotaxis tenuifolia (L.) DC.	cv, Frastagliata	11 (2n)	Wild rocket	Cavaiuolo et al., 2017; Reis et al., 2022
Eruca sativa Miller	unspecified	11 (2n)	Salad rocket	Bell et al., 2020

Table 1.1: Sequenced genomes and transcriptomes for leafy brassica crops used in this study

Like Arabidopsis, the genomes of cabbage, broccoli and rocket are diploid; however, a whole genome triplication event occurred after the split from the Arabidopsis lineage resulting in many duplicate and triplicate orthologues (Parkin et al., 2014; Lysak et al.,

2005) (Figure 1.1B). Nonetheless, there is considerable synteny between the reference sequences of leafy brassica crops and Arabidopsis (Figure 1.1C). Thus, Arabidopsis has strong potential to be used as a post-harvest model for leafy brassicas, and will allow comparison of this model system with new and existing datasets in brassica crops.

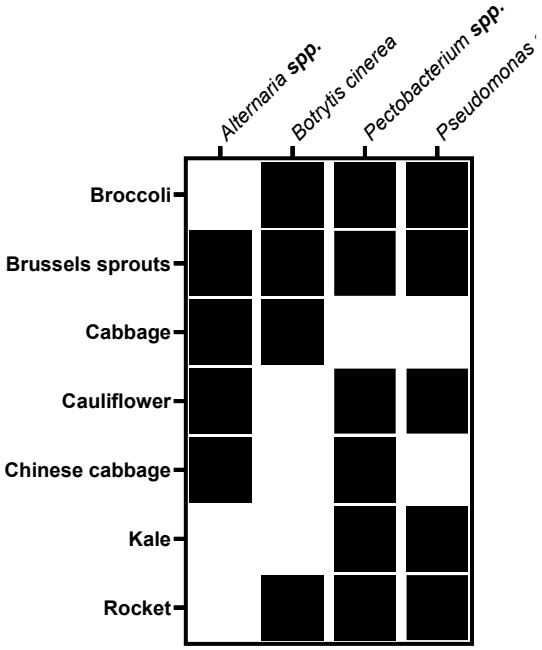


Figure 1.2: Key post-harvest diseases of selected leafy brassica crops; A black square denotes a pathogen or family of pathogens of concern in post-harvest storage, according to Gross et al.(2016).

1.3.3 Threats to health in post-harvest leafy vegetables

Stored crops can develop disease as a result of pathogen infection in the field or post-harvest. The extent of losses from storage diseases varies considerably based on post-harvest handling and climate. One study in Sri Lanka that followed post-harvest losses of cabbage from farm to consumers (Dharmathilake et al., 2020) showed that 82% of losses at the wholesale level were caused by removing damaged and diseased external leaves, and 14% were due to rotted plants; the pathogens were not, however,

identified. Indeed, there are few studies that are able to identify causative agents of loss at the distribution and consumer level, owing to the myriad end-users. The main causative agents of post-harvest disease in leafy brassicas are usually fungal and bacterial (Figure 1.2), and can be either hemi-biotrophs or necrotrophs (Gross et al., 2016). These two pathogen classes have different modes of infecting and feeding from host plants: hemi-biotrophs initially feed on living plant tissue, while necrotrophs kill the host cells to feed from their contents. In order to identify key foci for post-harvest crop improvement, it is important to establish if there are differences in post-harvest immune responses to biotrophic or necrotrophic pathogens compared to responses of soil-growing plants.

1.4 The plant immune system

Harvested plants contain the necessary molecular tools to launch immune responses, because each somatic cell is thought to contain the full receptor complement and signalling machinery needed to detect infection and launch an immune response (Maekawa et al., 2011). However, it is not clear how that machinery is affected by harvest stress. The immune system of plants is multi-layered with responses adapted to the nature of the pathogen threat; here we will focus on the plant cell components involved in the detection and hormonal pathways involved in the response to fungal and bacterial pathogens.

1.4.1 Pattern-triggered immunity

Plant cells are able to detect the presence of pathogen-associated molecular patterns (PAMPs), such as the flg22 peptide within bacterial flagellin, or chitin, a component of fungal cell walls. Detection of these PAMPs occurs at the plasma membrane of the cell through pathogen recognition receptors (PRRs), including FLS2 for flg22 (Gómez-Gómez et al., 1999) and CERK1 for chitin (Wan et al., 2008) (Figure 1.3). PAMP recognition triggers multiple cellular responses, collectively described as pattern-triggered immunity (PTI) (Jones and Dangl, 2006), including calcium influx (Blume et al., 2000), a burst of reactive oxygen species (Apostol et al., 1989), in some cases callose deposition (Luna et al., 2011) and induction of phosphorylation cascades (Asai et al., 2002). The phosphorylation cascades, through either mitogen-activated protein kinases (MAPKs) or calcium-dependent protein kinases (CDPKs) (Boudsocq et al., 2010), lead to altered sub-cellular localisation or activity of their target transcription factors (Popescu et al*.,* 2009) (Figure 1.3). These transcription factors upregulate early pathogen response genes, such as *FLAGELLIN-INDUCED RECEPTOR-LIKE KINASE 1* (*FRK1*) within 15 minutes of PAMP detection (Lyons et al., 2013). These early pathogen response genes strengthen the cell's defence against the pathogen threat through production of defensive metabolites and proteins, cell wall reinforcing components, and transcription factors that activate hormone-responsive immune pathways, including salicylic acid (SA) and jasmonic acid (JA) signalling (Zipfel et al., 2004).

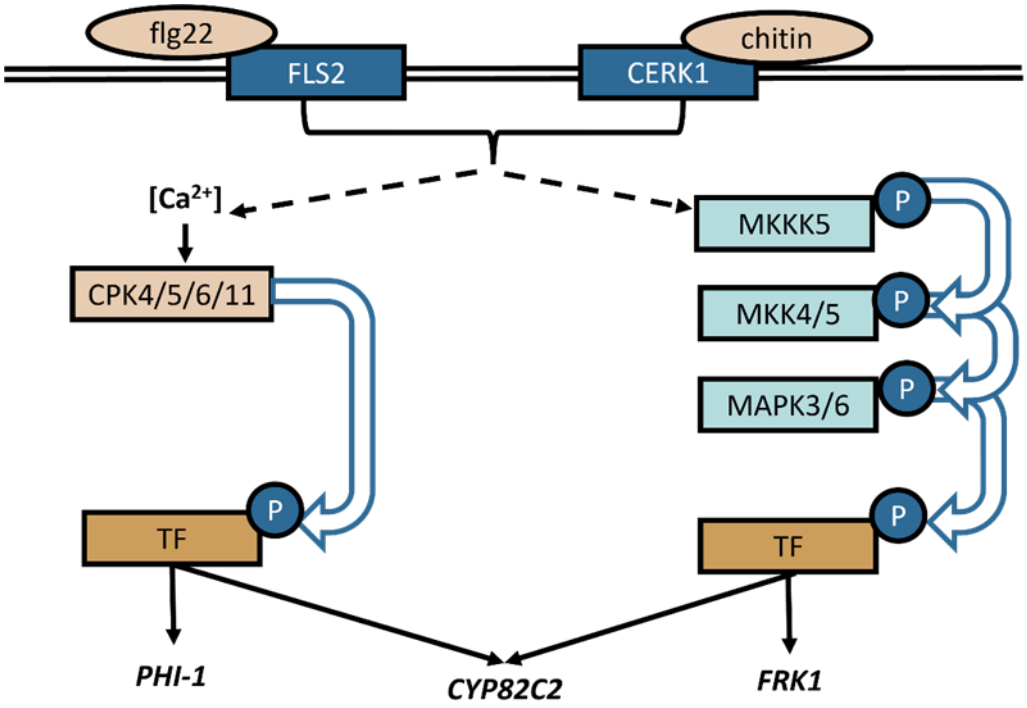


Figure 1.3: **PAMP detection and early signalling cascades** Activation of early pathogen response genes by CPDK and MAPK cascades triggered by perception of bacterial PAMP flg22 and fungal PAMP chitin. (Figure based on data from Asai et al., 2002; Boudsocq et al., 2010; and Bi et al., 2018)

1.4.2 Hormones in plant immunity

Activation of PTI results in the accumulation of immune-related hormones in the plant cell. Plant hormones have multiple and overlapping signalling nodes and functions in immunity and post-harvest physiology (Table 1.2), and can antagonise or enhance each other's activity (Altmann et al., 2020; Pieterse et al., 2009). This study will focus on two key immune hormones, SA and JA, that broadly defend against biotrophic and necrotrophic pathogens respectively, and are often mutually antagonistic (Glazebrook, 2005; Spoel and Dong, 2008).

Hormone	immunity	post-harvest physiology	References
abscisic acid (ABA)	**regulating stomatal opening**	**abiotic stress response**	Yin et al., 2013; Melotto et al., 2017; Ludford, 2002
auxin	negative impact on PTI	-	Naseem et al., 2015
brassinosteroid (BR)	control of PAMP response	can reduce senescence	Belkhadir et al., 2012; Ali et al., 2019
cytokinin (CTK)	biotroph defence	delays senescence	Albrecht & Argueso, 2017; Naseem et al., 2015; Ludford, 2002; Brenner et al., 2005
ethylene (ET)	**necrotroph defence**	**promotes senescence**	Adie et al., 2007; Ludford, 2002
gibberelin (GA)	managing SA-JA crosstalk	may delay senescence	Navarro et al., 2008; Ludford, 2002
jasmonic acid (JA)	**necrotroph defence**	**wounding response**	Glazebrook, 2005; Kimberlin, 2022
salicylic acid (SA)	**biotroph defence**	can delay degreening	Glazebrook, 2005; Yang et al., 2023b

Table 1.2: Selected phytohormones involved in immunity or post-harvest physiology; responses highlighted in bold indicate a known upregulation of hormonal levels in response to immunity and/or harvest in leafy crops.

1.4.3 The SA signalling pathway

The SA pathway can limit the spread of pathogens in their biotrophic stage by upregulating genes coding for anti-microbial proteins or peptides, such as *PATHOGENESIS-RELATED 1* and *2* (*PR1* and *PR2*) (Thomma et al., 1998). An accumulation of SA triggers activation of the transcription cofactor protein NON-EXPRESSOR OF PATHOGENESIS RELATED GENES 1 (NPR1). When levels of SA are low,

NPR1 is predominantly maintained in an oligomeric form in the cytosol. Pathogen-induced elevation of SA levels triggers monomerization of NPR1 and subsequent translocation into the nucleus (Kinkema et al., 2000; Mou et al., 2003; Tada et al., 2008), where it interacts with the TGA family of transcription factors to regulate SA-responsive gene expression (Zhang et al., 1999; Després et al., 2000; Zhou et al., 2000; Després et al., 2003) (Figure 1.4).

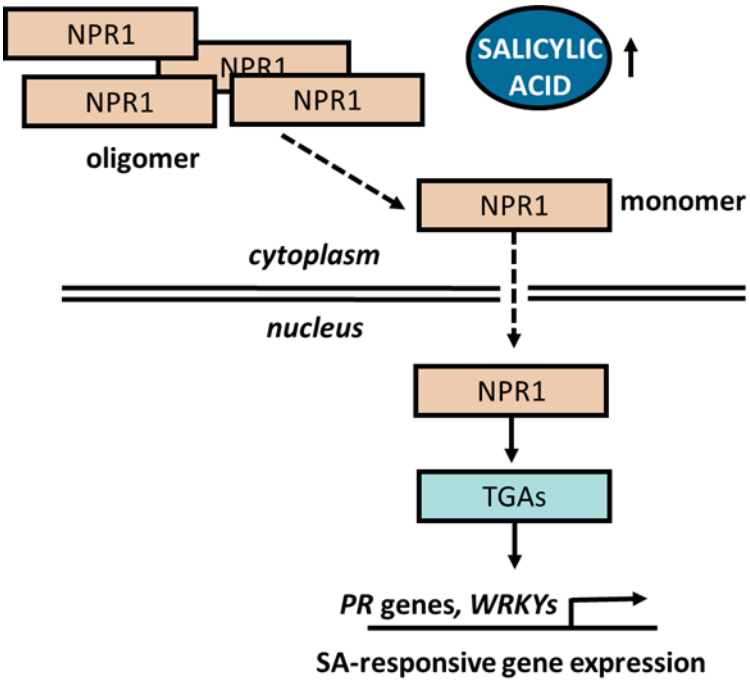


Figure 1.4: Simplified schematic of the SA signalling pathway

The SA pathway is key in protecting plants against hemi-biotrophic pathogens such as *Pseudomonas syringae,* a Gram-negative bacterium that can infect almost all crop plants (Xin et al., 2018). The bacteria enter leaves through stomatal openings or wounds, and multiply in the apoplastic space between cells (Katagiri et al., 2002). They initially feed biotrophically (i.e., without killing host cells) by repressing the host

immune response, while promoting the release into the apoplast of water and nutrients on which to feed (Cunnac et al., 2011). The infected leaves become increasingly chlorotic during this biotrophic phase. However, the bacteria eventually cause development of necrotic lesions in their hosts, hence their description as hemi-biotrophic. The SA signalling pathway contributes to host defence against *P.syringae* by stimulating the production of antimicrobial compounds, such as phytoalexins, which can inhibit bacterial growth (Tsuji et al., 1992; Vlot et al., 2009). In addition, sustained high levels of SA can trigger localised programmed cell death, which isolates biotrophic pathogens, like *P.syringae*, in a patch of dead cells, and consequently limits further spread (Fu et al., 2012).

1.4.4 The JA signalling pathway

The key immune pathway that regulates resistance to necrotrophic pathogens is controlled by the phytohormone JA. Activation of this pathway results in production of defensive compounds and enzymes, fortification of plant cell walls and downstream immune signalling (Hickman et al., 2017). JA is produced in response to damage by necrotrophs or wounding; basal levels are usually low, but rapid accumulation occurs within 30 seconds of mechanical damage to the plant (Glauser et al., 2009). It is biosynthesised in the thylakoid membranes of the chloroplast by enzymes that convert α-linolenic fatty acids (Schaller and Stinzi, 2009). Wounding, such as cutting during the process of harvesting, triggers a transient burst of elevated JA which returns to unwounded levels of expression within 24 hours (Reymond et al., 2000).

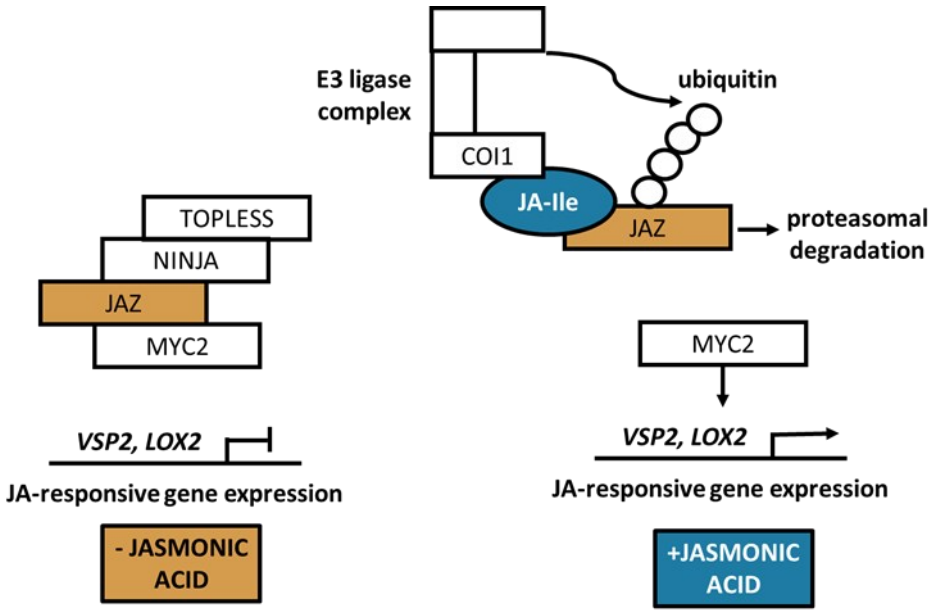


Figure 1.5: Simplified schematic of the JA signalling pathway

The level of JA in the plant cell regulates expression of JA-responsive genes by controlling the levels of transcriptional co-repressors (Figure 1.5). At low cellular JA levels, JA-responsive gene expression is repressed by JASMONATE ZINC-FINGER INFLORESCENCE MERISTEM (ZIM) DOMAIN (JAZ) proteins, which assemble co-repressor complexes that inhibit the activities of MYC transcription factors (Chini et al., 2007; Thines et al., 2007; Pauwels et al., 2015). JA-Ile promotes formation of a complex between JAZ proteins and CORONOTINE INSENSITIVE 1 (COI1), a protein that is part of an E3 ligase complex that promotes ubiquitination and proteasomal degradation of JAZ repressors (Chini et al., 2007; Thines et al., 2007; Sheard et al., 2010). JAZ degradation releases MYC transcription factors to activate the transcription of the JA-responsive genes, such as *VSP2* and *LOX2* (Lorenzo et al., 2004). MYC2 is able to dampen the JA response by upregulating expression of genes encoding its own

repressors: JAZ proteins and MYC2-TARGETED BASIC HELIX LOOP HELIX (bHLH) repressors (Chini et al., 2007; Liu et al., 2019b).

The JA pathway is key to defence against necrotrophic pathogens, such as *Botrytis cinerea* (Thomma et al., 1998). *B.cinerea* is a fungus that predominantly feeds as a necrotroph: it produces enzymes and reactive oxygen species that damage host cells, thereby releasing nutrients on which the fungus feeds. It is a broad-spectrum pathogen, infecting over 200 crop species (Williamson et al., 2007). Although used as a representative necrotrophic pathogen, there is an initial biotrophic or quiescent growth stage after the spore has germinated and the appressorium has breached the host's epidermis (Emmanuel et al., 2018). During this quiescent period, *B.cinerea* spreads through the apoplast in hyphal form, without visible symptoms on the leaf. It has been proposed that the necrosis and sporulation stage of the *B.cinerea* life-cycle is triggered by host stress responses, developmental stage or senescence (Shaw et al., 2016). Therefore, it could be hypothesized that harvested plants would be more susceptible to damage caused by *B.cinerea* infection.

1.4.5 SA-JA crosstalk

Crosstalk between plant hormone signalling pathways make immune signalling more robust and specific (Aerts et al., 2021). When SA or JA levels are elevated, the signalling pathways will antagonise each other (Mur et al., 2006). The SA signalling pathway represses JA signalling in multiple ways, including reduction of JA biosynthesis (Yuan et al., 2017), and NPR1-mediated suppression of MYC2 transcriptional activity (Nomoto et al., 2021). For its part, JA suppresses SA signalling by upregulating

expression of TFs that repress SA biosynthesis (Zheng et al., 2012; Gao et al., 2022). This antagonism is important for prioritisation and fine-tuning of immune responses that will best protect the plant against specific pathogen threats.

1.4.6 The ET signalling pathway

The ET signalling pathway is also involved in the response of plants to necrotrophic pathogens (van Loon et al., 2006). When ET accumulates in the cell, a cleaved fragment of ETHYLENE INSENSITIVE2 (EIN2) is translocated to the nucleus (Wen et al., 2012) (Figure 1.6), where it prevents proteasomal degradation of the ETHYLENE INSENSITIVE3 (EIN3) transcription factor (Wen et al., 2012; Guo and Ecker, 2003). EIN3 activates multiple ET-responsive genes, such as *ETHYLENE RESPONSE FACTORS* (*ERFs*) (Solano et al., 1998; Chang et al., 2013), which can trigger downstream defence signalling cascades (Oñate-Sánchez and Singh, 2002). The ET- and JA-signalling pathways can cooperate to regulate immune responses to necrotrophic pathogens through upregulation of genes such as *PLANT DEFENSIN1.2* (*PDF1.2*) (Penninckx et al., 1998), but MYC2 and EIN3 can also be mutually antagonistic (Song et al., 2014).

1.4.7 The ABA signalling pathway

Abscisic acid (ABA) is a key phytohormone in plant stress responses. Notably, when plants experience osmotic stress, ABA levels increase, and control closure of stomatal pores to reduce water loss through transpiration (Yin et al., 2013). Elevation of ABA in the cell leads to degradation of repressor proteins PROTEIN PHOSPHATASE 2Cs (PP2Cs), and allow subclass III SNF1-RELATED PROTEIN KINASE 2s (SnRK2s) to activate ABA-responsive transcription factors, such as ABA-RESPONSIVE ELEMENT (ABRE)-

BINDING FACTORS (ABFs) (Soma et al., 2021). ABA is most commonly associated with abiotic stress responses, but also interacts with immune signalling: elevated ABA levels promote degradation of NPR1 (Ding et al., 2016), which could dampen the SA pathway, and increase transcription of *MYC2* (Lorenzo et al., 2004), which could accentuate the JA pathway.

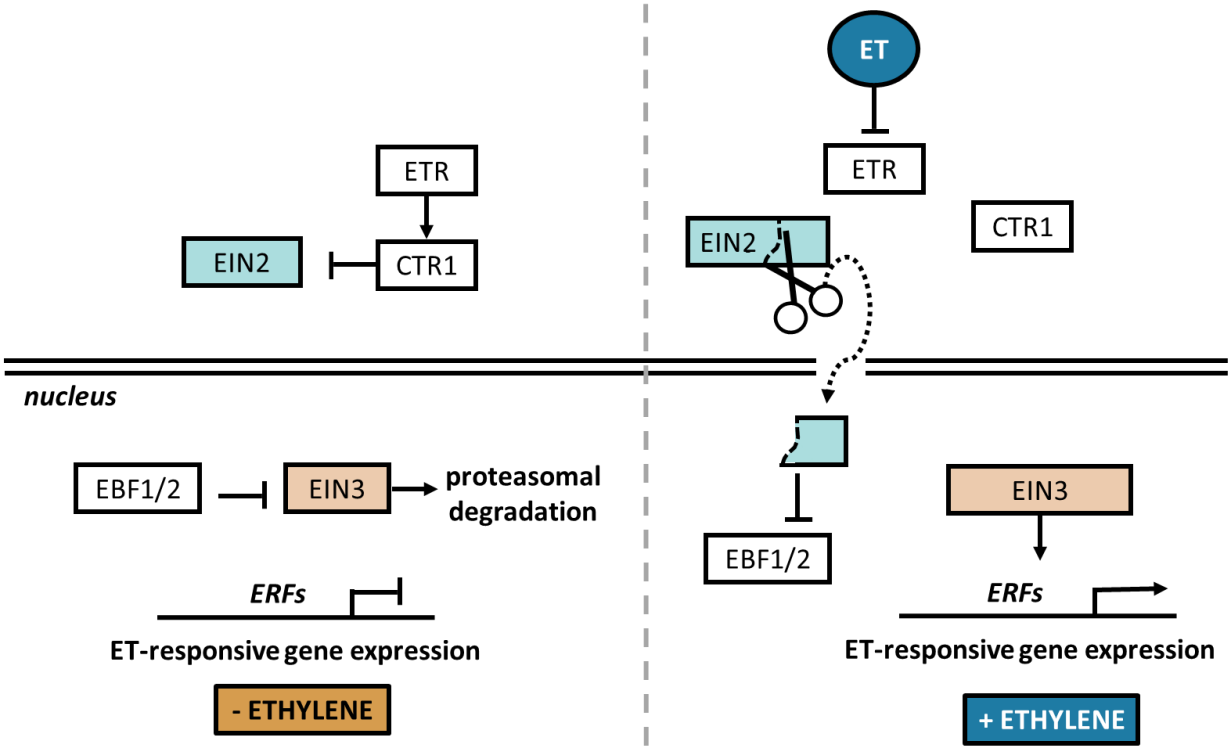


Figure 1.6 Simplified schematic of the ET signalling pathway: In the absence of ET, the ET receptors (ETRs) activate CONSTITUTIVE TRIPLE RESPONSE1 protein kinase (CTR1) (Kieber et al., 1993), which prevents cleavage of ETHYLENE INSENSITIVE2 (EIN2); EIN3-BINDING F-BOX1 and 2 (EBF1/2) target the ETHYLENE INSENSITIVE3 (EIN3) transcription factor to the proteasome for degradation, and ET-responsive genes are not expressed. When ET is present, it prevents ETHYLENE RECEPTOR (ETR) activation of CTR1, allowing EIN2 to be cleaved; the cleaved end is translocated to the nucleus where it prevents translation of EBF1 and 2, which allows EIN3 to activate ET-responsive genes.

1.5 Post-harvest stresses and their impact on immunity

Both *B.cinerea* and *P.syringae* have been extensively studied in Arabidopsis, even to the single-cell level (Tang et al., 2023; Zhu et al., 2023), and the corresponding JA and SA defence pathways are well characterised. However, to our knowledge there have been no studies on the impact of harvest on host susceptibility to these diseases, nor the impact on the SA and JA pathways. Experiments that have explored the effect of abiotic stress on immunity could provide indications about the impact of post-harvest physiology on immune pathways and disease susceptibility.

1.5.1 The impact of detachment of aerial organs on immunity

The harvest process of detaching the leafy rosette from the roots is likely to impact the immune response. Detached leaves are commonly used in laboratory disease assays for crop breeding and plant pathology studies (e.g. Arraiano et al., 2001; Miller-Butler et al., 2018). However, compared to intact plants, individually detached leaves have been shown to be more susceptible to the hemi-biotrophic fungus *Colletotrichum higginsianum* (Liu et al., 2007), while showing slower progression of *B.cinerea* symptoms (Dai et al., 2019). Interestingly, the rosette core itself, where the petioles attach to the stem, was suggested to have a protective role in disease resistance (Dai et al., 2019). The data from these studies suggest that separation of the rosette from the roots during harvest of leafy vegetables could result in a change in disease susceptibility compared to plants growing on soil.

1.5.2 The role of senescence in immunity

The accelerated, stress-induced senescence of harvested leafy crops is caused by nutrient starvation and is distinct from developmental senescence of a mature plant on soil (Ghimire et al., 2023). Although there is commonality in the machinery for breaking down and redistributing the chlorophyll and cellular components in both forms of senescence, SA is only thought to be involved in developmental senescence, whereas JA and ET have roles in both (Buchanan-Wollaston et al., 2005). Compared to wild type (WT), mutant plants with accelerated developmental senescence were generally more resistant to (hemi-) biotrophic pathogens, and more susceptible to necrotrophic pathogens (Zhang et al., 2020), but it is not clear if the same would be true for plants undergoing stress-induced senescence.

1.5.3 Interplay between osmotic stress and immunity

Osmotic stress causes changes in post-harvest leaf physiology that could affect disease outcomes. Plants reduce stomatal apertures of their leaves to reduce transpiration, but this can be a double-edged sword for pathogen infection: while it reduces pathogen ingress through stomata, it may increase apoplastic water content in which pathogens flourish (Hu et al., 2022). Although osmotic stress has variably been shown to exacerbate or attenuate disease symptoms (Aung et al., 2018), one recent study in intact Arabidopsis showed that low-level drought treatments rendered plants more susceptible to *Pseudomonas syringae* (Choudhary and Senthil-Kumar, 2022).

From these studies of the impact on immunity of detachment, senescence and osmotic stress, it is difficult to make predictions about how immunity will change in

harvested plants. The existing studies above show opposite effects on disease susceptibility of detachment and senescence. What is more, the overlapping nature of plant abiotic and biotic stress response pathways (Fujita et al., 2006), and their antagonistic or synergistic interactions mean that it is not possible to accurately predict the outcome of multiple stresses from their individual components (Rasmussen et al., 2013).

1.6 Strategies for improving post-harvest health

1.6.1 Existing strategies to improve post-harvest health

Understanding any weaknesses in post-harvest health and immunity will be critical for designing strategies to improve shelf-life and quality in storage. Existing treatments to extend the shelf-life of leafy vegetables have focused on chemical treatments, controlled atmosphere, and packaging (Mahajan et al., 2014). However, an increasing number of limitations are being imposed on chemical treatments, owing to concerns about health and environmental safety, such as the 2018 European Union ban on post-harvest use of the fungicide iprodione, which was used to control *B.cinerea* in stored cabbage (European Commission, 2015). Instead, there is a drive for sustainable strategies to improve post-harvest health, requiring lower energy and resource use, such as can be accomplished through the bioengineering of plants themselves.

1.6.2 The value of inducible gene expression systems for post-harvest health

The induction of a beneficial post-harvest trait specifically after harvest using an inducible gene expression system would be optimal in order to prevent yield penalties and unwanted side-effects in soil-growing plants. For example, immunity is energetically expensive: constitutive expression of a gene to address post-harvest weaknesses could divert resources away from growth and development (Bergelson and Purrington, 1996; Guo et al., 2018), and reduce the edible crop. Moreover, if the immune system of crops on soil is different to those in storage, a gene upregulated to boost post-harvest immunity could be detrimental to the crop's pre-harvest health.

Many systems of inducible gene expression in plants have been developed using exogenous or endogenous inducers, and driven by promoters derived from plants or other kingdoms, or synthetically composed of known enhancer elements (Table 1.3). Abiotic stress triggers have been used to drive gene expression, such as senescence (Gan and Amasino, 1995) and wounding (Rushton et al., 2002); however these systems are respectively too gradual and too transient to provide the rapid upregulation and continuous expression required to provide protection throughout the whole storage period of a post-harvest crop. This study will, therefore, design and test a harvest-inducible gene system to be used in improving post-harvest health.

Type of induction	inducer	Promoter	Promoter origin	Reference
Chemical	steroid (DEX)	LhGR	mammal	Samalova et al., 2005
	ethanol	*AlcA*	fungus	Caddick et al., 1998
	estrogen	XVE	mammal	Zuo et al., 2000
	peptide(dTALEs)	STAP	bacteria	Danila et al., 2022
Environmental	heat	*HSP18.2*	plant	Takahashi et al., 1992
	light	Ccas-CcaR	bacteria	Larsen et al., 2023
	cold	*RD29A-CBF3-cpl1-2*	plant	Feng et al., 2011
Abiotic stress	cold/drought	*ZmRXO1*	plant	Tao et al., 2015
	low phosphate	*ATPHT1.1*	plant	Belcher et al., 2020
	osmotic stress	*DLL*	plant	Polóniová et al., 2014
		SynP16	synthetic	Jameel et al., 2020
		BL1/BL2	synthetic	Kim et al., 2021
	salt stress	*SD16*	synthetic	Yang et al., 2021
	senescence	*SAG12*	plant	Gan and Amasino, 1995
Biotic stress	wounding/ infection	-	synthetic	Rushton et al., 2002
	wounding/ infection	*gst1*	plant	Barbosa-Mendes et al., 2009
	viral infection	*GWVSF*	synthetic	Huang and Li, 2020
	biotrophic infection	*pCaD*	plant	In et al., 2020
Hormone	ethylene	*EBS*	plant, and CRISPR gRNA	Kar et al., 2022
	salicylic acid	*FUASCsV8CP*	virus	Deb and Dey, 2019
	jasmonic acid	*SP-FF*	synthetic	Shokouhifar et al., 2019

Table 1.3: Examples of inducible gene expression systems used in plants

1.7 Key knowledge gaps and hypotheses

Key gaps in our understanding of post-harvest health in leafy crops are the impact of harvest on PTI, and on the SA- and JA-signalling pathways, and whether harvest

differently sensitises plants to biotrophic or necrotrophic pathogens. Based on the existing literature, there were two likely hypotheses:

1) *Harvested plants are more susceptible to both necrotrophic and biotrophic pathogens.* The plant energetic expenditure on abiotic stress responses would reduce their ability to respond to biotic stress, and cause an overall reduction in immune activation.
2) *Harvested plants are more susceptible to necrotrophic pathogens, but more resistant to biotrophs.* The accelerated senescence of harvested tissues could facilitate or trigger necrotrophic feeding, but impede the spread and feeding of biotrophs, which rely on living, productive host cells for their nutrition.

1.8 Study aims and objectives

Given the need for innovative bioengineering to improve post-harvest health and shelf-life of leafy brassicas, this study aims to explore harvest-induced changes in plant processes and immunity to identify traits that require optimisation in the post-harvest stage, and develop a harvest-inducible genetic system. To achieve this, this study will address the following objectives:

1) Using the model leafy brassica *Arabidopsis thaliana*, identify changes in PTI and immune hormone signalling in harvested plants.
2) Using new and existing RNA-seq datasets, compare the transcriptomes of harvested Arabidopsis rosettes and leafy brassica crops.

3) Identify, design and characterise harvest-inducible promoters to drive post-harvest upregulation of desirable traits.

This study focuses on fungal and bacterial pathogens as the main cause of damage to leafy vegetables: viral pathogens, oomycetes, insects and other pests are outside the scope of this study. Although there are many layers to plant defences against disease, such as non-host resistance, PTI, effector-triggered immunity (ETI) and viral RNA silencing, this study focuses on PTI as a common component of both PTI and ETI (Pruitt et al., 2021; Yuan et al., 2021; Ngou et al., 2021).

This study looks at post-harvest changes in leafy brassicas, including Arabidopsis, pointed cabbage, broccoli and salad rocket. The post-harvest biology of leafy crops is very different from that of fruit, such as apples and strawberries; seeds, such as rice; and underground storage organ crops, such as potatoes and carrots. Although post-harvest research in all of these types of crops is critical for food security, the developmental changes that occur to produce seeds, fruit and storage organs makes direct comparisons with their on-soil counterparts more challenging.

This study will bring new insights on the post-harvest immune system in leafy vegetables, and provide a foundation for future research into immunity in vegetable crop plants. This improved understanding could also feed into industrial efforts to combat diseases of stored vegetables, and focus efforts on the pathogens and immune pathways that most need to be addressed for post-harvest health. Harvest-inducible genes have the potential to form part of these efforts to improve post-harvest quality without compromising yield nor on-soil health. This study will provide proof-of-

concept for harvest-inducible promoters, which could then be used to drive post-harvest defence genes, or indeed genes improving nutrition or longevity of harvested plants, which could reduce food loss and waste.

Chapter 2 : Methods and materials

2.1 Plant materials and growth conditions

2.1.1 *Arabidopsis thaliana*

All *Arabidopsis thaliana* wild type and mutant lines were in the Colombia (Col-0) ecotype (Table 2.1).

Mutant genotype	Reference
efr-1 (SALK_044334)	Alonso et al., 2003
npr1-0 (SALK_204100)	Alonso et al., 2003
35S::NPR1-GFP (*npr1-1*)	Kinkema et al., 2000
35S::JAZ1-GS (*jaz1*)	Cuéllar Pérez et al., 2014
pMYC2::MYC2-FLAG (jin1-8)	Hou et al., 2010
pEIN3:EIN3-GFP-3xFlag (ein3-1) (line 6E)	Potuschak et al., 2003

Table 2.1: Mutant and transgenic genotypes used in this study and the paper in which they were created

2.1.2 Seed sterilisation

Arabidopsis seeds were sterilised immediately prior to sowing on soil by rehydrating in autoclaved ddH_2O for 30 minutes, and then washing twice in 70% ethanol, and twice in 10% filter-sterilised bleach, before five washes in ddH_2O. Seeds were then pipetted onto the surface of autoclaved soil and stored at 4°C for 3-7 days to ensure even germination. There was an extended bleach treatment for seeds to be grown on agar plates: 50% filter-sterilised bleach on rotation for ten minutes before the ddH_2O washes.

2.1.3 Plant growth

Plants were grown on soil that had been autoclaved at a maximum volume of 6.5 L soil with 1.5 L water. Soil was sand:vermiculite: Levington Advance F2 sphagnum moss peat-based compost (F20117800) (1:1:4). Arabidopsis plants were grown at 72 plants/tray in growth chambers at 21°C with long day conditions: 16 h light at 100 $\mu mol.m^{-2}.s^{-1}$ fluorescent tube lighting, and 65% humidity, followed by 8 h dark with 55% humidity.

2.1.4 Pointed cabbage

Three week-old seedling plugs of *Brassica oleracea* var. *capitata* cv. 'Regency' grown in the Kettle Produce Ltd, Fife nursey were transplanted into a growth chamber with natural light (average of 16 hours daylight/day) maintained at 18°C at night and 21°C during the day. Mature cabbages for the RNA-seq time course were collected as detailed below (Section 2.8.4).

2.2 Pathogen infection and quantification

2.2.1 *Botrytis cinerea* fungus culturing

Botrytis cinerea var. pepper (from Denby lab, York) was cultured on tinned apricot halves or V8® agar plates (autoclaved 20% V8® vegetable juice, 1.5% w/v agar, 2% w/v calcium carbonate) under sterile conditions. Spores from an existing culture or a glycerol stock were stabbed by pipette tip into the apricots or streaked on the plates, and then kept at room temperature (~18°C) for two weeks until full sporulation.

2.2.2 Fungal inoculation

Spores were removed after two weeks of fungal growth by adding 5 mL sterile ddH_2O and rubbing the fungal surface to release the spores into suspension. These suspensions were vortexed immediately before spore counting using a haemocytometer, and then diluted with sterile ddH_2O to 2x the required spore density. The spore suspension was further diluted 1:1 with filter-sterilised red grape juice to produce the final inoculum. Control plants were inoculated with 1:1 red grape juice: ddH_2O. A 10 µL drop of spore inoculum (containing 1000 spores) was added to the centre of an Arabidopsis leaf and allowed to air dry for one hour or until the droplet's surface tension had broken. The soil-growing plants were covered with a transparent lid to avoid spore dispersal onto control plants. A vent was inserted in the lids to prevent excessive humidity and covered with filter paper to avoid spore escape.

2.2.3 Quantification of *Botrytis cinerea* fungal growth

Three days after infection, inoculated leaves were photographed to assess the area of necrotic lesion, and then frozen in LN_2 to measure the level of fungal DNA in the sample. The area of necrotic lesion was measured using Fiji (Schindelin et al., 2012) as ($Area_{LEAF}$ thresholds: Hue = 0, Sat = 0, Bright = 46; $Area_{GREEN}$ threshold: Hue = 44, Sat =0, Bright = 46; $Area_{NECROTIC}$: drawn manually; $Area_{CHLOROTIC}$ calculated as:

$$Area_{CHLOROTIC} = Area_{LEAF} – (Area_{GREEN} - Area_{NECROTIC})$$

Data was analysed for significant difference using a one-way ANOVA ($p<0.05$).

The fungal DNA was extracted by grinding infected plant tissue and incubating at 65°C for 30 minutes in a water bath with 2% cetyltrimethylammonium bromide (CTAB). After cooling to room temperature the samples were centrifuged briefly before vortexing with equal volumes of chloroform:isopropanol (24:1). Samples were centrifuged at 3250 rpm for 15 minutes; the supernatant was added to equal volumes of isopropanol and incubated at room temperature for ten minutes. The DNA was pelleted at 3250 rpm for 15 minutes, and the pellet was washed with 70% ethanol. After a final centrifugation for ten minutes at 3250 rpm, the ethanol was removed, and the pellet allowed to air dry for three hours. The pellets were resuspended in sterile ddH_2O overnight at 4°C. Levels of fungal DNA were quantified against a standard curve of Bc*CUTA* expression using qRT-PCR.

2.3.4 *Pseudomonas syringae* culturing

Pseudomonas syringae pv. *maculicola* ES4326 (*Psm*) was streaked from a glycerol stock on LB agar + 50 µg/mL streptomycin ($strep_{50}$) + 10 mM $MgSO_4$ and incubated at 28°C overnight. A 5 mL overnight LB $strep_{50}$ culture + 10 mM $MgSO_4$ was inoculated with one colony from the streak plate and incubated at 28°C at 200 rpm; 1 mL from this overnight culture was used to inoculate 100 mL of LB + 10 mM $MgSO_4$ until spectrophotometer measurement of its optical density at 600 nm (OD_{600}) reached 0.6. This culture was then centrifuged for ten minutes at 4000 rpm and the pellet rediluted in 10 mM $MgSO_4$ to the required OD_{600} for plant infection. An OD of 0.2 in 0.005% Silwet® L-77 was used for spray, while an OD of 0.004 was employed for infiltration.

2.3.5 *Psm* disease quantification

Discs of 5 mm diameter were cut from leaves 5 and 6 with a leaf disc punch. Tissue was ground with tissue grinder sticks in 500 µL 10 mM $MgSO_4$ until homogenous, and a serial 1:10 dilution was set up to a minimum concentration of $1\text{^}10^{-6}$. 10 µL of each dilution was streaked on an LB $strep_{50}$ + 10 mM $MgSO_4$ plate and incubated at 28°C for two days. (Media recipes in Appendix A). Significant difference in colony number was calculated with one way ANOVA $p<0.05$.

2.3 Model harvest system

2.3.1 The Arabidopsis model system

Arabidopsis plants were grown on soil for 24 days. Plants for 'harvested' samples were cut at ZT4 with scissors just below the cotyledons, separating the basal leaf rosette from the roots. (Figure 2.1A). The rosette was then placed in a petri dish on a double layer of 85 mm diameter Whatman filter paper moistened with 3 mL sterile ddH_2O. Three rosettes were placed on each petri dish, and up to seven petri dishes were placed in 30 x 25 cm plastic trays lined with four layers of tissue dampened with 15 mL sterile ddH_2O to maintain humidity. Additional ddH_2O was added to filter paper each day as needed to maintain moisture. Trays were covered with a transparent plastic lid and returned to the growth chamber with the soil-growing plants. Under these conditions, plants were able to be used for assays for 4-5 days post-harvest (dph) (Figure 2.1B). For disease assays, early harvest (EH) samples were cut and placed on

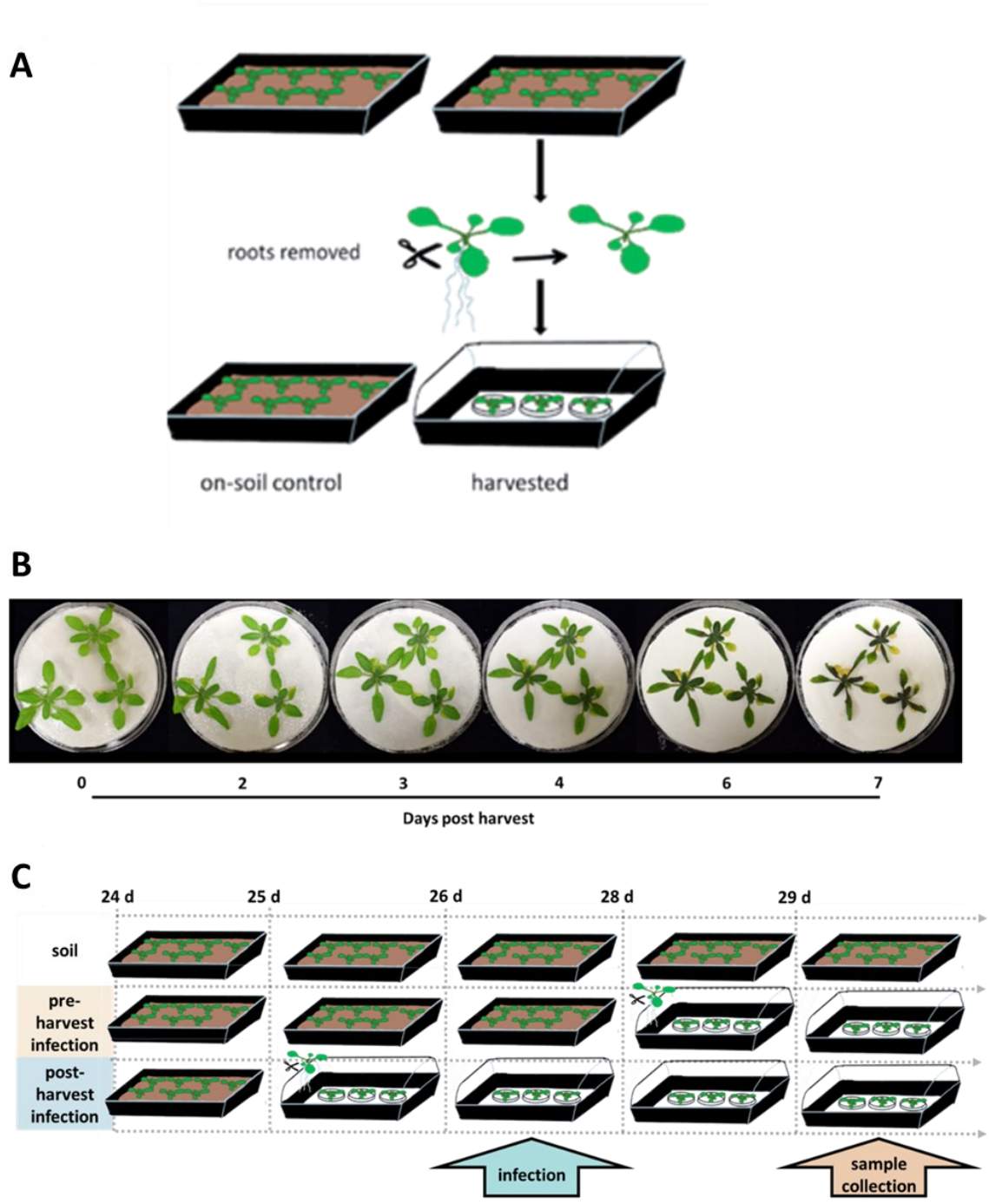


Figure 2.1: The model harvest system (**A**) The removal of roots and tray set-up (**B**) photographs of harvested Arabidopsis rosettes over seven days under model harvest conditions; (**C**) disease assay timings for pre-harvest infection (LH) and post-harvest infection (EH).

trays one day before infection; late harvest (LH) samples were cut and placed on trays one day after infection (Figure 2.1C). For hormone and elicitor assays, the rosettes were cut at ZT4 up to three days before treatment; the 'days post harvest' (dph) indicates how many hours prior to the chemical treatment the rosettes were put in trays.

2.3.2 The cabbage model system

Cabbage for post-harvest hormone assays had their rosette harvested at the 10-leaf stage, removing roots; lower leaves and were kept in covered 30x45 cm seed trays lined with damp tissue and two pieces of 24 cm diameter filter paper to maintain humidity. The harvested trays were misted with ddH_2O daily.

2.4 Protein extraction and western blotting

2.4.1 Standardisation of protein collection

Owing to the different levels of hydration of harvested and unharvested leaves, sample size for protein extraction could not be equalised by mass. In addition, the accumulation of dark pigmentation in the harvested leaves confounded equalising with the Bradford assay. Therefore, protein samples were collected by area, as a fixed quantity of leaf discs per replicate, and frozen in LN_2.

2.4.2 Protein extraction

Samples were frozen in LN_2 and ground to a fine powder in a TissueLyser for 3 x 30 s @ 20 beats/s. The ground tissue was vortexed in extraction buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.2% NP40, 5 mM EDTA, 0.1% Triton X-100, 50 µg/mL TPCK, 50 µg/mL TLCK, 0.6 mM PMSF) until homogenous, and centrifuged at 13 000 rpm for 15 min at 4°C. Supernatant from the spun samples was heated to 95°C for 10 minutes in SDS loading buffer (10% glycerol, 60 mM Tris pH 6.8, 2% SDS, 0.01% bromophenol blue, 1.25% BME), with or without 6 mM DTT.

2.4.3 Protein quantification

Equal volumes of the extracted protein were separated on an SDS-PAGE gel, and transferred by wet transfer overnight onto a nitrocellulose membrane. Protein was initially visualised using Ponceau stain, then blocked in milk buffer (5% milk powder, PBS, 1/1000 Tween-20) for at least one hour before immunoblotting. All antibodies used are listed in Table 2.2. The membrane was exposed to chemiluminescent substrate Thermo Scientific ™ SuperSignal™ West Pico PLUS or SuperSignal™ Dura for two minutes or ten minutes respectively, and imaged with a LI-COR® Odyssey Fc. Before reblotting for a different protein target, membranes were stripped of the antibody by incubating for 10 minutes in 0.5% β-mercaptoethanol and 1% SDS in PBS (warmed to 50°C) and then washed twice for 15 minutes in PBS + 0.1% Tween-20, before reblocking in 5% milk buffer before reprobing. S2 and GAPDH were used as loading controls.

Probing for	Type	Supplier	Cat.number	Ratio
Primary antibodies				
26S S2 (RPN1B)	rabbit	Abcam	ab98865	1:500
FLAG M2	mouse	Sigma	F1804-200ug	1:2000
GAPDH	rabbit	Sigma	G9545	1:5000
GFP	mouse	Roche	11 814 460 001	1:2000
NPR1	rabbit	Agrisera	AS12 1854	1:1000
p44/42 MAPK (Erk1/2) (137F5)	rabbit	Cell Signalling	4695	1:1000
Peroxidase Anti-Peroxidase (PAP)	-	Sigma-Aldrich	P1291	1:2500
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP®	rabbit	Cell Signalling	4370	1:4000
Secondary antibodies				
anti-rabbit HRP linked		Cell Signalling	7074S	1:2500
anti-mouse IgG HRP linked		Cell Signalling	7076S	1:2500

Table 2.2: Antibodies used in this study

2.5 RNA extraction

RNA was extracted from leaf tissue by grinding LN_2-frozen tissue to a fine powder before adding 0.5 mL RNA extraction buffer (100 mM LiCl, 10 mM EDTA, 100 mM Tris pH 8.0, 1% SDS) warmed to 80°C and 0.5 mL phenol:chloroform:isoamyl alcohol (25:24:1 v/v) (Invitrogen, UltraPure™ #15593031). Samples were vortexed vigorously

and centrifuged at 13 000 rpm at 4°C for five minutes. The aqueous phase was transferred to 0.5 mL cold chloroform:isoamyl alcohol (24:1), vortexed, and centrifuged as before; this was repeated. The RNA from the aqueous phase was precipitated overnight at 4°C in 2 M LiCl, and centrifuged at 13 000 rpm at 4°C for 15 minutes to pellet the RNA. The pellet was washed in 70% ethanol. All ethanol was removed before the RNA pellet was resuspended in 400 µL sterile ddH_2O. The resuspended RNA was re-precipitated at -20°C overnight in 0.13 M NaAc (pH 5.2) and 70% ethanol before pelleting again at 13 000 rpm in a centrifuge at 4°C for 15 minutes. The resulting pellet was washed in 70% cold ethanol; all ethanol was removed before resuspending the pellet in sterile ddH_2O. The RNA was quantified by Nanodrop Spectrophotometer (Thermo Scientific), and the 260/280 ratio was verified.

2.6 cDNA synthesis

Equal quantities (0.5-1 µg) of RNA were converted to cDNA using SuperScript™ II Reverse transcriptase (Invitrogen #18064022) with oligodT as per the manufacturer's instructions. cDNA was then diluted 1/10 with sterile ddH_2O before analysis by qRT-PCR. All primers used are detailed in Appendix A.

2.7 qRT-PCR

2.7.1 Primer testing

Primers for qRT-PCR were tested by amplification of cDNA in a standard PCR reaction (as below Section 2.11). Primers with a single strong band at the appropriate size were used for primer efficiency tests by qRT-PCR on a 1:2 or 1:10 dilution series of cDNA. Only primers with a >90% efficiency and single peak in their melt curve were used for future qPCR assays.

2.7.2 qRT-PCR

1-2 µL diluted cDNA were added to Power SYBR™ Green Master Mix (Thermo Fisher Scientific) with tested qPCR primers as per the manufacturer's instructions and run for 40 cycles with melt curve analysis in a StepOnePlus™ Real-Time PCR System (Applied Biosystems) or a QuantStudio™5 Real-Time PCR system. Gene expression levels were measured relative to housekeeping genes: At*UBQ5* for Arabidopsis and Bo*ACTIN* for pointed cabbage. Samples were run in triplicate; replicates with CT >±0.5 were excluded. Technical error of $2^{-\Delta CT}$ was calculated as the mean's 95% confidence interval.

2.8 RNA-seq sample preparation

2.8.1 Arabidopsis post-harvest RNA-seq

WT Col-0 *Arabidopsis thaliana* were grown in a growth chamber on soil as previously described. At 25 days old the t=0 began at ZT4. The early harvest (EH) plants were all harvested at t=0. For each replicate, leaves 5 and 6 from the plant were collected; leaves from 3 plants were pooled together for a total of six leaves. Quadruplet samples were taken at t=0, 12 h, 24 h, 48 h, 72 h and 96 h (Figure 2.2). Samples were snap-frozen in LN_2 and stored in a freezer at -80°C.

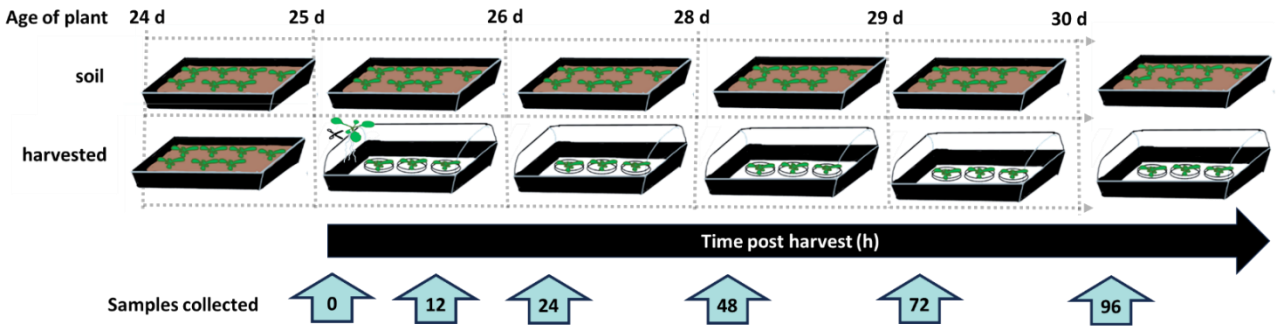


Figure 2.2: Arabidopsis post-harvest RNA-seq experimental set-up

For the accompanying *Botrytis cinerea*-infection samples, at t=24 h, leaves 5 and 6 of 'infected' samples had 1000 spores of *B.cinerea* added as previously described; 'control-infected' plants had a 10 µL drop of 1:1 red grape juice: ddH_2O added. 'Uninfected' samples had no liquid added. Samples of the 'infected' and 'control-infected' plants were only collected at t=72 h, i.e. 2 days post inoculation. An

additional late harvest (LH) sample set was collected only at t=72h, which were harvested one day after inoculation.

2.8.2 Arabidopsis post-harvest RNA-seq sample preparation

One sample from each time point was extracted simultaneously, as previously described (Section 2.5.1). Sample quantity and contamination were tested using a Nanodrop Spectrophotometer (Thermo Scientific). All samples were checked for 260/280 ≥ 2.0, and 260/230 ≥ 1.90. Any samples with 260/230 < 1.90 were cleaned with the Qiagen RNeasy kit. cDNA was made from each sample as previously described, and qRT-PCR run on *UBQ5* and *VSP2* genes to ensure similar expression levels between replicates. All samples had an RNA integrity number ≥ 6.4 tested by Agilent bioanalyser. Samples were sent on dry ice to BGI Hong Kong for sequencing.

2.8.3 Arabidopsis flg22 RNA-seq sample preparation

WT Arabidopsis Col-0 plants were grown on soil, and a subset was harvested one and two days before infiltration with 0.1 µM flg22 elicitor or control ddH_2O. The day before infiltration a transparent lid was placed ajar over the tray of plants to maximise stomatal opening, and thereby reduce infiltration damage. One hour post-infiltration, six leaves 4-6 from ≥3 plants were collected per replicate, and five replicates per timepoint. Samples were snap frozen in LN_2, and then ground to a fine powder. RNA was extracted using Qiagen RNeasy Plant Mini Kit (#74904) including the QIAshredder as per the manufacturer's instructions, with DNase I digestion on the columns using the Qiagen RNAse-free DNase set (#79254). Samples were eluted into 35 µL of RNAse free water (DEPC), and quantified using a nanodrop. cDNA was made from each

sample, and gene expression was tested for consistency between sample groups in advance of sequencing. The RNA was processed using an NEBNext Poly(A) mRNA Magnetic Isolation Module, and the library prepared using NEBNext® Ultra™ II DNA Library Prep Kit. The library was sequenced using NextSeq 500/550 High Output Kit v2 (75 Cycles) on NextSeq550 at Nagoya University.

2.8.4 *Brassica oleracea* var. *capitata* RNA-seq on-site field sampling

Brassica oleracea var. *capitata* cv. 'Regency' four week-old seedlings were sown out in the field by Kettle Produce Ltd in Balmalcolm, Cupar, Fife (56.25157, -3.143035) on 24 April 2022 at a density of 72 000 plants/hectare after an unusually dry growth season (Table 2.3; Figure 2.3A). The cabbages were harvested by hand on 7 July 2022 (air temperature 23°C, sunny, light breeze) when plants had been in the field for 74 days, and were at commercial harvesting size. Harvested cabbages were selected for nearest size to 30 cm x 20 cm, and damaged or diseased outer leaves were removed prior to transport. The 't=0' leaf samples were collected in the field and transported on dry ice before freezing in LN_2 and storage at -80°C. The remaining cabbages were transported to the laboratory and stored at 4°C

Month	Max temp (°C)	Min temp (°C)	Air frost days	Rain (mm)	Sun (hrs)
April	11.8	4.1	3	36.1	146.1
May	15.8	7.8	0	35.6	138.0
June	18.9	9.7	0	23.0	183.7
July	22.0	12.4	0	18.8	160.4

Table 2.3: Weather data from nearest weather station to field site (Leuchars, Fife, 56.377, -2.861) covering the field growth period of the cabbage for RNA-seq.

2.8.5 *Brassica oleracea* var. *capitata* RNA-seq experimental set-up

The post-harvest cabbages were surface-cleaned with sterile ddH_2O, and placed 2-3/covered tray at 4°C (Figure 2.3B). Replicate samples of ~10 cm^2 of leaf tissue (adjacent to the central rib) were collected on days 10, 20, 45 and 70 post-harvest, snap frozen in LN_2 and stored at -80°C (Figure 2.3C). Three replicates each of RNA from all infected and control-infected pointed cabbage were extracted. Gene expression of housekeeping gene Bo*ACTIN* and *BoVSP2* were measured to verify consistency between replicates. Samples were quality checked for RIN>6.5 by Agilent Bioanalyser as previously detailed. Three replicates of all time points were sequenced by BGI (Hong Kong) using the DNBseq platform.

2.9 RNA-seq analysis

2.9.1 Arabidopsis RNA-seq analysis

FastQC analysis was run on the raw reads of the RNA-seq analysis prior to removal of the adapter content (Andrews, 2010). The RNA-seq reads were trimmed for adapter content and aligned using HISAT2 (Pertea et al., 2016). These reads were normalised to the mean using DESeq2 (Love et al., 2014). The genes were filtered to remove those in which ≥4/15 samples had normalised RPKM (reads per kilobase of exon per million reads mapped) ≤ 1.0. Differentially expressed genes (DEGs) were identified as those with a fold change of ≥2.0, and a p-value ≤0.05 when corrected for multiple testing using the Benjamini-Hochberg test.

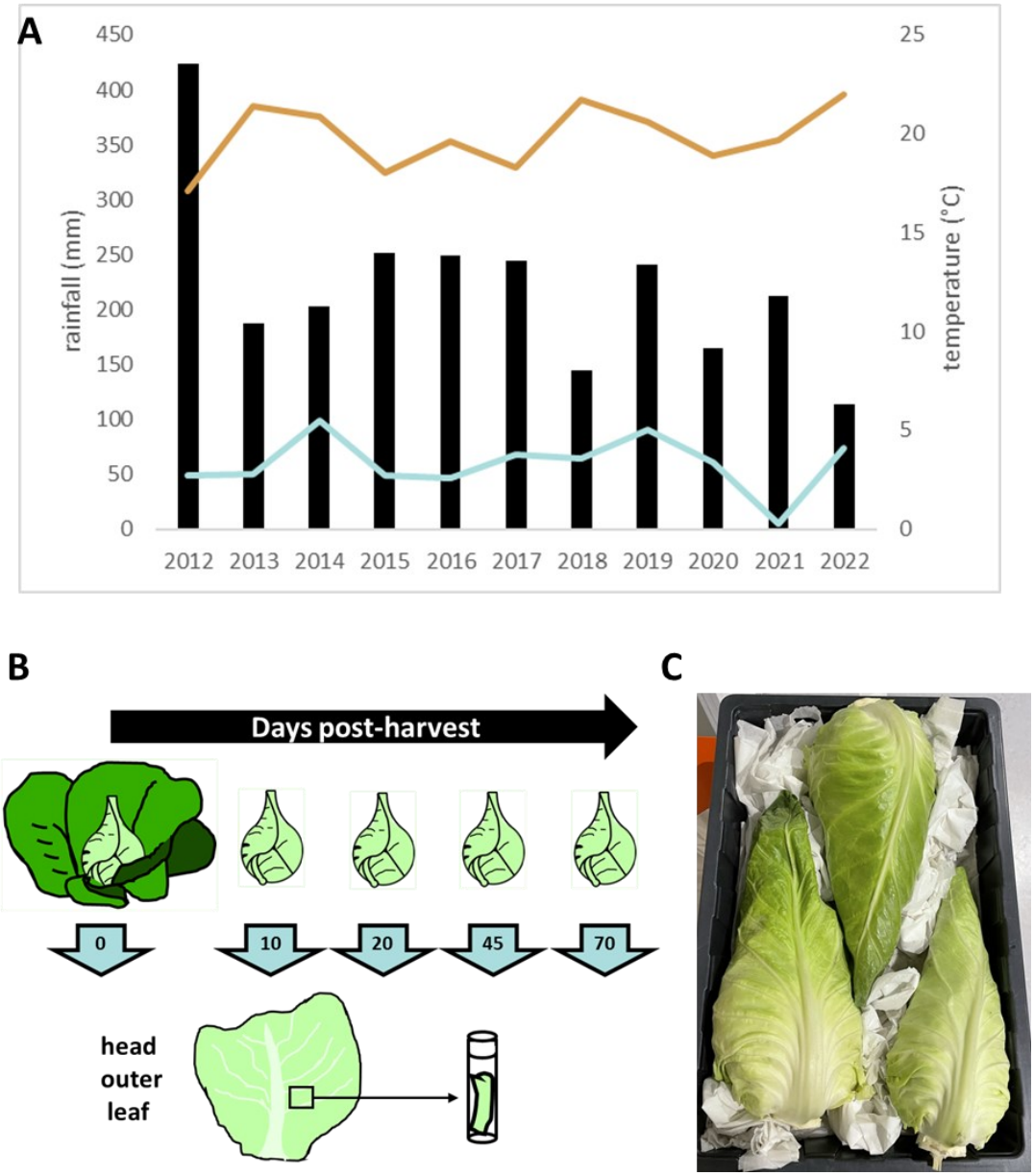


Figure 2.3: **Pointed cabbage plants for RNA-seq: (A)** The weather during the growth season (April-July) of pointed cabbage in Fife, Scotland (nearest weather station Leuchars (56.37-2.861)) for the ten years prior to sample collection. Black bars show total rainfall over the growth season; the red and blue lines show the minimum and maximum temperatures recorded during the growth season. (Met Office, Historic UK climate data, https://www.metoffice.gov.uk/research/climate/maps-and-data/historic-station-data, accessed 28/04/23); **(B)** sample collection for the RNA-seq experiment and **(C)** storage conditions.

Enriched GO biological terms of DEGs in the whole RNA-seq dataset were calculated using agriGO (http://systemsbiology.cau.edu.cn/agriGOv2) (Tian et al., 2017); the top 20 GO terms for both upregulated and downregulated GO biological terms were selected based on cumulative z-score across all post-harvest timepoints, and a FDR<0.05 at 96 hph

Gene expression profiles were clustered using Smoothing Spline Clustering (SSClustR) (Ma et al., 2006): nchain=5, threshold = 0.1, nclust was increased until the BIC score stopped decreasing at the optimal nclust of 35. The genes in each of these clusters were analysed for enriched GO terms using Shiny G0 0.77 (Ge et al., 2020), FDR cut-off 0.05, minimum pathway size 5, using *Arabidopsis thaliana* TAIR10 annotation (Lamesch et al., 2012). Figures were made in R.

2.9.2 Cabbage RNA-seq analysis

The raw RNA reads were trimmed for adapter content in Strand NGS (Strand NGS, 2016), and aligned and annotated to the reference *Brassica oleracea* genome (TO1000, Parkin et al., 2014). These reads were normalised using DESeq2 (Love et al., 2014). The genes were filtered to remove those in which ≥4/15 samples had normalised RPKM (reads per kilobase of exon per million reads mapped ≤ 1.0). Differentially expressed genes (DEGs) were identified as those with a fold change of ≥2.0 relative to t=0, and a p-value ≤0.05 when corrected for multiple testing using the Benjamini-Hochberg test. DEG expression profiles were clustered using Ward's algorithm (Ward, 1963).

2.9.3 BLAST comparison

For comparison between the brassica species, the cabbage RNA reads were BLASTed against the *Arabidopsis thaliana* coding sequences version TAIR10 (Lamesch et al., 2012) using weight matrix blosum62 (-3 for a nucleic mismatch, and +2 for a match, allowing gapped alignments). The *Brassica oleracea* TO1000 coding sequences downloaded from plants.ensembl.org were BLASTed against the Arabidopsis TAIR10 coding sequences (arabidopsis.org/blast). For published datasets, the Arabidopsis orthologues were adopted from the authors' BLAST analyses. For *Eruca sativa*, variety C was selected from the three unspecified varieties in the RNA-seq dataset because it had the highest number of DEGs (Bell et al., 2020). The GO terms were analysed in Shiny GO 0.77, and the top enriched GO terms were identified by enrichment false discovery rate (FDR)<0.05. The top 20 terms were selected based on smallest FDR.

2.10 Promoter analyses

2.10.1 Arabidopsis promoters

Enriched promoter motifs were analysed using a prediction program (Yamamoto et al., 2007) to assess relative appearance ratio of octamers (RAR):

$$\frac{\text{count in an activated promoter set/number of promoters in the set}}{\text{count in total promoters/number of total promoters}}$$

and then filtered to remove potential false positives ($p>0.05$), calculated by Statistical Motif Analysis in Promoter or Upstream Sequences

(https://www.arabidopsis.org/tools/ bulk/motiffinder/index.jsp), to provide RARf values. [This n-mer analysis was done by members of the Tada lab, Nagoya University.] Promoter motif sequences figures were made with WebLogo (https://weblogo.berkeley.edu/logo.cgi, Crooks et al., 2004). Known cis-regulatory elements were identified using the New PLACE software (Higo, 1999, www.dna.affrc.go.jp). Transcription factor regulatory databases were interrogated through Shiny GO 0.77 online tool (Enrichment FDR<0.05). Co-expression analysis was carried out in ATTEDII (Obayashi et al., 2022). TF pairing in harvest-inducible promoters was analysed using the online PMET programme (nero.wsbc.warwick.ac.uk, with promoter region 1 kb, including UTR, searched in JASPAR 2018 Core Plants Non-Redundant), with Benjamini-Hochberg multiple hypothesis testing, p value<0.01 (Rich-Griffin et al., 2020). Time course transcription factor inference was carried out using DREM 2.0 (Schulz et al., 2012) using TF target input from Agris (agris-knowledgebase.org; Yilmaz et al., 2011). RNA-seq datasets were compared using https://bar.utoronto.ca/eplant AtGenExpress (Austin et al., 2016).

2.10.2 *Brassica oleracea* promoter analysis

Developmental RNA-seq datasets were accessed through NCBI: cabbage datasets SAMN02404643 and SAMN02371508; broccoli dataset SAMN02443789. Broccoli transcription factor regulators were analysed in PlantTFDB (Jin et al., 2017). Promoter sequences for cabbage and broccoli were identified in the reference sequence (TO1000, Parkin et al., 2014) in NCBI. The sequence used was 1 kb upstream of the

suggested transcription start site of the gene, or up to the nearest transcribed gene upstream, whichever was shorter.

2.11 PCR

PCR for primer verification, colony PCR and genotyping was done in 1.25u GoTaq® (Promega #M3001), 1 X GoTaq® Green Reaction buffer (including 1.5 mM $MgCl_2$), 0.2 mM dNTPs, 0.2 mM forward and reverse primers and <0.1 µg template DNA. Thermo cycling was carried out as per the manufacturer's recommendations, and the products separated by electrophoresis on a 0.7-1.5% agarose gel (UltraPure™ Agarose (Invitrogen 16500-100) in 0.5X Tris-Borate-EDTA buffer (TBE)).

2.12 Design of synthetic constructs

2.12.1 Amplification of promoters

Genomic DNA from WT Col-0 plants was used as a template for amplifying 1 kb upstream of the putative transcription start site of the selected genes using PacBio DNA polymerase. These fragments were then purified by gel extraction (Qiagen QIAquick® Gel Extraction Kit #28704). A-tails were added to allow insertion of the fragment to the PCR™8 entry vector by incubating ~0.1 µg DNA template at 72°C for 20 minutes with GoTaq® G2 (1 u) (Promega M7841), dATP (0.2 mM) and 1x GoTaq® colourless reaction buffer. A fraction of the PCR product was added 1:1 with the pCR™8/GW/TOPO™ vector (Invitrogen™ K250020) and incubated overnight at room temperature before transformation into DH5α *Escherichia coli* cells.

2.12.2 Transformation of entry vectors into bacteria

An overnight pCR™8 vector/insert was transferred to *E.coli* DH5α chemically competent cells by heat shock methodology. The insert was incubated on ice for 30 minutes with the *E.coli* before heat-shock at 42°C for one minute in a water bath, and then returned to ice for one minute before the addition of 200 µL S.O.C. medium. Cultures were rotated 200 rpm at 37°C for one hour before plating on LB agar + spectinomycin (50 µg/mL) plates, which were kept at 37°C overnight.

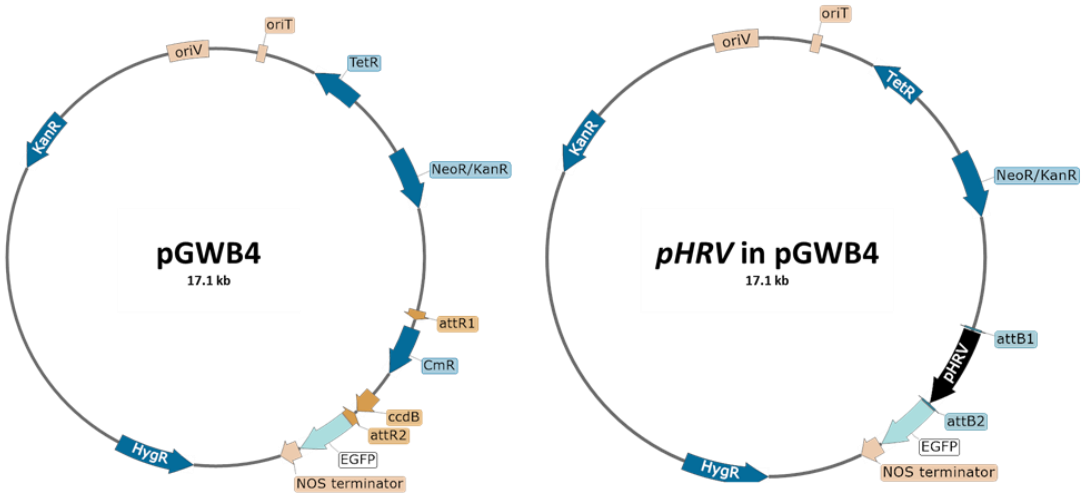


Figure 2.4 Gateway cloning of harvest-inducible promoters The Gateway plasmid pGWB4 with hygromycin (HygR) and kanamycin (KanR) resistance cassettes; **(B)** promoter sequences were inserted upstream of the sGFP cassette. Figures made in Snapgene (Snapgene, 2023).

2.12.3 Testing for successful insertion of construct

Colonies were initially checked by colony PCR. Small samples of individual bacterial colonies were placed in 50 µL sterile ddH_2O, vortexed and heated to 95°C for 10

minutes. 0.5 µL of these samples were used in GoTaq® PCR as above and separated by electrophoresis on a 1.5% agar TBE gel with 1/2000 SYBR Safe DNA gel stain (Invitrogen). Colonies that showed positive bands were grown in 5 mL LB agar $spec_{100}$ overnight before extracting the plasmid by QIAprep Spin Miniprep kit (Qiagen). The colonies were checked by restriction digest and then sent for Sanger sequencing to verify the presence of the correct fragment.

2.12.4 Transfer from entry to Gateway destination vector

The promoter insert within the pCR™8 vector was transferred into a Gateway destination vector pGWB4 using Gateway™ LR Clonase™ II Enzyme mix (Invitrogen™ #11791020) as per the manufacturer's protocol (Figure 2.4), and transformed into *E.coli* competent cells as above with kanamycin 50 µg/mL for selection of positive colonies.

2.12.5 Transformation of destination vector into *Agrobacterium tumefaciens*

Agrobacterium tumefaciens strain GV3101 (250 µL) was transformed by mixing with 2 µg of plasmid DNA on ice for 5 minutes. The mixture was frozen in LN_2 for 5 minutes, then warmed at 37°C in a water bath for 5 minutes. Each tube had 1 mL LB liquid added, and was incubated at 18°C at 200 rpm for 2-4 hours. Cultures were spun down at 4000 rpm in a centrifuge, and the top 800 µL removed before plating the bacteria on LB agar with 50 µg/mL gentamycin, 25 µg/mL rifampicin and 50 µg/mL. Plates were incubated at 28°C for 2 days, and individual colonies were then cultured overnight at 28°C in 5 mL LB liquid culture with 50 µg/mL gentamycin, 25 µg/mL

rifampicin and 50 µg/mL of kanamycin before plasmid DNA isolation by QIAprep Spin Miniprep kit (Qiagen). Cultures were checked for presence of insert by PCR.

2.13 Transient gene expression in Arabidopsis

npr1-0 and *efr* plants were used for transient expression to reduce the plant resistance to the infiltrated *Agrobacterium* (Zipfel et al., 2006). Some of the transient expression experiments were done by Josie Pritchard under my supervision in the Spoel lab as part of her BSc Honours Project. Plants were grown for 25 days and kept in the dark for 16 hours prior to infiltration. The *Agrobacterium tumefaciens* cultures (all in strain GV3101) were streaked three days prior to infiltration on LB plates with 50 µg/mL kanamycin (selecting for insert), 25 µg/mL gentamycin (selecting for Ti plasmid) and 25 µg/mL rifampicin (selecting for *Agrobacterium* genome). *Agrobacterium* without an insert was also streaked as a negative control. *35S::GFP* was prepared as a positive control. The day before infiltration, the streaked *Agrobacterium* strains were used to inoculate 15 mL of LB + Kan_{50}, Gen_{25}, and Rif_{25}, which was incubated overnight at 28 °C at 200 rpm in the dark. The next morning, the overnight culture was diluted with the same media to make a 25 mL culture of absorbance OD_{600} of 0.3. Acetosyringone (100 µM) was added for virulence gene induction. The cultures were incubated at 28 °C and 200 rpm until the culture reached OD_{600} of 0.6 (~2 hours). The culture was centrifuged at 4,000 × *g* for 10 min, and then resuspended in 15 mL induction medium (0.1% $(NH_4)_2SO_4$, 0.45% KH_2PO_4, 1% K_2HPO_4, 0.05% sodium citrate, 0.2% sucrose, 0.5% glycerol, 1 mM $MgSO_4$, at pH 5.7) supplemented with antibiotics (Kan_{50}, Gen_{25}, Rif_{25}) and 200 µM acetosyringone. The culture was incubated at 30 °C and 200 rpm for 3–4

h, and then pelleted at 4,000 × *g* for 15 min. The pellet was resuspended in 5 mL of 10 mM $MgSO_4$ (pH 5.7) and centrifuged at 4,000 x g for 15 minutes. The bacterial suspension was resuspended with infiltration medium supplemented with acetosyringone at 200 µM to an OD_{600} of 0.4, and then infiltrated through abaxial stomata of leaves 3 and 4 of the 25 day-old plants using a needle-less 1 mL syringe. The plants were placed in the dark overnight. Two days after infiltration, the 1 day post-harvest (1 dph) plants were harvested and placed on damp filter paper in covered trays. Three days post-infiltration, the levels of GFP were assessed by confocal microscopy as detailed in general methods, and measurements were made of protein, DNA and RNA levels of the GFP and kanamycin resistant genes.

2.14 Creation of stably transformed lines of Arabidopsis

2.14.1 Transforming Arabidopsis by floral dip methodology

The successfully-transformed Agrobacterium colonies were cultured with rotation for two days at 28°C in 5 mL LB with 50 µg/mL gentamycin, 25 µg/mL rifampicin and 50 µg/mL of kanamycin, and then recultured overnight in 200 mL of the same LB medium under the same growth conditions. The overnight cultures were pelleted in a centrifuge for 10 minutes at 6000 rpm, and resuspended in 400 mL of infiltration media (5% sucrose, 0.02% Silwet L-77). Soil-growing Arabidopsis plants at the early-flowering stage were dipped into this infiltration medium for 30 seconds and kept dark overnight to encourage bacterial growth (Zhang et al., 2006). Seeds from these dipped

plants were selected for on ½ MS plates with 30 µL/mL hygromycin (Cambridge bioscience #H9726) and 50 µL/mL ampicillin. Seeds were cold-stratified for one week, exposed to light for 10 hours, and then kept dark for 4-5 days: transformed, hygromycin-resistant seedlings had root growth and stood upright, whereas non-transformed plants had minimal roots, or lay flat on the agar. Transformed seedlings were maintained in normal lab daylight for five days, then transferred to ½ MS + 50 µg/mL ampicillin plates to be grown in long day cycles for 10 days, before final transfer to soil.

2.14.2 Testing transformed lines

The hygromycin-resistant plants were genotyped to test for the presence of the relevant insert. A ~3 mm^2 section of a leaf was placed in 5 µL DNA extraction buffer (200 mM Tris-HCl (pH 7.5), 25 mM EDTA, 250 mM NaCl, 0.5% SDS adjusted to pH 8) + 45 µL TE buffer (10 mM Tris + 1 mM EDTA pH 8.0) (Edwards *et al.*, 1991). Tissue was ground for 1 minutes @ 17 beats/s in a TissueLyser, and then centrifuged briefly. Up to 0.5 µL of leaf DNA extract was added to 10 µL PCR reaction (section 2.11 above) and amplified for 30 cycles before visualisation by gel electrophoresis. From plants that had the insert confirmed by genotyping, seeds were collected for testing offspring ratios and developing homozygous insert lines.

2.15 Confocal microscopy

Individual leaves were placed abaxial side-up on slides adhered by double-sided tape. The settings of the Leica TCS SP8 microscope were x20 objective (HCX APO L U-V-I 20x/0.50 WATER), pinhole 1 AU, 488 nm laser (OPSL intensity 2%). GFP fluorescence was collected @503-532 nm (Gain 31, HyD); chlorophyll collected @ 685nm - 758nm (Gain 10, HyD); brightfield collected using PMT (Gain 392.8). Multiple images were taken per leaf. Levels were initially calibrated using wild type plants.

2.16 Hormone and elicitor treatments

2.16.1 Arabidopsis hormone and elicitor treatments

Leaves of Arabidopsis mutants *35S::NPR1-GFP* (*npr1-1*) in Col-0 background were sprayed with 0.5 mM salicylic acid (sodium salicylate [Sigma Life Science S3007]) or a water control. After 6 or 24 hours three biological replicates were pooled per sample: whole leaves for RNA or leaf discs for protein analysis. For NPR1 oligomer analysis, protein samples were split for +DTT and -DTT samples to observe total NPR1 protein and NPR1-redox state separately. These samples were separated by electrophoresis on 8% SDS-PAGE gels as detailed in general methods. *35S::JAZ1-GS* and *pMYC2::MYC2-FLAG* (*jin1-8*) were sprayed with 0.1 mM methyl jasmonate (MeJA) in 0.5% ethanol, and leaves five and six from six separate plants were pooled into two replicates four hours post-spray. MeJA is volatile, so lids were placed over treated plants, and separation was maintained between control and sprayed trays. Control plants were sprayed with 0.5% ethanol. *pEIN3::EIN3-GFP-3xFlag* (*ein3*) were sprayed

with 0.1 mM ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC), and collected at 4 hours post-spray. For elicitor assays, plants were sprayed (in MAPK experiments) or infiltrated with 0.5 µM flg22 peptide, or 1 µM chitin hexaose suspended in sterile ddH_2O. When sprayed, 0.005% Silwet was added to expedite contact with the leaf surface.

2.16.2 Cabbage hormone and elicitor treatments

Owing to the low numbers of plants, one replicate was used per sample for the assay optimisations. Three half leaves were infiltrated per plant (7 leaf stage) with 0.5 µM flg22, 0.5 mM SA, 0.1 mM MeJA or control of SDW. For flg22 treatments one leaf was collected at 30, 60 and 180 minutes; SA and MeJA treated leaves were collected at 4 hours post-infiltration. RNA was extracted and expression levels quantified by qRT-PCR as previously detailed.

2.17 MAPK phosphorylation assay

From 22 days old, WT-Col-0 plants had their roots removed as per our harvest model system. Plants had their rosettes placed on damp filter paper at 3, 2 or 1 days before treatment. At ~ZT4, plants were sprayed with 0.005% Silwet + 0.5 µM flg22, 1 µM chitin hexaose or the control spray of ddH_2O + 0.005% Silwet. After 20 minutes, leaf discs from leaves 5 and 6 from three separate plants were collected and snap frozen in LN_2. Protein was extracted as previously detailed, but using an extraction buffer designed to not disrupt phospho-linkages (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1%

NP40, 5 mM EGTA, 0.1 mM DTT, 50 µg/mL TPCK, 50 µg/mL TLCK, 0.5 mM PMSF, 1 x Halt™ phosphatase inhibitor cocktail (Thermo Scientific)). 20 µL of the extracted protein was separated (70 V for 4 hours) on a 10% SDS-PAGE gel, and transferred overnight onto nitrocellulose membrane. The membrane was incubated overnight at 4°C with 1/4000 α-phosphorylated MAPK antibody in PBS + 1/1000 Tween-20 (Cell SignallingTechnology® Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP® Rabbit mAb #4370). The membrane was washed with milk buffer and incubated with 1/2000 α-Rabbit antibody in 5% milk buffer for one hour. The membrane was exposed to chemiluminescent substrate (Thermo Scientific ™ SuperSignal™ West Pico PLUS) for two minutes, and visualised (acquisition 2 minutes) using a LI-COR™ Odyssey Fc. After stripping, the membrane was washed in PBS+1/1000 Tween, and incubated overnight @ 4°C with 1/2000 antibody for total MAPK (Cell SignallingTechnology® p44/42 MAPK (Erk1/2) (137F5) Rabbit mAb #4695). The membrane was exposed to chemiluminescent substrate (Thermo Scientific ™ SuperSignal™ Dura) for ten minutes, and visualised (acquisition 10 minutes) using a LI-COR™ Odyssey Fc.

2.18 ChIP qPCR

2.18.1 Cross-linking proteins to chromatin

0.96 g of 25 day-old *35S::NPR1-GFP* (*npr1-1*) or WT(negative control) seedlings were collected per sample, rinsed with water and gently dried on paper towels before being crosslinked by vacuum infiltration (≥-70 kPa) in 37 mL 1% formaldehyde for 2 x 15

minutes. Crosslinking was quenched by addition of 2.5 mL glycine (2 M) and further vacuum infiltration for 5 minutes. Leaf tissue was washed with ice cold 1X PBS, dried, and snap frozen in LN_2 for storage at -80°C. Parallel RNA samples were collected to verify expression of the genes under investigation by ChIP.

2.18.2 Isolation of nuclei and chromatin shearing

Samples were ground to a fine powder in a LN_2-chilled mortar and pestle, and 2.5 ml nuclei extraction buffer (100 mM MOPS pH 7.6, 10 mM MgCl2, 0.25 M sucrose, 5% Dextran T-40, 2.5% Ficoll 400) freshly supplemented with 1X protease inhibitors and 40 mM β-ME was added. Samples were filtered twice through Miracloth and centrifuged for 5 mins at 10,000 g at 4°C. The pellet was resuspended in 75 µl nuclei lysis buffer (50 mM Tris-HCl pH 8, 10 mM EDTA pH 8, 1% SDS). Each sample had 225 µL ChIP dilution buffer (-Triton) (16.7 mM Tris-HCl pH 8, 167 mM NaCl, 1.2 mM EDTA pH 8, 0.01 % SDS) added, and were incubated on ice for 30 minutes before sonication in Diagenode BioRuptor Plus for 10 cycles of high power 30s ON / 30s OFF. After sonication, 365 µl ChIP dilution buffer (-Triton), 200 µl ChIP dilution buffer (+Triton) (16.7 mM Tris-HCl pH 8, 167 mM NaCl, 1.2 mM EDTA pH 8 , 0.01 % SDS, 1.1% Triton X-100) and 35 µl 20% Triton were added to each sample, and they were centrifuged twice at 13,000 rpm at 4°C for 5 mins to remove cellular debris, each time retaining the supernatant. An input sample of 20 µl (1/50 volume from 1 ml) was collected and stored separately.

2.18.3 Removing proteins that bind non-specifically to beads

Protein A beads (30 µL/sample) were washed with 1 ml ChIP dilution buffer (+Triton) and collected by centrifugation at 5,000 rpm for 30s at room temp. Beads were resuspended in ChIP dilution buffer (+Triton) (100 µL/sample) and 100 µl of suspended beads were added to 900 µL of ChIP supernatant. These samples were incubated with rotation for 1 h 20 mins at 4°C. To remove the beads with non-specific proteins bound, samples were centrifuged at 5,000 rpm for 30s and the supernatant retained.

2.18.4 Binding tagged proteins to beads

Samples were incubated overnight at 4°C with 1.8 µL ab290 α-GFP antibody. A new set of Protein A beads (30 µL/sample) were washed with 1 ml ChIP dilution buffer (+Triton), spun down and resuspended in ChIP dilution buffer (+Triton) (100 µL/sample). The samples were incubated with rotation at 4°C with 30 µL of the beads for two hours. The beads were then washed to remove non-specifically bound proteins. Between each wash step, samples were rotated for five minutes at 4°C, and beads were allowed to settle to the base of the tube. Beads were washed sequentially with 1 ml low salt wash buffer (0.1% SDS, 1% Triton X-100, 20 mM Tris-HCl pH 8, 2 mM EDTA pH 8, 150 mM NaCl), 1 ml high salt wash buffer (0.1% SDS, 1% Triton X-100, 20 mM Tris-HCl pH 8, 2 mM EDTA pH 8, 500 mM NaCl), 1 ml LiCl wash buffer (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 10 mM Tris-HCl pH 8, 1 mM EDTA pH 8) and 1 ml 0.5X TE buffer (5 mM Tris-HCl pH 8, 0.5 mM EDTA pH 8). After the final wash step, the beads were retained for elution of specifically bound chromatin fragments.

2.18.5 Elution and purification of specifically bound DNA

Two rounds of 250 µL of elution buffer was added to each sample set of beads, briefly vortexed, and incubated at 65°C for 15 minutes with shaking. Beads were spun down @3.8 g for 2 minutes, and supernatant saved for a total of 500 µL of eluate. 10µL of the input samples were added to 490 µL of elution buffer, and processed identically to ChIP samples in all subsequent steps. Samples were incubated overnight @ 65°C with 1200 rpm shaking with 20 µL of 5 M NaCl to dissociate the bound proteins from the beads. Sample were then incubated at 45°C for 1 hour with 30 µL protein degradation buffer (0.67 M Tris-HCl [pH 6.5]; 0.17 M EDTA) and 0.4 U of proteinase K (Thermo Scientific™ #26160) to remove proteins from the isolated DNA.

2.18.6 DNA precipitation

Equal amounts of the sample were added to phenol:chloroform:isoamyl alcohol (25:24:1) and vortexed vigorously. After centrifugation for five minutes @ 16 000 g, the upper aqueous layer was extracted, and incubated at -80°C for one hour with 20 µg glycogen and 180 µL 3 M NaAc and 1.35 mL 96% EtOH. Samples were centrifuged for 30 minutes at 16 000 g, and the resultant pellet was washed with 70% EtOH. All EtOH was removed, and the pellet resuspended in 50 µL sterile ddH_2O.

2.18.7 ChIP qPCR analysis

qRT-PCR was run as previously detailed using promoter-specific primers (Appendix A) in triplicate for each input and IP sample. From the qRT-PCR results, mean % input of the IP samples were calculated as follows:

$$\text{input } Ct_{adj} = Ct - \log_2(500)$$

$$\text{IP } Ct_{adj} = Ct - \log_2(10)$$

$$\text{Mean \% input} = 100 * 2^{(\text{input } Ct_{adj} - \text{IP } Ct_{adj})}$$

2.19 Statistical analysis and graphics

Statistical analyses were carried out in GraphPad Prism version 10.0.0 (GraphPad, 2023). In *P.syringae* disease assays, each leaf-disc was a biological replicate. Normal distribution of the data was assessed using the Shapiro-Wilk test (alpha=0.05) owing to the small sample size (<50). If variance was similar between the sample sets (standard deviation ratio <4:1), an unpaired t-test was used when comparing two samples, and a one-way ANOVA with post-hoc Tukey's multiple comparisons was used for >2 sample sets. For *B.cinerea* lesion size analysis, the data was not normally distributed, so the Kruskal-Wallis test was used to compare sample sets.

Graphics were made in GraphPad and RStudio (RStudio, 2023); graphing code trouble-shooting was carried out in ChatGPT (OpenAI, 2023). RNA-seq data was analysed in StrandNGS software (Strand NGS, 2023).

CHAPTER 3: HARVEST RECALIBRATES PLANT DEFENCES AGAINST PATHOGENS WITH DISTINCT LIFESTYLES

3.1 INTRODUCTION

The health of post-harvest crops is crucial for global food security and human health (Hammond et al., 2015). Previous research has predominantly focused on the impact of post-harvest abiotic stress on crop quality or fruit health. However, post-harvest changes in plant immunity have been little studied in leafy vegetables. Studies on leafy vegetables have shown that harvest induces a transient wounding response (Torres-Contreras et al., 2018), senescence (Page et al., 2001), osmotic stress responses (Ben-Yehoshua and Rodov, 2003), secondary metabolite production (Bell et al., 2020), and downregulation of photosynthesis (Spadafora et al., 2019). However it is not possible to predict the impact of these multiple abiotic stresses on the post-harvest immune system, owing to the overlapping and sometimes antagonistic nature of plant abiotic and biotic stress response pathways (Fujita et al., 2006; Rasmussen et al., 2013).

Harvest may impact on the ability of plants to detect and respond to pathogen threats. Post-harvest tissues contain the necessary molecular tools to resist disease: each somatic plant cell is thought to contain the full receptor complement and signalling machinery needed to detect infection and launch an immune response. Therefore, plant tissues could still resist infection even after separation from the roots or stem. The detection of pathogen-associated molecular patterns (PAMPs), such as the flg22 peptide from bacterial flagellin or chitin from fungal cell walls, occurs at the plasma membrane of plant cells through pathogen recognition receptors (PRRs),

including the flg22-responsive FLAGELLIN-SENSITIVE2 (FLS2) (Gómez-Gómez et al., 1999), and chitin-responsive CHITIN ELICITOR RECEPTOR KINASE1 (CERK1) (Wan et al., 2008). PAMP recognition triggers multiple cellular responses, collectively described as pattern-triggered immunity (PTI), including a rapid burst of calcium ions (Ca^{2+}) and reactive oxygen species (ROS), and induction of mitogen-activated protein-kinases (MAPKs) and calcium-dependent protein kinases (CDPKs) to activate downstream transcription factors (TFs) that upregulate early pathogen response genes (Asai et al., 2002), such as *FRK1* and multiple *WRKY* transcription factor genes (Boudsocq et al., 2010). Many of these early pathogen response genes in turn activate hormone-responsive immune pathways, including salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) signalling. Plants are able to modulate these downstream hormone-responsive immune pathways depending on the lifestyle of the pathogen: broadly-speaking, the antagonistic SA and JA/ET pathways are upregulated in response to (hemi-) biotrophic and necrotrophic pathogens, respectively (Glazebrook, 2005). It is important to understand how harvest impacts these immune pathways in order to identify potential targets for promoting post-harvest health.

Using *Arabidopsis thaliana* as a post-harvest Brassicaceae (brassica) family model system, here I examined if and how harvest alters PAMP-responsive and hormone-responsive immune pathways. Because harvested tissues experience abiotic stresses and have limited resources due to detachment from the roots, it was expected that immune responses may not be prioritised in these tissues. Moreover, premature senescence caused by harvest stress may favour necrotrophic pathogens that promote

cell death to feed. Surprisingly, however, this study demonstrates that harvest affects distinct parts of the immune system in different ways. PAMP-induced expression of early pathogen response genes was dramatically dampened by harvest even though upstream MAPK signalling was intact. Similarly, harvest suppressed the activation of SA-dependent responses, which was due to a reduction in levels of SA signal transduction components. By contrast, JA signalling components were increased after harvest and supported enhanced JA-mediated immune responses. Together, these findings show that harvest dramatically recalibrates different plant immune sectors. These data will inform future strategies to improve post-harvest crop health.

3.2 RESULTS

3.2.1 Harvest does not affect PAMP perception

The earliest plant immune responses to disease depend on efficient pathogen detection and activation of PTI, and as such many PTI components are pre-made in the cell, ready for activation. To study the effect of harvest on these PTI components, we analysed their expression in an *Arabidopsis thaliana* model system for post-harvest leafy brassicas (see Chapter 2). Within hours, harvested leaves downregulated the expression of genes encoding for PRRs, as well as CDPK and MAPK cascades, with the exception of *MPK6* and *MKK5* ($\log_2$FC<0, Figure 3.1A-B). This general downregulation of PTI-signalling components suggests post-harvest immune signalling may be dramatically impacted.

The MAPK phosphorylation cascade is a key link between PAMP perception and activation of early pathogen response TFs. Phosphorylation of MAPK3/4 and MAPK6 was measured 20 minutes after spraying with flg22 or chitin elicitors to assess the impact of harvest on MAPK signalling. Both flg22- and chitin-induced phosphorylation of MPK3/4 and MPK6 were elevated in harvested plants relative to soil-growing plants (Figure 3.1C). Despite the lower post-harvest levels of mRNA of MAPK cascade components (Figure 3.1B), total MAPK protein levels remained constant in harvested plants relative to those growing on soil. These data suggest that pathogen perception and early signalling are not compromised in harvested tissues, and may even be at a higher level compared to their soil-growing counterparts.

3.2.2 Harvest suppresses downstream PTI responses

Activation of MAPKs leads to the phosphorylation and activation of downstream TFs that promote expression of early pathogen response genes. Expression analyses demonstrated that at 12, 72 and 96 hours after harvest, over one third of TFs phosphorylated by MAPK3, 4 and 6 (selected from Popescu et al., 2009) were downregulated relative to soil-growing plants ($log_2FC < -0.5$) (Figure 3.2A). By contrast, at 24 hours post-harvest over one third of these TFs were upregulated ($log_2FC > 0.5$). Even though the MAPK phosphorylation cascade was intact in post-harvest plants, the abundance of downstream TF targets could have changed the level of early pathogen response gene induction, owing to the short time between pathogen perception and gene induction. Thus, we examined if harvest impacts PTI-induced transcriptional reprogramming.

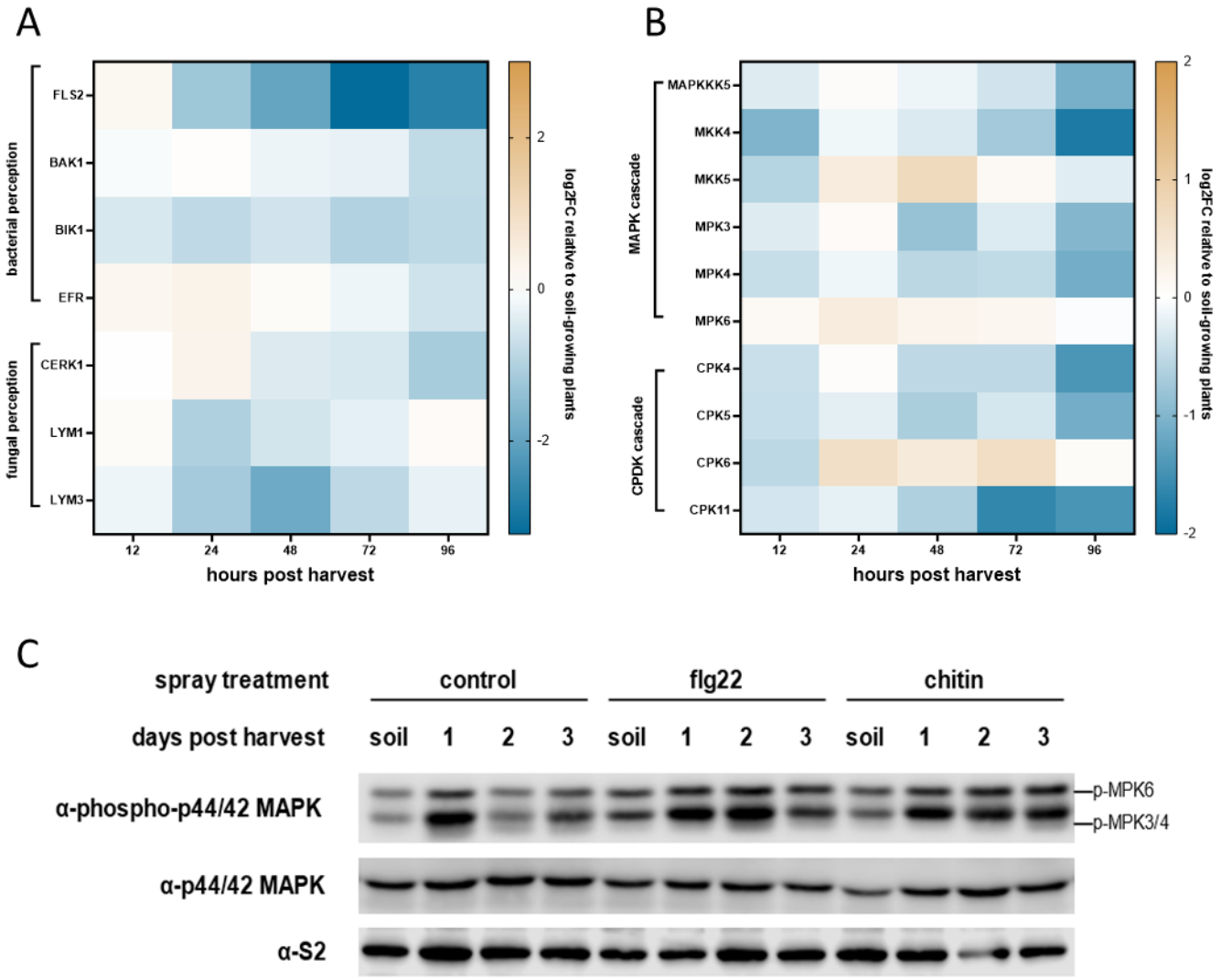


Figure 3.1: Reduced transcription of PTI components in harvested tissue does not affect MAPK activity. Gene expression in post-harvest Arabidopsis (log_2FC relative to soil) of **(A)** pattern recognition receptors (PRRs) involved in responding to bacterial PAMP flg22 and fungal PAMP chitin; and (**B**) key components of the CDPK and MAPK cascades, identified by Boudsocq et al (2010) as being involved in flg22 signalling. (**C**) phosphorylation of MAPK3/4 and MAPK6 proteins 20 minutes post spray with 0.1 µM flg22, 0.1 µM chitin hexaose or water control; probed with α-phospho MAPK for phosphorylation levels compared to α-MAPK for total MAPK levels, and α-S2 as a loading control (Blot is representative of N=4).

Detection of PAMPs, such as the bacterial elicitor flg22, leads to changes in early pathogen response genes within one hour (Asai et al., 2002). Thus, RNA-seq was used to identify post-harvest transcriptional changes in WT plants one hour post-

infiltration with 0.1 µM flg22. While flg22 induced the reprogramming of 3778 genes in soil-growing plants, harvested plants showed a dramatically muted response (Figure 3.2B). Of the MAPK-dependent early pathogen-responsive genes (Boudsocq et al., 2010), I observed decreased levels of induction of PTI marker genes, such as *FRK1* and *CYP81F2*, in harvested plants relative to those on soil (Figure 3.2C). Interestingly, *PHI-1,* which is controlled solely by the CDPK pathway, showed the inverse gene expression pattern compared to *FRK1*, which is controlled only by the MAPK pathway, suggesting that harvest may have differential impacts on the CDPK- and MAPK-dependent PTI signalling branches.

To gain an understanding of how harvest attenuates the PTI response, flg22-induced genes differentially expressed between harvested and unharvested plants were divided into 16 clusters according to gene expression patterns (Ward linkage analysis distance < 0.15) (Figure 3.3A-B). Over one-third of the differentially expressed genes (DEGs) exhibited stronger flg22-responsive upregulation on soil relative to harvested plants (Figure 3.3B, clusters 8, 14-16). The most enriched Gene Ontology (GO) terms for genes in these clusters related to immunity, phosphorylation, and response to bacteria and PAMPs. There were, however, clusters showing strong flg22-induced gene upregulation at all post-harvest timepoints (clusters 9 and 10), which were most enriched for genes annotated with GO terms for immunity, including SA biosynthesis, and defence responses (76 defence genes in total).

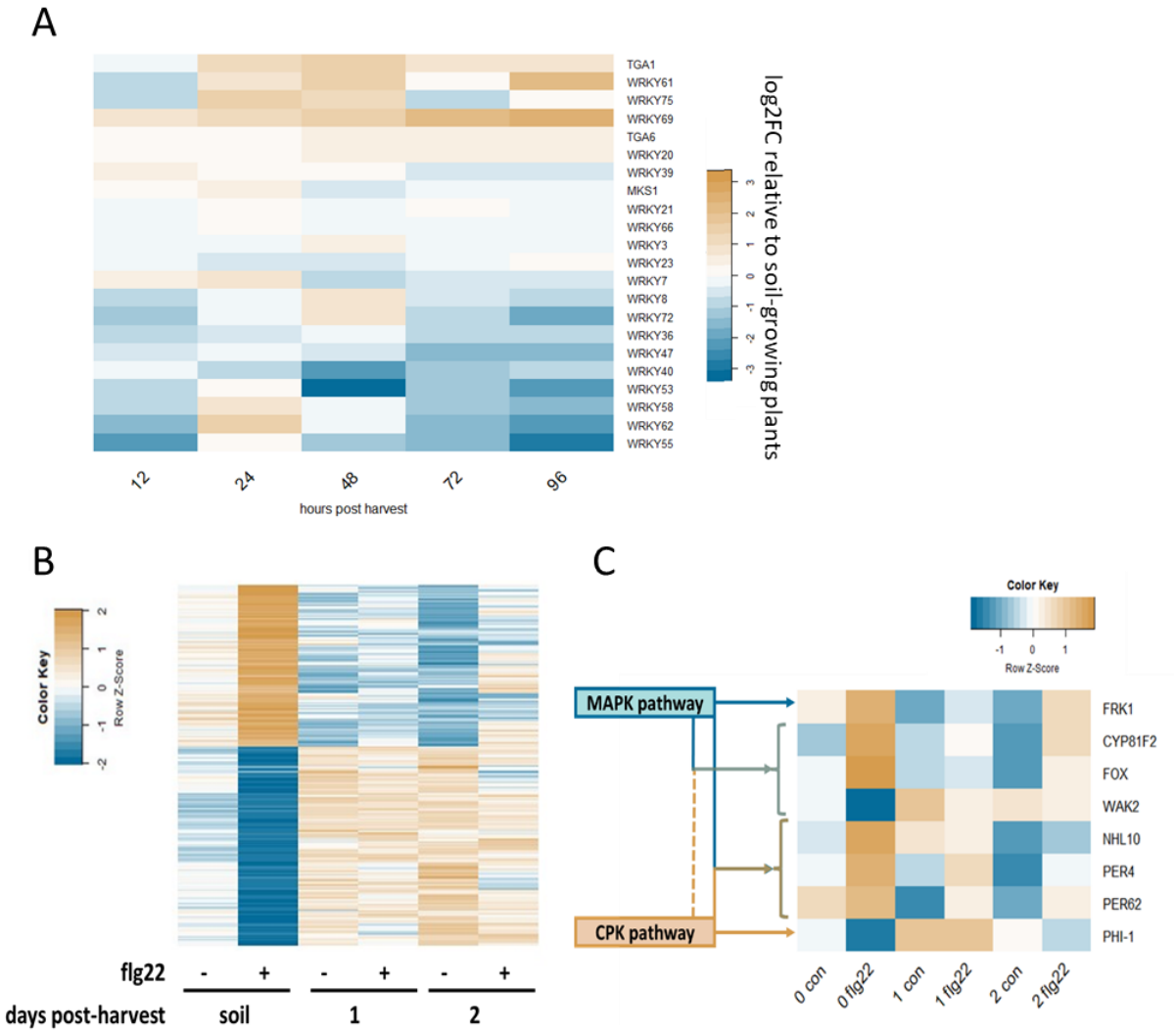


Figure: 3.2: Harvest impacts the extent of PTI-induced transcriptional reprogramming. (A) Relative expression of transcription factors thought to be phosphorylated by MAPK3/4/6 (Popescu et al., 2009) from Arabidopsis RNA-seq dataset (log_2FC of harvested/soil). **(B-C)** Arabidopsis plants were harvested 1 and 2 days prior to infiltration with 0.1 µM flg22 or a water control; samples for RNA extraction were collected in triplicate 1 hpi and analysed by RNA-seq. Normalised gene expression of (**B**) significant DEGs in the soil flg22 v soil control samples (log2FC > 1, One-way ANOVA, Benjamini-Hochberg correction for multiple hypothesis testing, p(corr)<0.05); heatmap is scaled by row: orange shows higher expression, and blue lower expression); and (**C**) a subset of genes identified in Boudsocq et al (2010) as early pathogen response genes.

The overlap between the flg22-induced response and that of the abiotic stress of harvest is evident in clusters 11-13: genes in these clusters are upregulated by flg22 on soil, and are already induced in control-treated, harvested samples. The most enriched GO terms in these three clusters relate to hypoxia and senescence, protein folding and JA responses, all of which are responses common to both biotic and abiotic stress. Although the peak activation of the hormone response pathways would not be expected as early as one hour after elicitor detection, some SA- or JA-responsive genes were upregulated in response to flg22 on soil (Figure 3.3C) and showed a reduced induction post-harvest. Taken together, these findings demonstrate that harvest strongly suppresses large sectors of the PTI transcriptional response and potentially affects downstream immune hormone response pathways.

3.2.3 Harvest suppresses activity of the SA pathway

When a biotrophic pathogen is detected by the cell, PTI will trigger upregulation of the SA-responsive immune pathway. In order to identify whether harvest impacted mRNA levels of key SA pathway regulators, the untreated, post-harvest RNA-seq dataset was analysed. Initially, mRNA levels of the SA receptor and transcriptional coactivator, *NPR1,* were not significantly different ($log_2FC<-1$) in harvested tissues relative to soil-growing plants, but four days post-harvest downregulation was observed (Figure 3.4A). This suggests that harvest does not immediately impact the transcriptional control of *NPR1*. In order to test whether NPR1 levels were being impacted by harvest at a

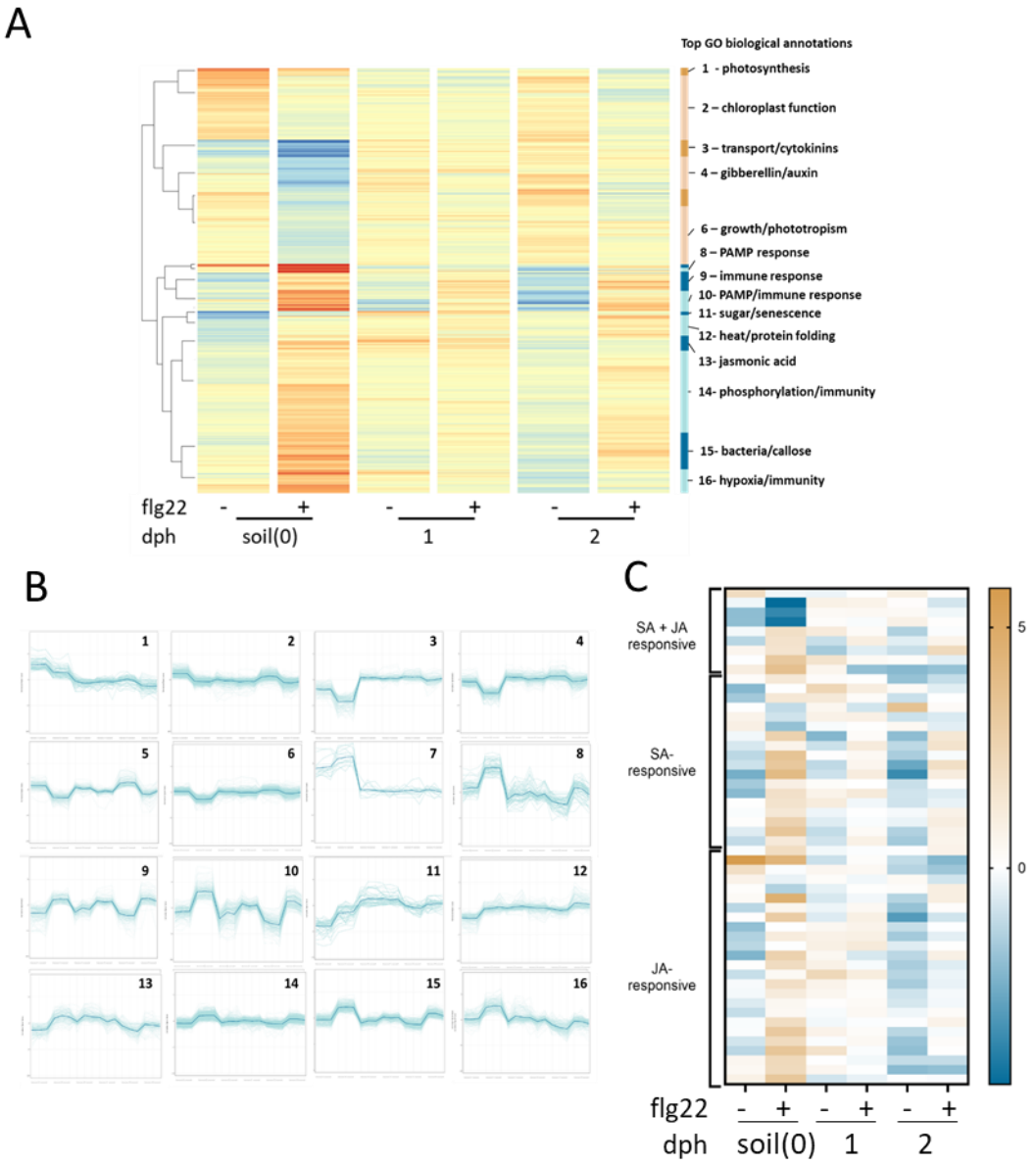
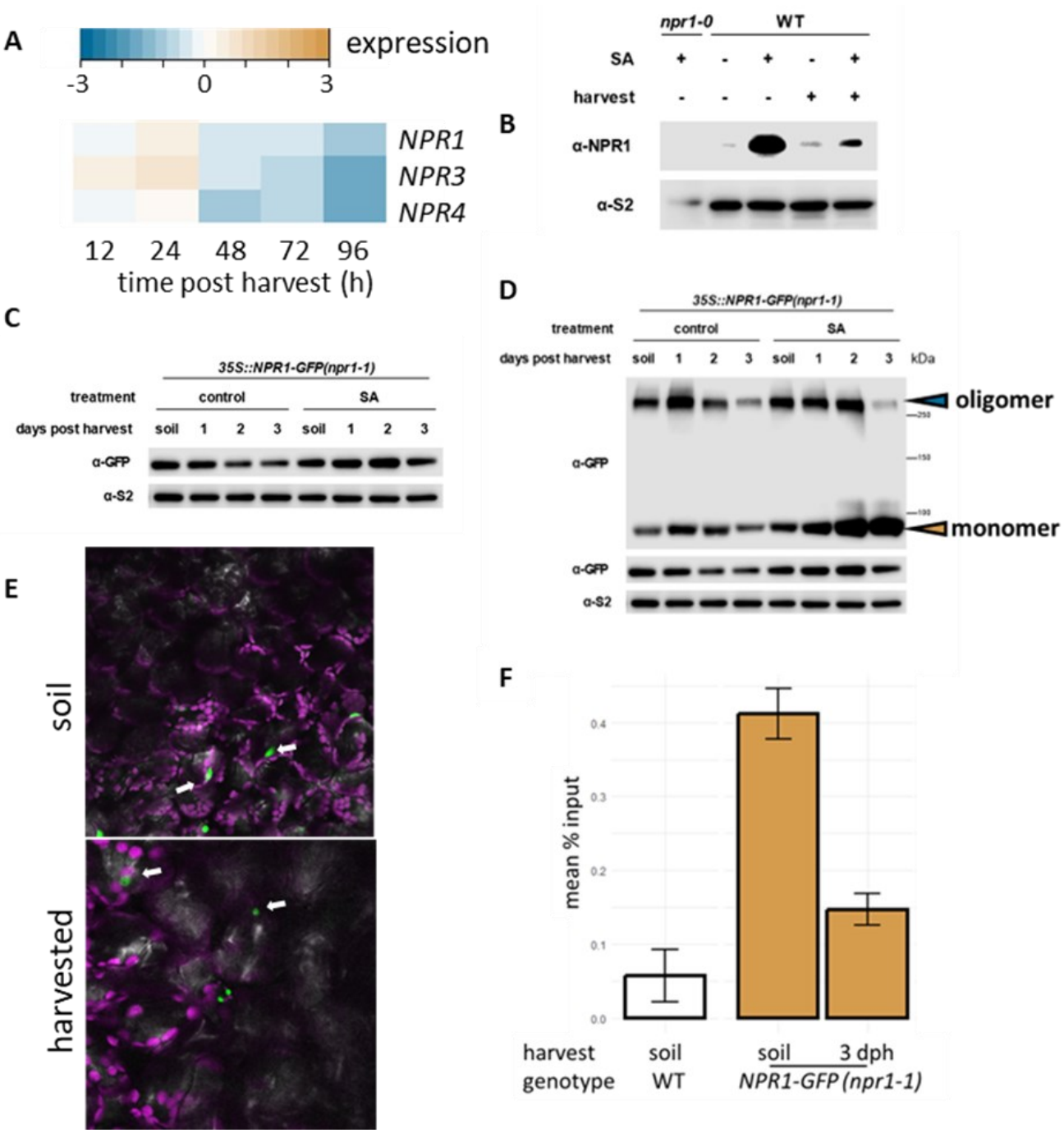


Figure 3.3 Changes in PTI-induced transcription post-harvest. (A-B) All DEGs from RNA-seq of 0.1 µM flg22 infiltrated leaves (one-way ANOVA p<0.05, FDR<0.05) are clustered by Ward linkage analysis. **(A)** Heatmap shows gene expression normalised to the median across all samples. Clusters were defined as groups of genes with an average linkage distance < 0.15 (dendrogram of clustering on the left of the graph). The key GO biological annotation themes from the top 20 GO terms (GO Shiny 0.77) are marked on the right next to the cluster number; clusters 5 and 7 did not have significantly enriched GO terms. dph = days post-harvest (**B**) Cluster profiles on normalised gene expression. (**C)** DEGs annotated with GO terms for SA or JA-response.

protein rather than transcriptional level, soil-growing and harvested plants were sprayed with 0.5 mM SA and after four hours endogenous protein levels of NPR1 were probed. Remarkably, SA-sprayed plants accumulated much higher levels of NPR1 protein on soil relative to the harvested plants (Figure 3.4B). To test if this was due to post-transcriptional regulation of the NPR1 protein, the levels of the constitutively expressed NPR1-GFP protein (in *npr1-1* background) were observed after harvest. There was no substantial difference between the uninduced and SA-induced levels of NPR1-GFP protein in harvested compared to unharvested plants (Figure 3.4C). These data suggest that harvest reduces the levels of endogenous NPR1 by reducing the levels of SA-responsive transcription.

Figure 3.4: Harvest impacts SA-responsive NPR1 accumulation and binding (overleaf). **(A)** *NPR* gene expression from RNA-seq data of untreated plants post-harvest (orange is high, blue is low; log_2FC of harvested/soil-growing plants). **(B)** Protein levels of NPR1 in WT plants on soil (harvest -) or two days after harvest (harvest +) following treatment with water spray control (SA-) or 0.5 mM SA spray (SA+) detected with α-NPR1 antibody, with α-S2 as a loading control (blot is representative of N=3). (**C**) The same experiment with the overexpressing *35S::NPR1-GFP* (*npr1-1*) line and plants on soil, or 1-3 days post-harvest (blot is representative of N=4). (**D**) NPR1-GFP protein levels in non-reduced (-DTT) (top α-GFP panel) and reduced (+DTT) protein samples (bottom α-GFP panel), with α-S2 as a loading control [NB. total NPR1 same blot as Figure 3.4B]. (**E)** Localisation of NPR1-GFP protein in leaf cells in soil-growing plants, and two days after harvest in *35S::NPR1-GFP* (*npr1-1*). The pseudo-coloured green spot is the signal from the expected wavelength of GFP; the pink signal corresponds to the expected chloroplast auto-fluorescence . White arrows point to two nuclei in each image. (**F**) Percentage of PR1 promoter (*as-1* site) DNA bound to NPR1-GFP protein relative to the total input amount of DNA from chromatin immunoprecipitation of overexpressor line *35S::NPR1-GFP* (*npr1-1*) plants cross-linked 4 hours post 0.5 mM SA spray (white bars indicate WT negative controls and orange bars indicate *35S::NPR1-GFP* lines; error bars show 95% CI of technical error; graph is representative of N=2).



SA triggers part of the NPR1 protein pool to undergo a conformational switch from cytoplasmic oligomers to nuclear monomers that interact with TGA TFs to upregulate SA-responsive gene expression (See Chapter 1, Figure 1.4) (Kinkema et al., 2000; Mou et al., 2003; Tada et al., 2008). Therefore, the impact of harvest on SA-

induced monomerization of NPR1-GFP was assessed. Four hours following SA spray, harvested plants exhibited equal or higher levels of NPR1 monomerisation (Figure 3.4D), and a strong NPR1-GFP signal was observed in the nuclei of both soil-growing and harvested plants (Figure 3.4E). This indicates that SA-induced monomerisation and translocation of NPR1 are intact in harvested plants.

Once the NPR1 monomer is present in the nucleus, it can indirectly bind to the promoters of genes that it regulates, such as *PR1.* To examine if harvest alters the ability of NPR1 to associate with chromatin, SA-induced localisation of NPR1-GFP to its *PR1* target gene promoter was assessed. Preliminary data from these experiments suggests that lower levels of NPR1-GFP associated with the *PR1* promoter of harvested plants relative to their soil-growing counterparts (Figure 3.4F). In summary, there was little difference between untreated soil-growing and harvested plants in terms of NPR1 protein abundance, conformation and localisation until exogenous SA was added. Upon SA treatment, however, harvest significantly impaired NPR1 accumulation and probably also reduced NPR1 binding to SA-responsive gene promoters.

Next, the effect of harvest-induced impairments in NPR1 accumulation and chromatin association on the expression of its target genes was studied. Plants were sprayed with 0.5 mM SA and mRNA levels of SA-responsive genes were measured after six hours. As expected, harvest had a striking impact on SA-responsive genes. The levels of mRNA of SA-responsive genes, such as *PR1* and *WRKY18*, were induced at much lower levels relative to soil-growing plants (Figure 3.5A-B).

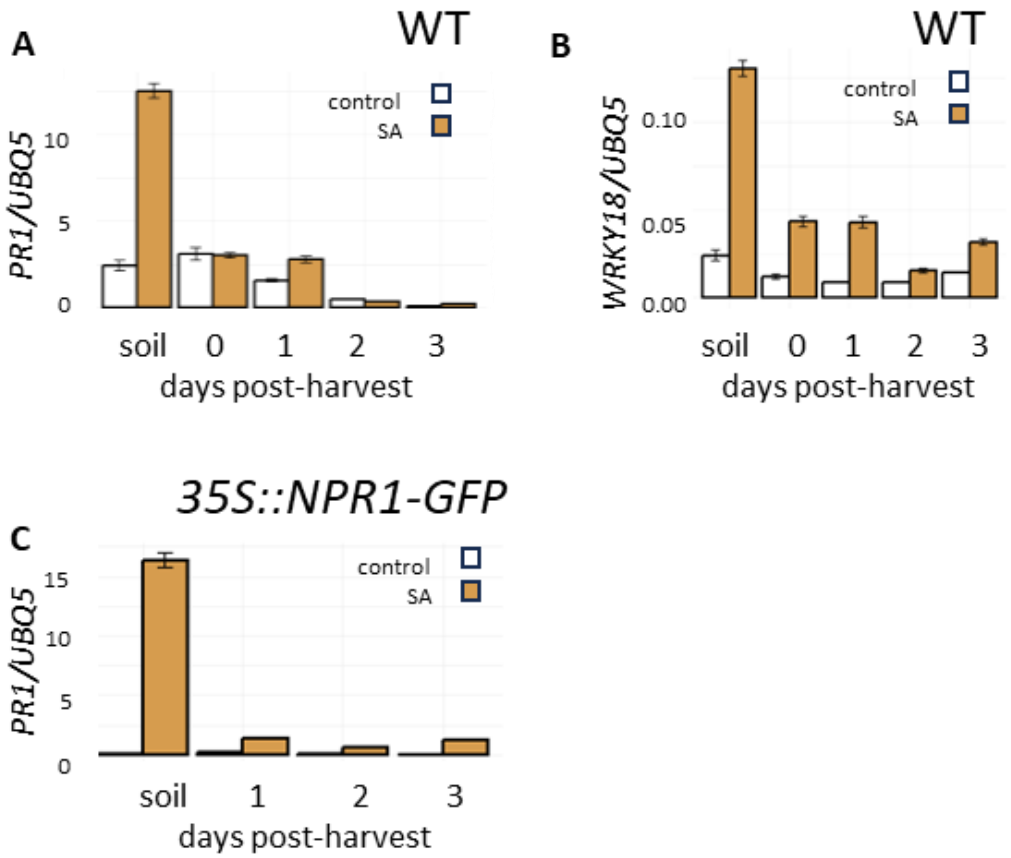


Figure 3.5: **Harvest represses SA-responsive gene expression** qRT-PCR of mRNA levels of SA-responsive genes (**A**) *PR1* and (**B**) *WRKY18* in WT plants relative to housekeeping gene *UBQ5* 6 hours after 0.5 mM SA spray; 0 days post-harvest plants were harvested at the same time as being sprayed with SA. Data is representative of N=3 and each sample consisted of 6 pooled leaves from ≥4 plants with error bars showing technical standard error. (**C**) *PR1* expression relative to housekeeping gene *UBQ5* expression in *35S::NPR1-GFP* (*npr1-1*) plants (representative of N=2).

To investigate whether higher *NPR1* expression could restore SA-induced gene expression in harvested plants, the same experiment was carried out in the overexpressing line *35S::NPR1-GFP* (*npr1-1*). Interestingly, reduced SA-mediated induction of *PR1* was also seen in this constitutively expressing line (Figure 3.5C). Therefore, the reduced induction of NPR1-dependent target genes is likely due to harvest-induced changes in both NPR1 activity and protein abundance.

To test the impact of harvest-mediated suppression of SA signalling on immunity to biotrophic pathogens, plants were infected with the hemi-biotroph *Pseudomonas syringae* pv. *maculicola* ES4326 (*Psm*). To replicate the way that this pathogen infects plants through the stomata, *Psm* was inoculated by spraying. *In planta* bacterial content was measured at 6 hpi to verify an equal baseline level of bacteria entering the leaf. Lower levels of bacteria were detected at 6 hpi in harvested plants (t test, $p<0.1$ for 2/3 technical replicates) (Figure 3.6A). This suggests that harvest-induced stress and associated closing of stomata provides plants with some protection against pathogen ingress.

To circumvent changes in pathogen ingress, *Psm* infections were then carried out by pressure infiltration, which ensures all leaves receive a similar inoculum. To assess whether pre- or post-harvest inoculation impacted disease progression, one set of plants was harvested one day before infection with *Psm* (early harvest) and another harvested one day after infection (late harvest); both were compared to plants growing on soil. At 2 dpi there were significantly higher levels of *Psm* in harvested plants relative to the soil-growing plants (Figure 3.6B). To understand the potential role of gene expression in contributing to enhanced susceptibility of harvested plants, *Psm*-responsive genes (as classified in Zhu et al., 2023) were analysed in the RNA-seq dataset of untreated plants. More than 40% of *Psm* susceptibility-associated genes were significantly upregulated post-harvest ($1<\log_2FC$), and, conversely, the expression of all selected genes associated with immunity to *Psm* was downregulated after harvest ($-1>\log_2FC$ at 96 h post-harvest), (Figure 3.6C).

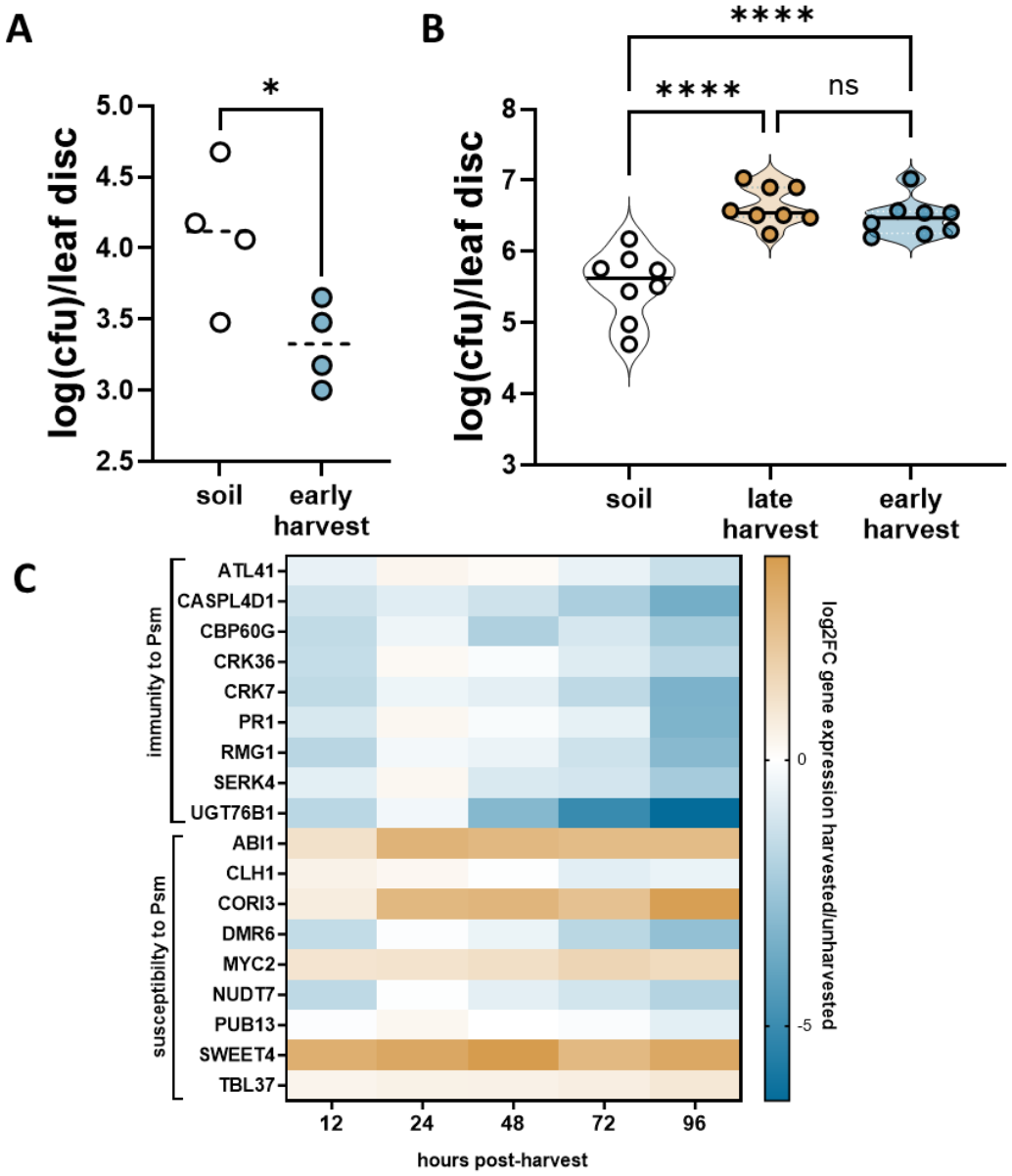


Figure 3.6: Harvest impacts plant susceptibility to hemi-biotrophic bacteria *Pseudomonas syringae.* (**A**) 6 hours post-spray levels of *Pseudomonas syringae* (*Psm*) in each leaf disc.Data compared using t test, p = 0.025. Each point represents data from one leaf; line shows the mean. Early harvest plants were harvested one day prior to spray. Late harvest plants are not represented here, as after 6 hours, they had not yet been harvested. N=3 (**B**) 48 hours post-*Psm* infiltration; one-way ANOVA, post-hoc Tukey test p<0.0001 (****), LH-EH samples showed no significant difference. (**C**) log_2FC of harvested/unharvested expression of genes associated with immunity to *Psm* or susceptibility to *Psm* (as classified in Zhu et al., 2023) from post-harvest RNA-seq dataset.

It is likely that the combined effect of reduced SA and PTI responses contributed to the increased susceptibility of harvested plants to this hemi-biotrophic bacteria.

3.2.4 Harvest enhances activity of the JA pathway

Given the harvest-mediated suppression of PTI and SA signalling, I next established if harvest also suppressed other immune pathways. In addition to SA signalling, plants utilise JA signalling to extend protective immunity to necrotrophic pathogens and herbivorous insects. An increase in intracellular JA facilitates the degradation of JAZ transcriptional repressors, thereby freeing up MYC transcription activators to stimulate JA-responsive gene expression (Chini et al., 2007) (See Chapter 1, Figure 1.5). To test whether harvest impacted on transcriptional control of these key JA pathway components, RNA-seq data of untreated, harvested plants was analysed. Post-harvest levels of *JAZ1* repressor mRNA were transiently downregulated (log_2FC relative to soil <-1) at two days post-harvest, but upregulated (log_2FC>1) by four days post-harvest (Figure 3.7A). This suggests that harvest might lead to transient de-repression of the JA pathway.

Protein levels of the JAZ1 repressor are affected by both the transcription of the *JAZ1* gene and the stability of the protein. To test the impact of harvest on JAZ1 protein stability independent of transcription rates, I measured JAZ1 protein levels in unharvested or harvested plants expressing *35S::JAZ1-GS.* In plants untreated with MeJA, JAZ1-GS protein levels markedly increased after harvest at all timepoints tested (Figure 3.7B). Despite these higher post-harvest levels of JAZ1 protein, four hours after spray with 0.1 mM MeJA, JAZ1 protein was degraded to similarly low levels in the

harvested and soil-growing plants (Figure 3.7B). These data suggest that JAZ1 protein was stabilised in harvested plants in the absence of additional JA. The rate at which JAZ repressors are degraded in response to JA could impact upon the speed of JA-mediated transcriptional responses. To test whether the higher levels of JAZ repressor in the harvested plants would take longer to degrade, samples of JAZ1 protein were taken from harvested and unharvested plants over the course of two hours after MeJA treatment. JAZ1 protein levels were below detectable levels within 30 minutes of JA treatment in both soil-grown and harvested plants (Figure 3.7C). Therefore, the enhanced accumulation of JAZ1 repressors in harvested plants is unlikely to have a significant impact on the rate of downstream gene activation under conditions that promote JA accumulation.

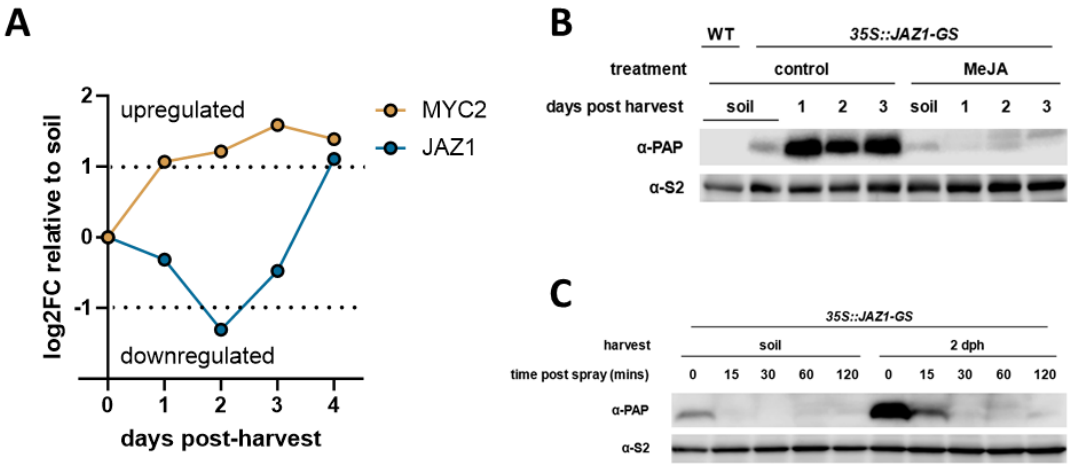


Figure 3.7: **Harvest impacts levels of JA-signalling pathway components. (A)** Levels of *MYC2* (orange) and *JAZ1* (blue) mRNA in untreated, harvested plants (log_2FC relative to soil growing plants; up- and down-regulated are log_2FC >1 and <-1 respectively). (**B**) Levels of JAZ1 protein in *35S::JAZ1-GS* plants sprayed with 0.1 mM MeJA in 0.05% EtOH or control spray of 0.05% EtOH *to* compare harvested and unharvested JAZ1 levels (blot is representative of N=3); and (**C**) to test the timing of JAZ1 degradation (N=2, α-S2 was used as a loading control.) α-PAP probes for the GS tag on JAZ1.

Next, the effect of harvest on MYC transcription activators was examined. To test whether harvest impacted the transcription of *MYC2*, mRNA levels in untreated, harvested plants were measured. *MYC2* was significantly upregulated at all post-harvest timepoints in untreated plants ($log_2FC>1$ relative to soil) (Figure 3.7A). Transgenic *pMYC2::MYC2-FLAG(jin1-8)* plants treated with or without 0.1 mM MeJA were then used to examine whether this higher post-harvest transcription corresponded with elevated levels of MYC2 protein. While harvest did not consistently alter MYC2-FLAG protein levels in untreated plants, harvest strongly promoted the accumulation of this protein in MeJA-treated plants (Figure 3.8A). Increased levels of MYC2 coupled with rapid degradation of the JAZ1 repressor in harvested tissues could increase the expression of JA-responsive genes after harvest.

MYC2 is the key TF involved in activating JA-responsive gene expression (Dombrecht et al., 2007). To investigate if MYC2 accumulation leads to enhanced JA responses in harvested tissues, JA-induced gene expression was examined post-harvest. After 24hrs of treatment with MeJA, the JA-responsive genes *VEGETATIVE STORAGE PROTEIN 2* (*VSP2*) and *LIPOXYGENASE 2* (*LOX2*) showed much higher expression in harvested plants, while *VSP2* mRNA levels were also higher in harvested plants at 6 hrs post-MeJA treatment (Figure 3.8B-E). The peak of JA-responsive gene induction was at two days post-harvest. Collectively, these data show that the elevated levels of MYC2 transcription factor in post-harvest tissues correspond to an enhanced induction of JA-responsive genes.

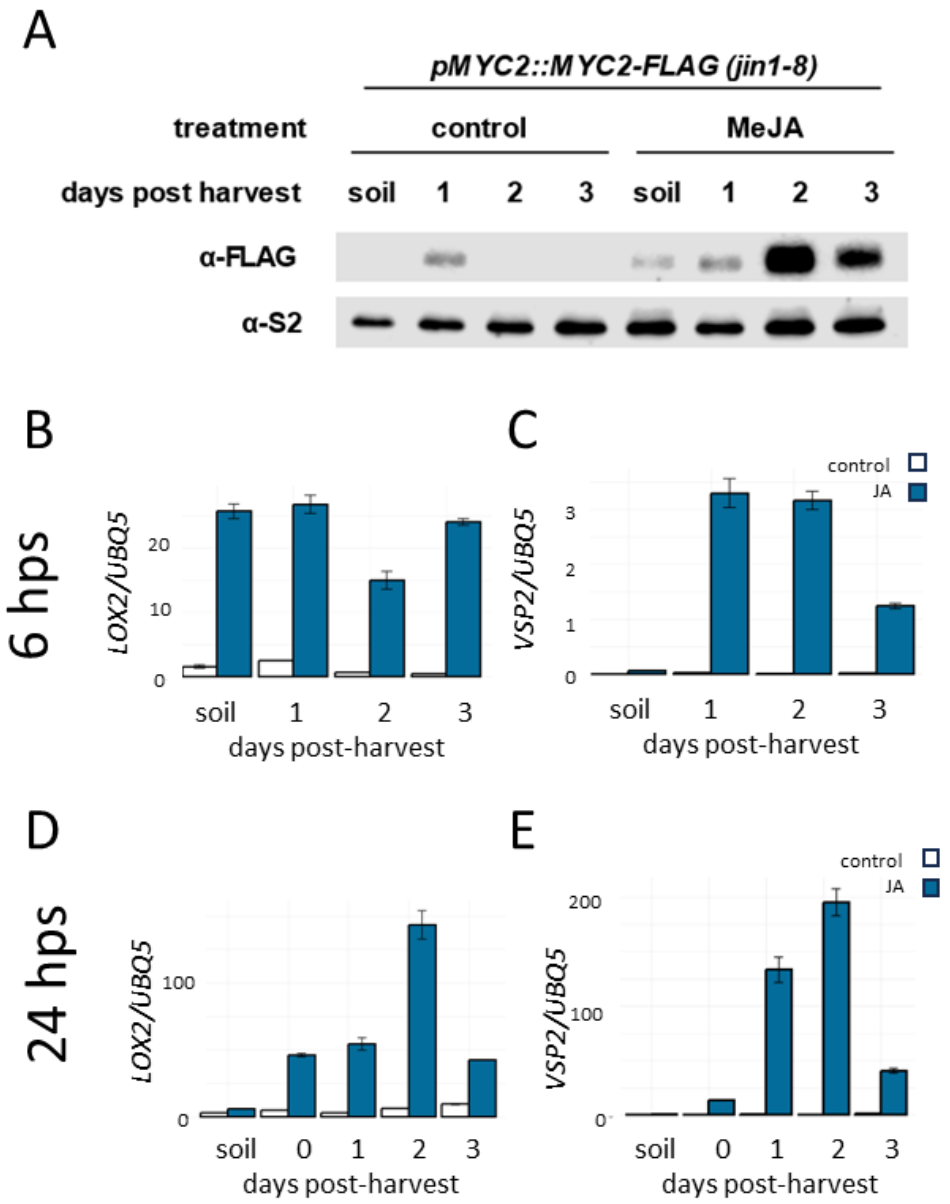


Figure 3.8. **Harvest increases JA-responsive gene expression.** (**A**) Levels of MYC2 protein in *pMYC2::MYC2-FLAG* (*jin1-8*) plants 4 hours post-spray with 0.1 mM MeJA; S2 protein levels were used as a loading control (blot representative of N=2). **(B-E**) JA-responsive gene expression in 0.1 mM MeJA treated plants. (**B-C**) 6 hps (representative of N=3) and (**D-E**) 24 hps (representative of N=2), relative to housekeeping gene *UBQ5*. Error bars show technical error CI 95%; 0 dph timepoint was harvested at the point of MeJA treatment.

Whereas *VSP2* and *LOX2* expression can be upregulated by JA signalling alone, expression of *PLANT DEFENSIN 1.2* (*PDF1.2*) requires the simultaneous activity of both ET- and JA-responsive signalling proteins (Penninckx et al., 1998). To identify how these combined pathways were impacted by harvest, *PDF1.2* mRNA levels were measured 6 hours after MeJA treatment. Surprisingly, given the increased induction of post-harvest JA-responsive genes, JA/ET-responsive *PDF1.2* upregulation was abolished in harvested plants (Figure 3.9A). This suggests that the JA- and ET- pathways are not acting synergistically in harvested plants.

The ET pathway can work in concert with the JA-responsive pathway to control plant defences to necrotrophic pathogens, but the pathways can also be mutually antagonistic depending on the nature of the threat (Lorenzo et al., 2003). Notably, MYC2 is known to promote degradation of the key ET-responsive transcription factor ETHYLENE INSENSITIVE3 (EIN3) and binds to it to suppress its transcriptional activity (Zhang et al., 2014). To test the impact of harvest on this key regulator of the ET pathway, I measured EIN3 protein in harvested and unharvested *ein3* mutant plants expressing transgenic *pEIN3::EIN3-GFP-FLAG.* Irrespective of treatment with the ET precursor 1-aminocyclopropane-1 carboxylate (ACC), harvest did not have a significant effect on EIN3 protein levels (Figure 3.9B). This suggests that harvest does not lead to significant destabilisation of EIN3 protein. To test if transcriptional activity of EIN3, rather than its protein level, was affected by harvest, mRNA levels of EIN3-dependent target genes were measured. *ETHYLENE RESPONSE FACTOR 1* and *2* (*ERF1*, *ERF2*) did not show a markedly changed response in harvested tissues treated with ACC (Figure

3.9C-D). Taken together, these data suggest that harvest does not have a large impact on ET signalling.

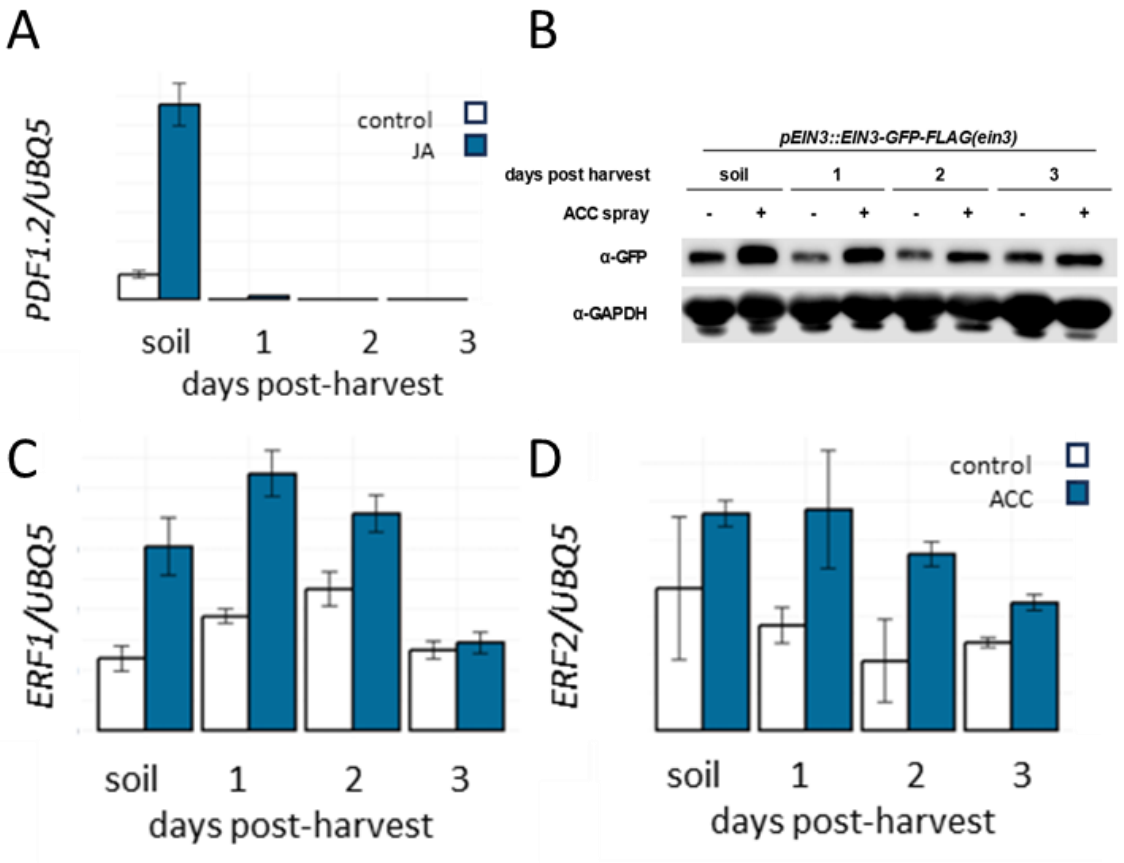


Figure 3.9: ET signalling is unaffected by harvest. (A) *PDF1.2* gene expression in 0.1 mM MeJA treated plants 6 hps. **(B)** EIN3-GFP protein levels in *pEIN3::EIN3-GFP-FLAG* (*ein3*) plants 4 hps with 0.1 mM ACC. **(C-D)** mRNA levels of ET-responsive genes 6 hps with 0.1 mM ACC or water control (N=1) (qPCR data shown relative to housekeeping gene *UBQ5*; error bars show technical error CI 95%).

Because harvested tissues exhibited enhanced JA signalling, the effect of harvest on the necrotrophic fungus *Botrytis cinerea* was investigated. Plants were inoculated with *B.cinerea* spores, and disease progression was assessed three days later by measuring necrotic lesion area and levels of fungal DNA in the leaves. One set

of plants was harvested first and then inoculated one day later (early harvest-EH); the remaining plants were inoculated while growing on soil, and a subset of these (late harvest- LH) were harvested one day later. The LH plants had the largest mean necrotic lesions (Figure 3.10A), although not significantly larger than soil-growing plants, and the highest levels of fungal DNA in their leaves (Figure 3.10B). This suggests that harvest after on-soil infection renders plants more susceptible to *B.cinerea*. By contrast, the lowest level of *B.cinerea* infection was in the EH plants, which had significantly smaller lesion size than the LH plants (Figure 3.10A) and less fungal DNA than both the soil and LH plants (Figure 3.10B). Together, these data suggest that harvested tissues are rendered more susceptible to pre-existing *B.cinerea* infections but are more resistant to new *B.cinerea* infections.

To gain more insight into how harvest changed immune responses to *B.cinerea*, the transcriptome of harvested (EH and LH) and unharvested (soil) plants were compared two days after *B.cinerea* infection. Of the 1845 genes upregulated in soil-growing plants in response to *B.cinerea* infection, 69% had higher expression in infected LH plants relative to soil, while 80% were expressed at a higher level in EH infected plants (Figure 3.10C). Similarly, of the 568 genes downregulated on soil in response to *B.cinerea* infection, 63% had lower expression in infected LH plants, and ~80% of genes had lower expression levels in infected EH plants. Taken together, these data suggest that harvest amplifies differential gene expression in response to *B.cinerea* infection.

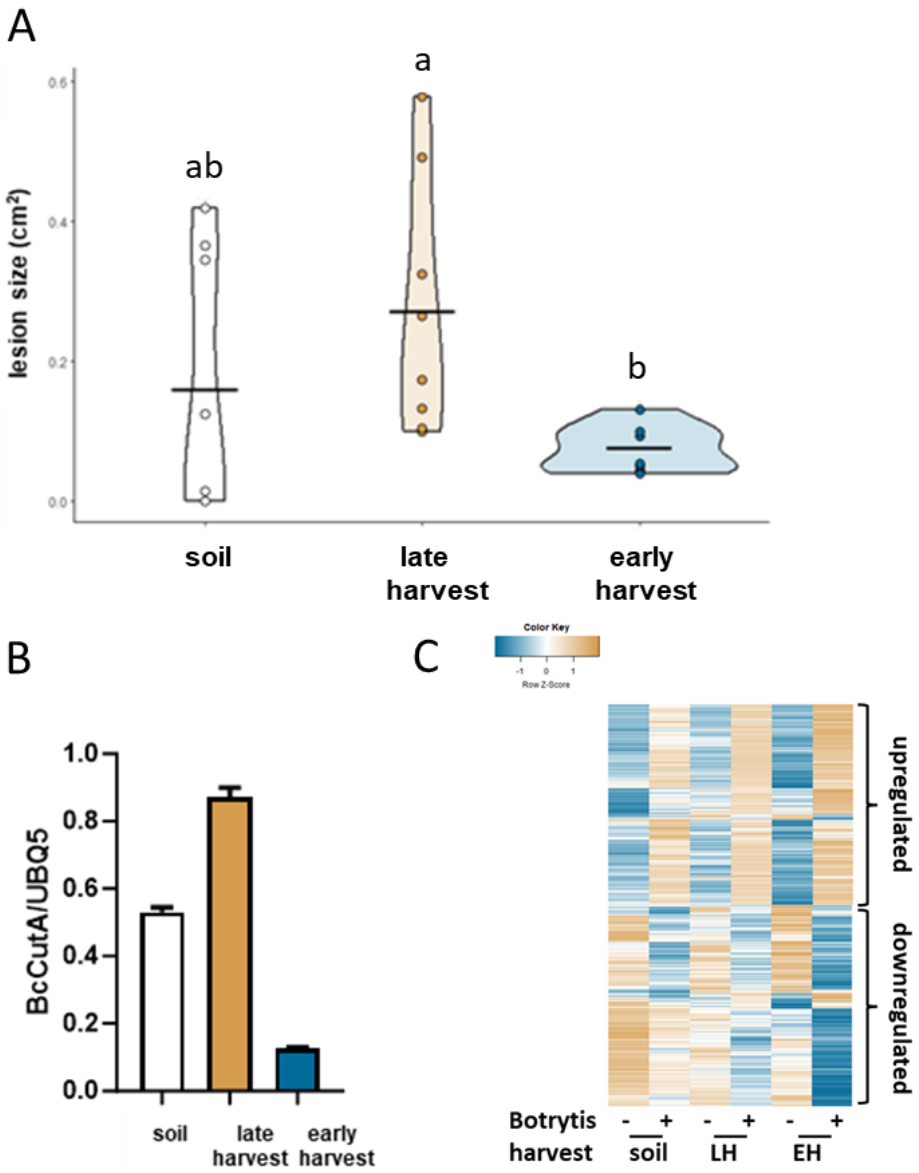


Figure 3.10: **Harvest impacts plant susceptibility to necrotrophic fungus *Botrytis cinerea*.** Soil-growing, late harvest (LH) and early harvest (EH) plants were infected with *Botrytis cinerea*. Three days post inoculation we measured (**A**) necrotic lesion area (letters represent significant difference in post-hoc Dunn's test following Kruskal-Wallis test, with $p<0.05$) and (**B**) *B.cinerea* DNA levels relative to plant housekeeping gene *UBQ5* mRNA levels (representative of N=2: error bars show technical error (CI=95%) in qRT-PCR). **(C)** Transcriptomic changes were measured two days post-inoculation; heatmap shows normalised gene expression of DEGs in response to *B.cinerea* inoculation on soil (one-way ANOVA, $-1>\log_2 FC>1$, Benjamini-Hochberg multiple testing correction, $p(corr)<0.05$; n=3; orange shows upregulation, blue shows downregulation).

3.3 DISCUSSION

The aim of this study was to characterise the impact of harvest on the immune system of plants in the post-harvest period. An *Arabidopsis* model harvest system was used to examine the impact of harvest on components of PAMP-triggered and hormone-mediated immune pathways. Harvest reduced the induction of early pathogen response genes and dampened the SA pathway, which correlated with increased post-harvest susceptibility to the hemi-biotrophic bacteria *Psm*. By contrast, harvest boosted the JA response, which resulted in a significant difference in the symptoms and growth of necrotrophic *Botrytis cinerea*, depending on whether the plant was infected post-harvest or on soil.

This study showed that PTI was attenuated in harvested plants, even though MAPK cascade components and activity remained constant post-harvest (Figures 3.1 & 3.2B-C). This suggests that PAMP perception is likely not affected by harvest but instead, signalling downstream of MAPK signalling is suppressed. MAPK cascades activate PTI through activation of TFs, such as MAP KINASE SUBSTRATE1 (MKS1) and WRKY72, that control early pathogen response gene expression (Popescu et al., 2009). Although expression of some of these TFs was reduced at three days post-harvest (Figure 3.2A), the impact of harvest on flg22-responsive genes was seen much earlier, at one day post-harvest. Therefore, transcriptional control alone of these TFs is unlikely to explain the attenuated PTI in harvested tissues. The interaction of MAPKs with their target TFs can also be affected by the presence of post-translational modifiers, such as small ubiquitin-like modifier (SUMO) (Miller et al., 2010). It has been proposed that

differential SUMOylation of TFs, such as WRKY3 and WRKY72, can switch their roles from activating to repressing gene expression (van den Burg and Takken, 2010). It would, therefore, be interesting to investigate the post-harvest levels of post-translational modifications on key MAPK target TFs to assess their contribution to attenuated PAMP-responsive gene expression in harvested tissues. Moreover, assessing the association of PAMP-induced TFs with the promoters of early pathogen response genes may further reveal how harvest impacts PTI-associated gene expression.

Harvest prevented the SA-responsive accumulation of NPR1 protein, suppressed NPR1-dependent target genes, and rendered plants more susceptible to hemi-biotrophic *Psm* (Figures 3.4, 3.5 & 3.6). The redox state of NPR1 and its localisation were unchanged by harvest, but less NPR1 may be associated with the *as-1* elements of promoters of SA-responsive genes like *PR1* (Figure 3.4). Previous studies have shown that NPR1 monomers that enter the nucleus when SA levels are low are targeted for proteasomal degradation by a Cullin3-RING E3 ligase (CRL3) in complex (Fu et al., 2012; Spoel et al., 2009). Interestingly, elevated ABA levels, as often found in post-harvest tissues (Guo and Gan, 2014; Ludford, 2002) , promote CRL3-mediated degradation of NPR1 (Ding et al., 2016). Thus, ABA-enhanced degradation of NPR1 in the nucleus could be responsible for the lower levels of post-harvest endogenous NPR1.

If increased degradation of NPR1 were the only factor controlling the post-harvest changes in the SA pathway, we would have expected higher SA-responsive gene

expression in plants over-expressing *NPR1-GFP*. However, even though NPR1-GFP protein levels remained broadly similar between soil-growing and harvested plants (Figure 3.4C), and NPR1 was normally localised in the nucleus of over-expressing lines (Figure 3.4E), SA-responsive gene upregulation was still greatly impaired by harvest (Figure 3.5C). Therefore, it is likely that NPR1 activity is also suppressed in harvested plants. Indeed, in preliminary experiments harvest strongly decreased SA-induced association of NPR1 with its *PR1* target promoter (Figure 3.4F). In the absence of SA, NPR1 may associate with the WRKY70 repressor bound to the *PR1* promoter and only switch to bind to the neighbouring TGA binding site (i.e. *as-1* element) upon SUMOylation (Saleh et al., 2015). Further ChIP analysis could identify whether NPR1 adopts the repressive complex in harvested tissues, resulting in suppression of SA-responsive gene expression. The combined reduced effectiveness of PTI and SA signalling in harvested plants likely contributes to observed increased susceptibility to the hemi-biotrophic pathogen *Psm* (Figure 3.6B)

The JA pathway would be expected to show the inverse response to the SA pathway, given their broadly antagonistic interaction (Pieterse et al., 2009). Indeed, this study observed that harvested plants displayed enhanced JA signalling, which was associated with elevated JA-responsive MYC2 protein levels (Figure 3.8). MYC2 protein levels have been suggested to be controlled by the ubiquitin-proteosome system: once MYC2 has transcriptionally activated its target genes, it is marked by ubiquitin for degradation by the proteasome (Jung et al., 2015; Chico et al., 2014; Chico et al., 2020). This proteolytic degradation of MYC2 is thought to be reduced by light, JA, and the

activity of the deubiquitinating enzymes, UPB12 and UBP13, which remove ubiquitin from MYC2 (Chico et al., 2014; Jeong et al., 2017). Interestingly, UBP12 and UBP13 proteins are themselves stabilised by elevated ABA levels (Liu et al., 2022), which are often present in harvested tissues. Therefore, the impact of elevated JA and ABA in harvested tissues could be responsible for the elevated MYC2 protein levels and its activity in upregulating JA-responsive genes.

There are two JA-responsive signalling branches: one controlled by JA via MYC2, and the other controlled by both JA and ET via ERF1 (Lorenzo et al., 2004; Song et al., 2014). Harvest caused enhanced JA-responsive induction of genes in the MYC2 pathway, such as *VSP2*, but repressed induction of *PDF1.2* in the ERF1 pathway, despite no marked impact of harvest on the ET pathway (Figures 3.8B-E). Elevated ABA is thought to enhance the MYC2 pathway and suppress the ERF1 pathway (Anderson et al., 2004). It is possible, therefore, that the interplay of ABA and JA in harvested tissues leads to the differences observed in these two JA-responsive pathways.

The JA pathway is important for defence against necrotrophic pathogens in general, and specifically against *Botrytis cinerea* in Arabidopsis (Zhang et al., 2017). Enhanced post-harvest JA signalling likely contributes to the reduced *B.cinerea* fungal growth seen in harvested tissues (Figure 3.10B). *B.cinerea*-responsive gene expression was also amplified post-harvest (Figure 3.10C), suggesting that harvest primed immune responses against this necrotroph. Taken together, it is tempting to extrapolate from our findings that harvest increases resistance to necrotrophic pathogens while conversely, increasing susceptibility to (hemi-)biotrophic pathogens.

It is likely that the impact of harvest on pathogen infection goes beyond changes in PTI and immune hormone signalling pathways. In harvested tissues, the response to low water and nutrient availability is likely to affect pathogen ingress and growth. Harvested leaves close their stomata to reduce water loss (Thomson, 2005), which may reduce entry of pathogens, such as *Psm,* that use stomata as entry points. Indeed, we observed lower *Psm* infection rates in harvested tissues that were spray inoculated (Figure 3.6A). In addition, lower apoplastic water levels of harvested tissues would likely be unfavourable for the initial stages of bacterial and fungal proliferation (Freeman and Beattie, 2009; Melotto et al., 2008; Fillinger and Elad, 2016). Reduced host resources in harvested plants, such as sugar availability, may also limit pathogen growth (Yamada et al., 2016). The level of infection seen in harvested plants may therefore be the result of a less favourable host environment for the pathogen, as well as changes in immune signalling pathways.

Overall, our study has shown that harvested tissues are markedly different from soil-growing plants in terms of their disease susceptibility, and the effectiveness of PTI and hormone-dependent immune pathways. These new insights are crucial for designing strategies to improve post-harvest crop health, while not impinging on plant welfare in the field. To address the weaknesses of post-harvest immunity, good targets for engineering would be PTI-induced TFs, such as *WRKY40* and *WRKY33*, and downstream components of the SA pathway, such as *PR1*. Though care should be taken that the latter does not compromise enhancement of the JA response in harvested tissues. This improved understanding of post-harvest immunity in the

Arabidopsis model leafy brassica will provide a basis for further study in agriculturally relevant crops, and inform novel bioengineering strategies.

Chapter 4: Comparative transcriptomic analysis identifies key post-harvest responses in brassicas

4.1 Introduction

Leafy brassica vegetables, such as cabbage, broccoli and rocket, are important sources of micronutrients and fibre (Hedges and Lister, 2006). As a group, leafy brassicas are the third most important vegetable crop globally in terms of production, exceeded only by tomatoes and onions; the trade in cabbage and broccoli alone is worth >$18 and $15 billion USD, respectively (FAO, 2021). High water content, surface area and respiration render leafy crops particularly vulnerable to post-harvest damage and quality loss by wilting, pathogens and mechanical damage (Kader and Saltveit, 2002). As such, considerable research in post-harvest brassicas has led to optimisation of storage and pre-harvest conditions to minimise post-harvest losses (e.g. Spadafora et al., 2016; Able et al., 2005; Fernández-Léon et al., 2013; Janssens et al., 2022), and enhance nutrient content (Dewhirst et al., 2017).

A striking feature of leafy brassica crops is their range of phenotypes within closely related species and even between varieties. Domestication and breeding programmes have selected for traits that have made the crop plants so distinctive, such as a proliferation of inflorescences for broccoli (*Brassica oleracea* var. *italica*); or the formation of a compact, protective core of leaves (leaf heading) in cabbages (*Brassica oleracea* var. *capitata* L.) (Cheng et al., 2016). Within the single species of *Brassica oleracea,* the range of storage life extends from 15 days to 6 months, with

longer shelf-lives being correlated with lower post-harvest respiration rates (Gross et al., 2016). Some brassicas, such as cabbage and turnip (*Brassica rapa* subsp. *rapa*), have a biennial life cycle. These crops develop a storage organ to enable overwintering with reduced metabolic activity and flower the following year (Brummell and Toivonen, 2018). For cabbages, this storage organ is the compact head of leaves in which reduced metabolism contributes to their long storage life. By contrast, other aerial organ crops, such as broccoli and rocket, have relatively high post-harvest metabolism and respiration, and consequently, reduced storage life. These differences make leafy brassicas ideal for comparative studies of post-harvest transcriptomes to identify how closely related organisms exhibit such a large range of post-harvest phenotypes. However, only a few isolated transcriptomic studies of post-harvest leafy brassicas have been published (See Chapter 1, Table 1.1), and comparative transcriptomic studies of post-harvest leafy brassicas are entirely lacking.

Harvested tissues undergo physiological changes, including reduction in respiration (Kader and Saltveit, 2002) and accelerated senescence (Brummel and Toivonen, 2018). Elevated levels of reactive oxygen species (ROS) and ethylene (ET) often accompany senescence (Tan et al., 2020; Ludford, 2002). Moreover, abscisic acid (ABA) has been associated with delayed senescence in post-harvest tissues (Miret et al., 2018). During post-harvest senescence, chlorophyll and antioxidants are degraded, and harvested plants exposed to light accumulate secondary metabolites, such as anthocyanin (Socquet-Juglard et al., 2016). These changes will occur at varying rates depending on the storage life of the harvested tissue.

In this study, the post-harvest stress transcriptomes of the brassicas Arabidopsis, cabbage (*Brassica oleracea* var. *capitata*), broccoli (*Brassica oleracea* var. *italica*) (Ahlawat et al., 2022) and rocket (*Eruca sativa*) (Bell et al., 2020) will be compared. Comparative analysis of expression in different brassica species is non-trivial: although there is considerable synteny between the reference genomes of broccoli, cabbage and Arabidopsis, a whole genome triplication event following the split from the Arabidopsis lineage results in many duplicate and triplicate orthologues (Parkin et al., 2014; Lysak et al., 2005) (See Chapter 1, Figures 1.1B-C). Therefore, I will use Arabidopsis orthologues and their associated genetic tools to compare expression of specific genes between the datasets. Together, these data uncover the post-harvest gene expression profiles and associated cellular processes of diverse brassicas with markedly different storage lives, and demonstrate the relevance of utilising the Arabidopsis model system to identify key processes in brassica crops.

4.2 Results

4.2.1 Post-harvest Arabidopsis undergoes transcriptional reprogramming

The model brassica *Arabidopsis thaliana* was used to create a model harvest system of leafy brassicas (see methods in Chapter 2). The soil-grown plants had their roots removed at 24 days old and were placed on damp filter paper in a covered tray at 21°C in long-day conditions, during which time I observed the visual changes that took place over the course of post-harvest storage. As post-harvest storage time increased, the

Arabidopsis leaf tissues gradually lost turgor and leaf colour darkened. By 7 days post-harvest (dph) the plants could no longer be used for experimentation due to dehydration (Figure 4.1A). This suggests that loss of water by transpiration, even in the relatively humid conditions in which they were stored, limited the storage life of Arabidopsis.

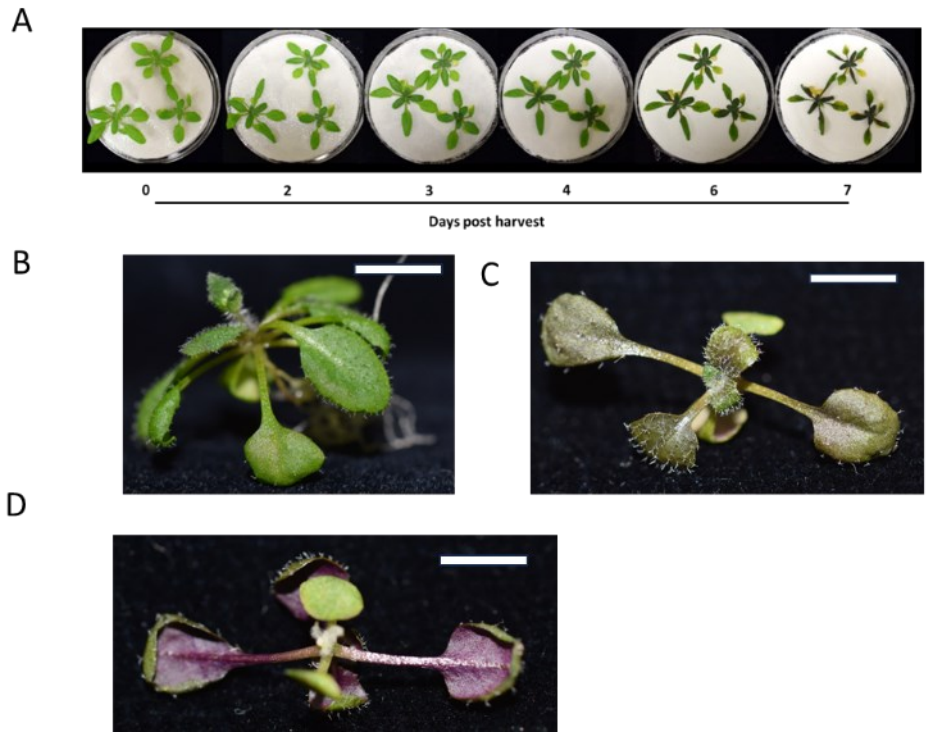


Figure 4.1: Harvested Arabidopsis rosettes show dehydration, and changes in development and leaf pigmentation (A) Photographs of harvested Arabidopsis rosettes over the course of seven dph on damp filter paper in 90 mm diameter petri dishes in normal model conditions; **(B-D)** harvested Arabidopsis rosettes kept for ten days after harvest on sealed agar plates showing **(B)** root regrowth, **(C)** the adaxial leaf surface, and **(D)** the abaxial leaf surface. (White bar indicates 5 mm scale).

To restrict water loss, I grew Arabidopsis on agar plates for 14 days and then removed their roots, after which the rosettes were transferred to fresh agar plates sealed with parafilm. Ten days after harvest, most of the rosettes had regrown roots

(Figure 4.1B), and those that had not were darker green on the top side (adaxial) of their leaves and had a striking purple pigmentation on the leaf undersides (abaxial) (Figure 4.1C-D). The ability of the harvested rosettes to regrow roots made the sealed agar plates unsuitable for the post-harvest model system, but gave an interesting insight into the changes in development and pigmentation that could occur given sufficient time post-harvest. For all subsequent experiments, the harvested rosettes were maintained on damp filter paper (see Chapter 2 for full method).

Harvest exposes rosettes to a combination of abiotic stresses, including wounding, and a lack of nutrients and water. In order to identify the impact of these stresses on the post-harvest transcriptome, mRNA samples from leaves of the harvested Arabidopsis rosettes and their soil-growing counterparts were collected at six timepoints over the course of four days after harvest for RNA sequencing. All eleven treatments in triplicate together produced a total of 1330 Mb of high quality reads (Q30, Appendix B), with a mean per sample of 40.3 Mb, and 20,667 mapped genes and 33,598 transcripts. These data show that expression of over three-quarters of all Arabidopsis genes were mapped in this dataset. Of these 20,677 mapped genes, differentially expressed genes (DEGs) were defined as those with a $\log_2$FC >1 (upregulated) or <-1 (downregulated) in harvested tissues relative to soil-growing samples from the same time point ($p<0.05$, two-way ANOVA, Benjamini- Hochberg multiple hypothesis testing with *p* correction) (Figure 4.2A-B).

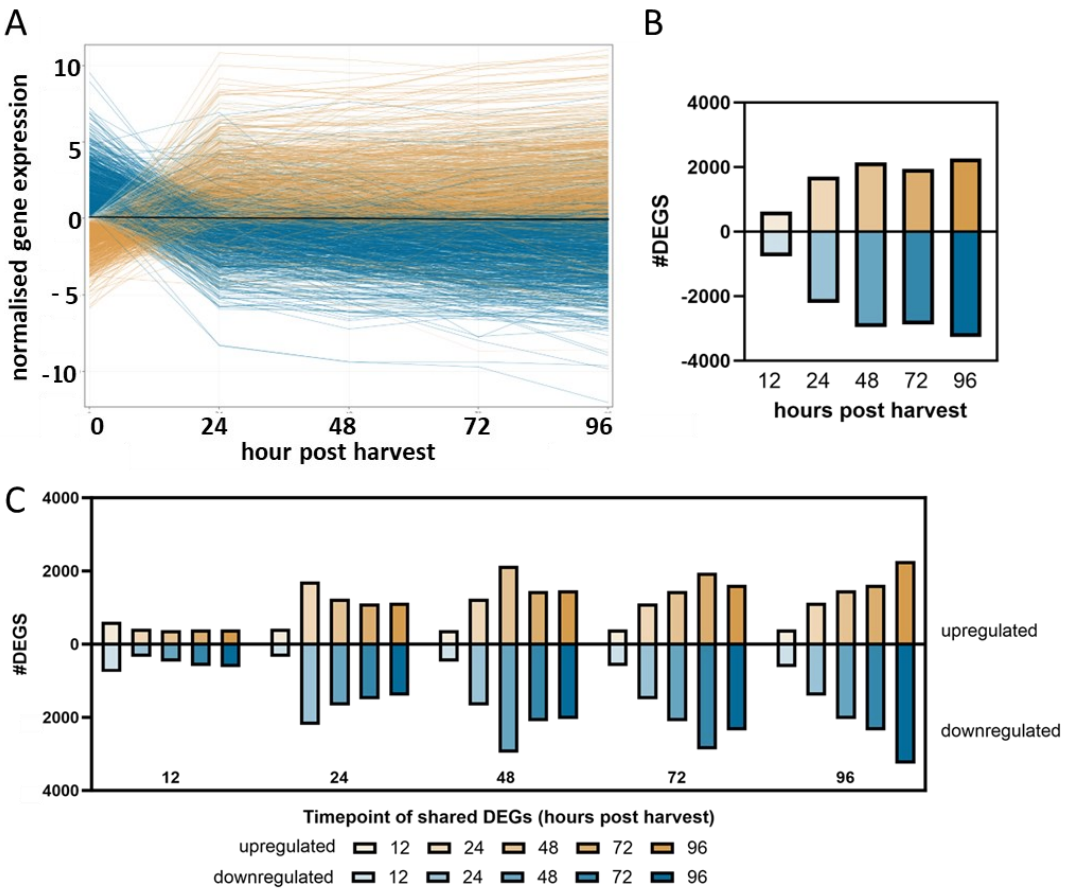


Figure 4.2: Harvested Arabidopsis undergo a significant transcriptional shift (A) Transcriptional profile of normalised gene expression of DEGs in post-harvest Arabidopsis; **(B)** total DEGs (one-way ANOVA $p<0.05$) at each post-harvest timepoint relative to soil-growing plants (negative numbers show downregulated, positive show upregulated) **(C)** shared DEGs between timepoints (orange upregulated; blue downregulated)

A combined total of 8402 DEGs were identified across all timepoints. This indicates a substantial transcriptomic shift in harvested tissues, as >30% of Arabidopsis genes were differentially expressed.

It might be expected that with increasingly scarce resources, harvested tissues would generally reduce gene expression (Zhang et al., 2023). In order to understand the overall pattern in expression change in harvested tissues, I compared the number

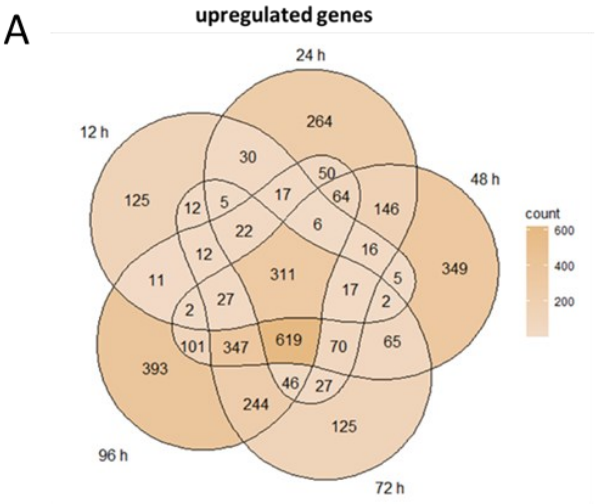
of up- and -down-regulated genes at each post-harvest timepoint. There was an average of 37% more downregulated compared to upregulated DEGs across all timepoints, and the ratio of down- to up-regulation increased with time post-harvest (Figure 4.2B). However, the number of upregulated genes also increased as harvest continued, with 96 hours post-harvest (hph) having both the highest number of upregulated (2271) genes as well as downregulated (3269) (Figure 4.2B). These data suggest that the length of time post-harvest correlates with the number of DEGS, and that, although there were relatively more downregulated genes in harvested tissues, there was more gene upregulation than expected.

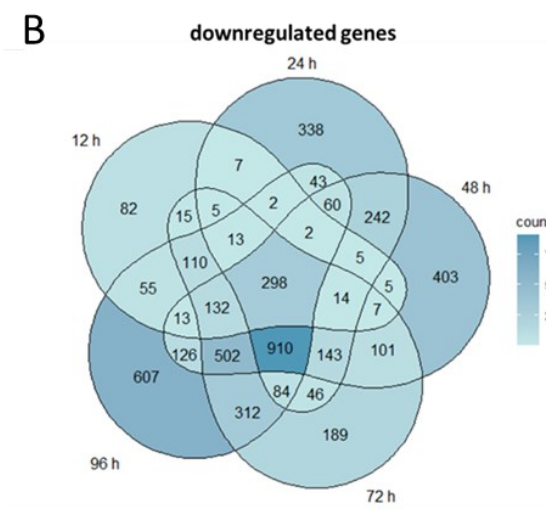
It is not clear from DEG numbers alone whether the genes involved are shared between timepoints or are unique. In order to identify the dynamics of the post-harvest time course, I quantified common DEGs at each timepoint. From 48 hph onwards, more than two-thirds of DEGs were shared between the samples (Figure 4.2C). Excluding 12 hph, there were 930 shared upregulated genes at all timepoints, and 1208 shared downregulated genes. This suggests that over half of the DEGs at 24 hph did not subsequently change their expression relative to their soil counterparts. Thus, transcriptome reprogramming occurred relatively early in post-harvest tissues. However, samples in the earliest post-harvest timepoint (12 hph) shared the least DEGs with the other post-harvest timepoints: 311 up-regulated and 296 down-regulated (Figures 4.2A-B). This relatively low similarity could be due to the short time since the shock of harvest, or to the fact that the 12 hph samples were collected in the evening, whereas other samples were collected in the morning. Over 20% of

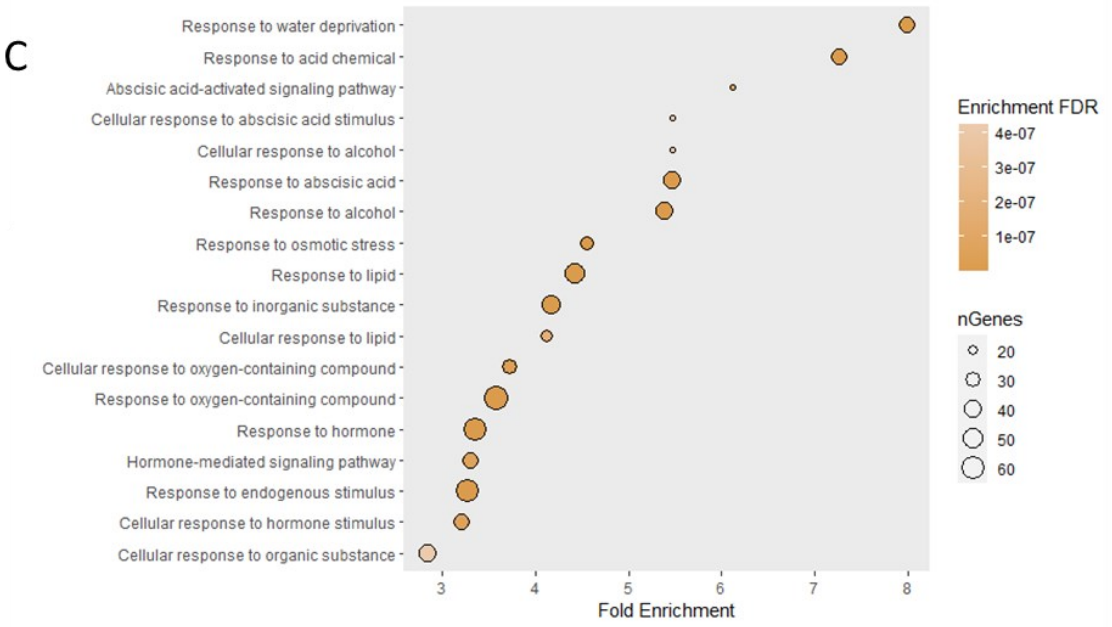
Arabidopsis genes show diurnal oscillation in their transcription (Yang et al., 2020), so this may have affected the relative fold change of some genes at 12 hph. Nonetheless, the subset of genes that are shared across all post-harvest timepoints could shed light on the sustained post-harvest response in Arabidopsis.

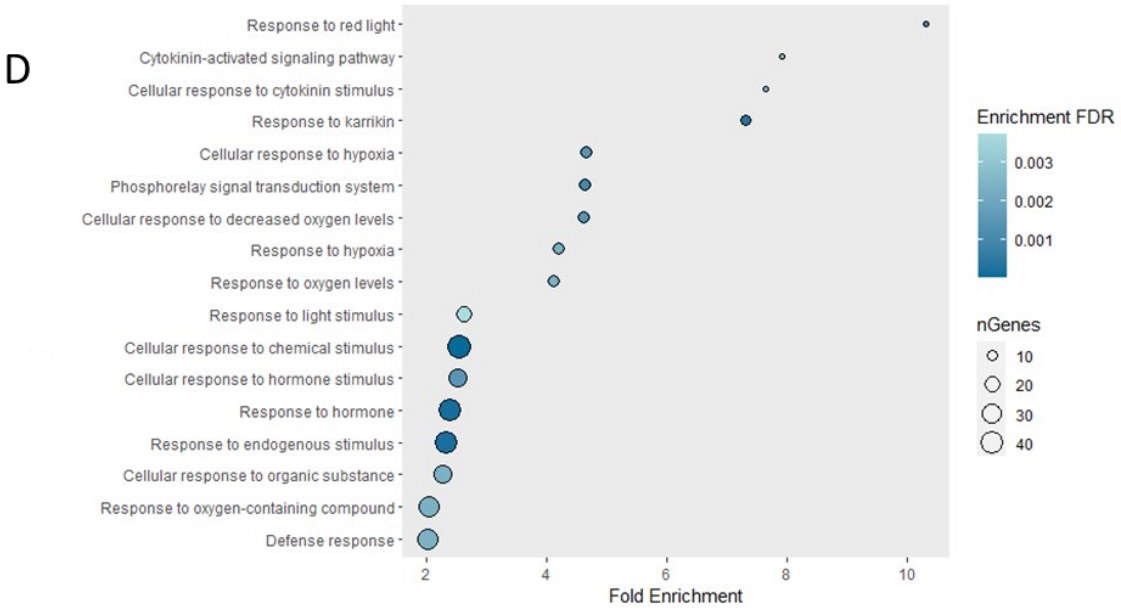
Gene ontology biological process (GO BP) annotation provides a systematic description of the responses or pathways with which each Arabidopsis gene is associated (Berardini et al., 2004). In order to identify biological processes affected throughout the whole post-harvest time course, I used Shiny GO 0.77 (Ge et al., 2020) to search for significantly enriched (False Discovery Rate (FDR) <0.05) GO BP terms in the subset of DEGs shared at all timepoints (Figure 4.3A-B). Common upregulated genes (Figure 4.3A) were significantly enriched in GO BP terms related to abiotic stress, particularly dehydration, osmotic stress and response to hormones, notably ABA (Figure 4.3C). These data highlight the early and continuous abiotic stress responses expressed by harvested tissues. By contrast, the 296 common downregulated genes (Figure 4.3B) were most significantly enriched for GO terms related to responses to light, oxygen, and the growth and development hormones auxin and cytokinin (Figure 4.3D). This suggests that harvested tissues suppress energy-expensive growth-related activities, such as photosynthesis and cell division, from the earliest stages of post-harvest storage.

Figure 4.3 (overleaf): Genes and processes common to all post-harvest timepoints in Arabidopsis Venn diagrams of common **(A)** upregulated and **(B)** downregulated genes between post-harvest timepoints. **(C-D)** Most enriched GO BP terms for genes in all post-harvest samples that are significantly **(C)** upregulated and **(D)** downregulated (Enrichment FDR<0.05).


A
upregulated genes
24 h
12 h
48 h
96 h
72 h
264
30
50
125
12
5
17
64
146
22
6
12
16
11
311
5
349
27
17
2
2
101
347
619
70
65
393
46
27
244
125
count
600
400
200


B
downregulated genes
24 h
12 h
48 h
96 h
72 h
338
7
43
82
15
5
2
60
242
13
2
110
5
55
298
403
14
5
132
7
13
126
502
910
143
101
607
84
46
312
189
coun


C
Response to water deprivation
Response to acid chemical
Abscisic acid-activated signaling pathway
Cellular response to abscisic acid stimulus
Cellular response to alcohol
Response to abscisic acid
Response to alcohol
Response to osmotic stress
Response to lipid
Response to inorganic substance
Cellular response to lipid
Cellular response to oxygen-containing compound
Response to oxygen-containing compound
Response to hormone
Hormone-mediated signaling pathway
Response to endogenous stimulus
Cellular response to hormone stimulus
Cellular response to organic substance
3
4
5
6
7
8
Fold Enrichment
Enrichment FDR
4e-07
3e-07
2e-07
1e-07
nGenes
20
30
40
50
60


D
Response to red light
Cytokinin-activated signaling pathway
Cellular response to cytokinin stimulus
Response to karrikin
Cellular response to hypoxia
Phosphorelay signal transduction system
Cellular response to decreased oxygen levels
Response to hypoxia
Response to oxygen levels
Response to light stimulus
Cellular response to chemical stimulus
Cellular response to hormone stimulus
Response to hormone
Response to endogenous stimulus
Cellular response to organic substance
Response to oxygen-containing compound
Defense response
2
4
6
8
10
Fold Enrichment
Enrichment FDR
0.003
0.002
0.001
nGenes
10
20
30
40

To increase the sensitivity and relevance of GO term identification in a dataset, gene expression levels can be taken into account (Kim and Volsky, 2005). As such, I analysed enriched GO terms across the Arabidopsis post-harvest time course using Parametric Analysis of Gene Set Enrichment (PAGE) (Kim and Volsky, 2005) through agriGOv2 (Tian et al., 2017). The most enriched GO BP terms in upregulated genes further reinforced the abiotic stress responses identified in the shared gene set (Figure 4.3C), including the ABA pathway and osmotic stress response (Figure 4.4A). Moreover, PAGE analysis also identified enriched GO terms related to reproductive stages, such as fruiting, seeds and embryonic development. These data suggest that harvest may trigger a switch from the vegetative to the reproductive stage.

Metabolism of flavonoid secondary metabolites was also among the most enriched GO terms, most notably anthocyanin biosynthesis (Figure 4.4A). Anthocyanin is thought to be produced in stressed leaves as an osmoregulator, an antioxidant or for protection from UV-B (Gould, 2004). Anthocyanin accumulation is likely responsible for the purple pigmentation seen in harvested leaf rosettes (Figure 4.1D). Intriguingly, upregulated genes were also enriched in RNA polymerase II transcription annotation (Figure 4.4A). RNA polymerase II is specifically involved in mRNA transcription of protein coding genes, as opposed to ribosomal RNA or tRNA (Yang et al., 2023a). This would suggest that there was an increase in transcription of mRNA post-harvest. By contrast, downregulated genes were significantly enriched for GO BP terms related to defence and immunity (Figure 4.4B). Interestingly, the immunity-related enrichment was significant

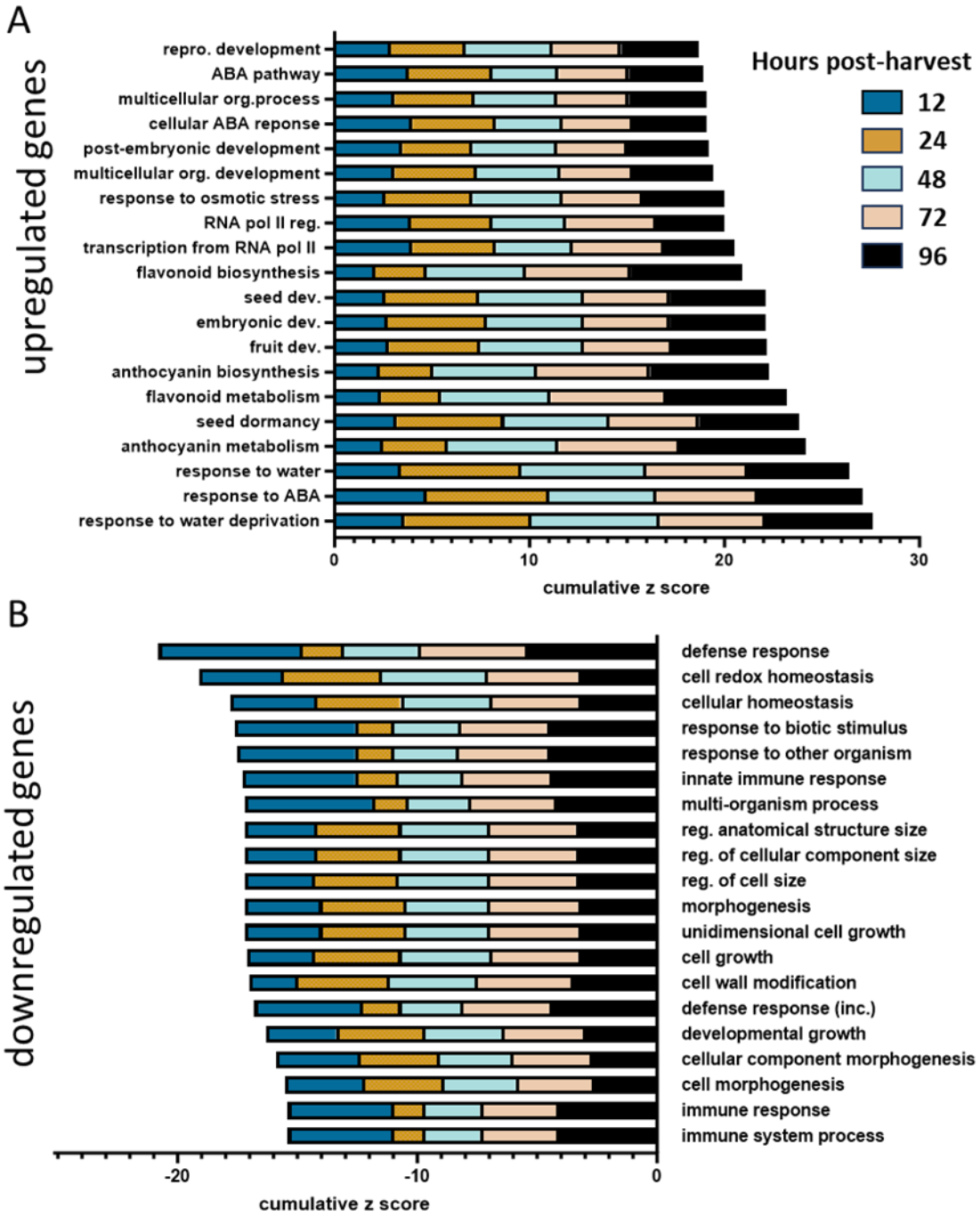


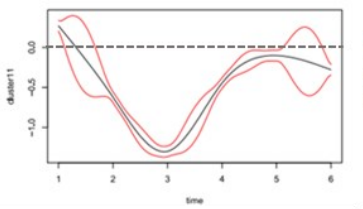
Figure 4.4: Biological processes enriched in post-harvest Arabidopsis The twenty most enriched GO BP terms in **(A)** upregulated and **(B)** downregulated genes in post-harvest Arabidopsis identified using Parametric Analysis of Gene Set Enrichment (PAGE) through agriGOv2. (Top GO biological terms were selected based on cumulative z-score [comparing $\log_2$FC against a normalised distribution] across all post-harvest timepoints, and FDR<0.05 at 96 hph).

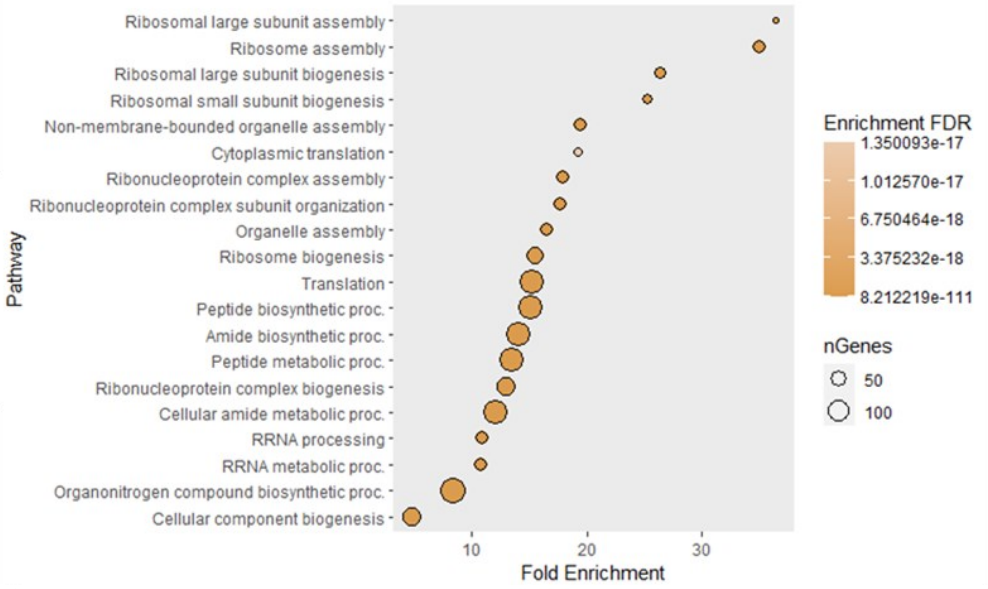
(FDR<0.05) only at 12, 72 and 96 hph, and not 24 and 48 hph. This suggests that there was a transient suppression of immunity-related genes immediately after harvest, which occurred again later in post-harvest storage. GO terms related to cell size and growth were significantly enriched in the downregulated genes (FDR<0.05) at all post-harvest timepoints. This suggests that vegetative growth is suppressed post-harvest. There was also a significant enrichment of genes related to cell redox homeostasis in the downregulated gene set. This suggests that harvested tissues either had less requirement or less ability to regulate the delicate redox balance in their cells. Overall, this analysis indicates that post-harvest Arabidopsis exhibits active transcriptional reprogramming, prioritising abiotic stress responses and reproductive development, and downregulating biotic stress responses and growth.

The dynamics of gene expression over the course of the four days post-harvest could further illuminate the processes and pathways regulated in response to the multiple stresses of harvest. In order to group meaningfully co-expressed genes, I clustered the DEGs using Smoothing Spline Clustering (SSC) (Ma et al., 2006), which identified 35 profiles to optimally account for the range in post-harvest gene expression (Figure 4.5A-C, Appendix C). To understand the biological processes associated with these expression profiles, I again identified significantly enriched GO BP terms (FDR<0.05) in each cluster (Appendix D). Profiles that showed general up- or down-regulation broadly supported the PAGE GO term enrichment analysis (Figure 4.4). Of particular interest were profiles with transient up- or down-regulation over the course of post-harvest storage.

A

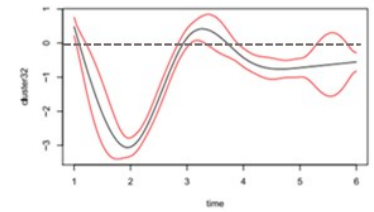
Cluster 11

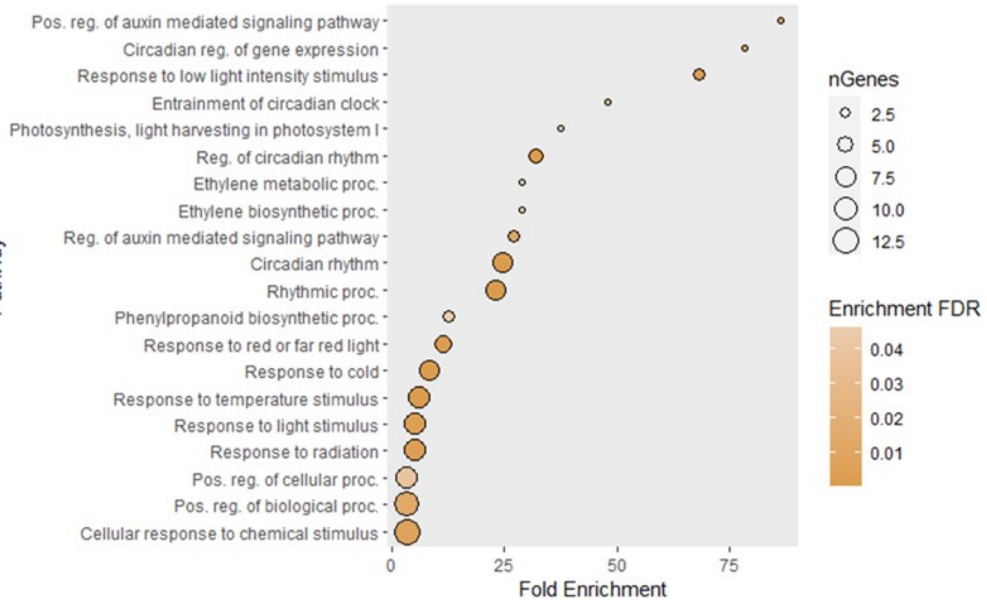




B

Cluster 32





C

Cluster 23

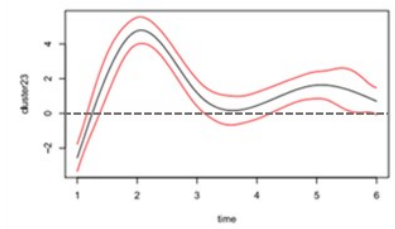


Figure 4.5 (previous page): Selected SSC clustered gene expression profiles of post-harvest Arabidopsis Mean expression profiles and associated top 20 enriched GO BP terms (FDR<0.05) of Arabidopsis post-harvest RNA-seq gene clusters **(A)** 11, **(B)** 32 and **(C)** 23 (black line = mean, red line = 95% confidence bands; dashed line at y=0); timepoints 1-6 are 0, 12, 24, 28, 72 and 96 hph, respectively) (made in SSClust, Ma et al., 2006); full cluster profile in Appendix C.

The 264 genes in cluster 11, were downregulated after harvest until 24 hph, and then recovered to almost pre-harvest levels from 48 hph onwards (Figure 4.5A). The most enriched GO biological terms in cluster 11 related to the energy-rich process of ribosome formation and protein translation. This suggests that there may be a transient dip in protein production after harvest. The 71 genes in Cluster 32 had lowest expression at 12 hph (Figure 4.5B), and were enriched for GO terms related to photosynthesis, auxin signalling and circadian rhythms. These data suggest that the harvested tissues may have a transiently downregulated response to light or circadian rhythms immediately after harvest. The small cluster of 20 genes in cluster 23 had a peak of expression at 12 hph; these genes were significantly enriched for GO BP terms related to ion transportation and starvation response (Figure 4.5C). Although only few genes were present in this profile cluster, it included three key transcription factors (TFs) involved in the response to iron deficiency: *BASIC HELIX-LOOP-HELIX 38*, *39* and *100* (Kurt and Filiz, 2018); these genes are believed to be negatively regulated by jasmonic acid (JA) (Cui et al., 2018). Overall these data suggest that, in the first two days post harvest, Arabidopsis undergoes transient differential expression, mostly downregulation, of some key cellular processes, after which the post-harvest transcriptome is relatively constant.

In summary, the post-harvest Arabidopsis RNA-seq data analyses show that a significant transcriptomic shift takes place post-harvest, with upregulation of abiotic stress responses, the ABA pathway, and anthocyanin production, and down-regulation of growth and biotic defences.

4.2.2 Harvest may impact the development and metabolism of cabbage

There are multiple brassica crops closely related to Arabidopsis, including cabbage, rocket and broccoli (Chapter 1, Figure 1.1B). Unlike Arabidopsis, however, some of these brassicas survive in storage for months, rather than days or weeks. To explore the transcriptional changes underlying these differences in shelf-lives, I collected RNA samples of field-grown pointed cabbage (*Brassica oleracea* var. *capitata* cv. 'Regency') over the course of 70 days post-harvest in the dark and at 4°C (Chapter 2, Figure 2.3B). All five treatments in triplicate together produced 84 Gb of high-quality reads (Q30), with an average of 5.6 Gb per sample, 31,913 genes mapped and 44,127 transcripts (Appendix E). This showed that the dataset covered about half of the 62,232 known cabbage genes (Guo et al., 2021). To see how the overall expression profile of the post-harvest cabbage differed over time, I used Pearson's correlation coefficient to make pairwise comparisons. Samples showed the closest correlation with those collected at the same or proximal timepoints (Figure 4.6A)

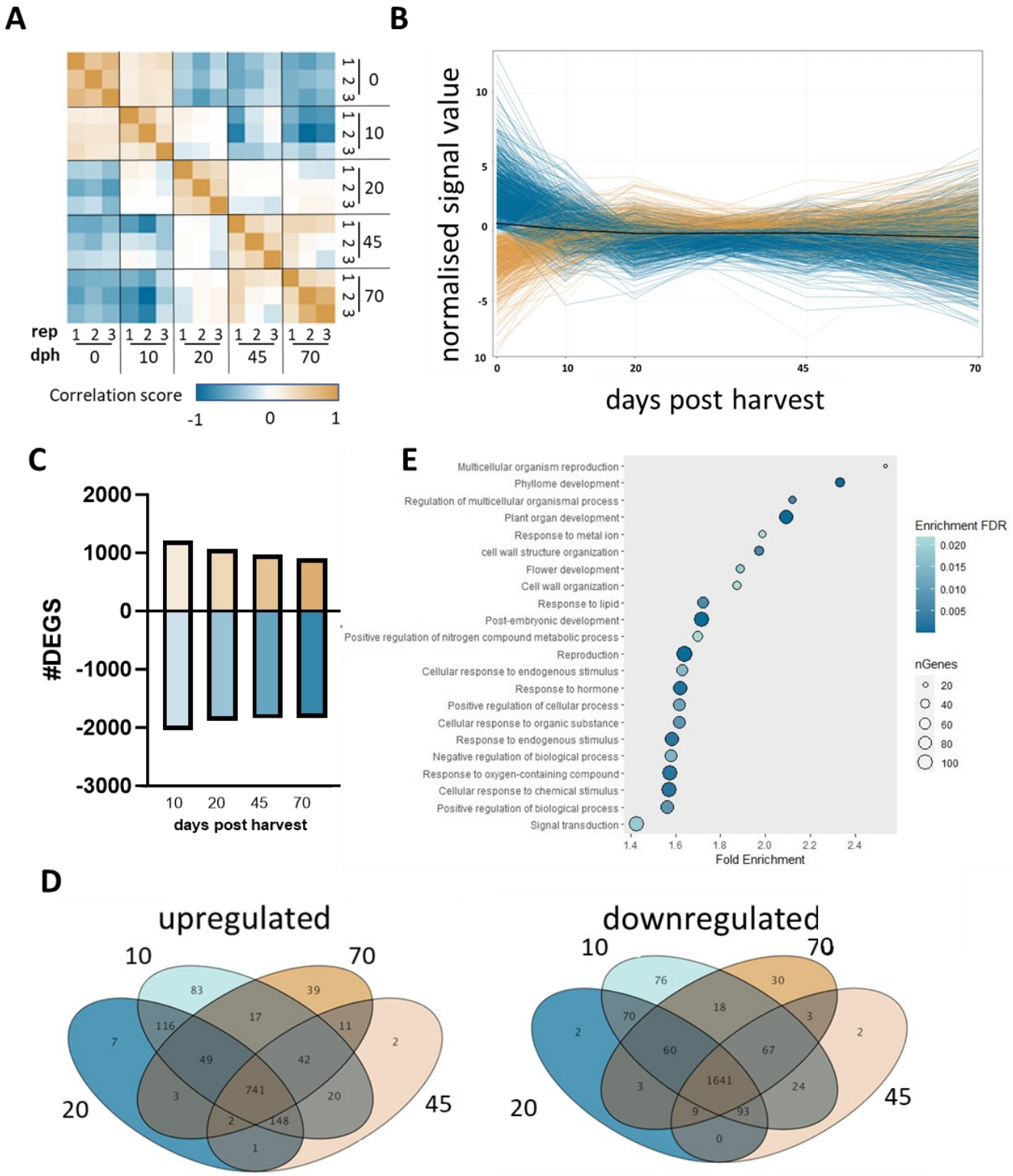

A
B
C
D
E
rep
dph
Correlation score
normalised signal value
days post harvest
#DEGS
upregulated
downregulated
Enrichment FDR
nGenes
Fold Enrichment
Multicellular organism reproduction
Phyllome development
Regulation of multicellular organismal process
Plant organ development
Response to metal ion
cell wall structure organization
Flower development
Cell wall organization
Response to lipid
Post-embryonic development
Positive regulation of nitrogen compound metabolic process
Reproduction
Cellular response to endogenous stimulus
Response to hormone
Positive regulation of cellular process
Cellular response to organic substance
Response to endogenous stimulus
Negative regulation of biological process
Response to oxygen-containing compound
Cellular response to chemical stimulus
Positive regulation of biological process
Signal transduction

Figure 4.6 (previous page): DEG expression in post-harvest pointed cabbage
(A) Pairwise correlation between sample replicates calculated using Pearson's coefficient (orange shows stronger correlation, dark blue weaker correlation); **(B)** transcriptional profile of normalised gene expression of DEGs in post-harvest cabbage; **(C)** total DEGs ($p<0.05$ two way ANOVA, $1>\log_2FC<-1$/timepoint 0 with Benjamini-Hochberg multiple hypothesis testing p correction) at each post-harvest timepoint relative to soil-growing plants (negative numbers show downregulated, positive show upregulated) **(D)** overlapping DEGs that are up- or down-regulated at all-post-harvest timepoints; **(E)** the top 20 most significantly enriched (FDR<0.05) GO BP terms from genes downregulated at all timepoints post-harvest.

The soil-growing samples were positively correlated with the 10 dph samples, but then negatively correlated from 20 dph onwards. This suggests that an interesting shift in the post-harvest transcriptome occurs between 10 and 20 dph.

The changes in gene expression of the stored cabbages relative to the samples directly analysed after collection from the field represent the transcriptional impact of harvest on cabbage. Comparisons of the transcriptomes of stored post-harvest cabbage to those directly analysed after collection from the field identified 3,280 DEGs across all post-harvest timepoints (Figure 4.6B-C). This equates to ~10% of mapped genes being differentially expressed after harvest. In order to chart the changes in gene expression post-harvest, I identified the number of up- and down-regulated genes at each post-harvest timepoint. There was a similar number of DEGs in all of the timepoints and on average 50-60% more downregulated than upregulated genes (Figure 4.6C). This indicates that gene repression is a predominant feature of harvest-induced transcriptional reprogramming in cabbage.

To see if there was a set of common genes between the timepoints, I compared each set of up- and down-regulated genes. There were 741 upregulated genes shared

between all timepoints, which represents >60% of upregulated genes in each sample (Figure 4.6D). There was a large number of shared downregulated genes: 1,641 genes, or >80% of those at each timepoint (Figure 4.6D). The common processes across the post-harvest time points were then analysed by identifying enriched GO BP terms and Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway annotation (Kanehisa et al., 2023) in the shared DEGs. The cabbage genome is not as well annotated as the Arabidopsis genome with GO terms and KEGG annotation. Therefore, I used Arabidopsis orthologues of the cabbage genes (identified in Liu et al., 2014) to search for significantly enriched GO BP and KEGG annotations in the post-harvest cabbage dataset. To identify processes that were differentially regulated throughout the post-harvest period, I selected up- or down-regulated genes across all timepoints, and analysed their GO term enrichment. There were 425 up-regulated genes with Arabidopsis orthologues common to all time points, but there were no significantly enriched (FDR<0.05) GO terms. On the other hand, there were 965 shared downregulated genes that were significantly enriched for GO BP terms related to reproduction, development, and general cell maintenance processes (Figure 4.6E). These data suggest that harvested cabbage suppressed its growth and reproduction throughout the storage period.

In order to identify which processes might be differentially regulated over the course of post-harvest storage, I analysed the enriched GO BP terms of Arabidopsis orthologues of cabbage DEGs at each individual timepoint. For upregulated genes, enriched GO BP terms were only found in the 10 and 20 dph datasets; the most

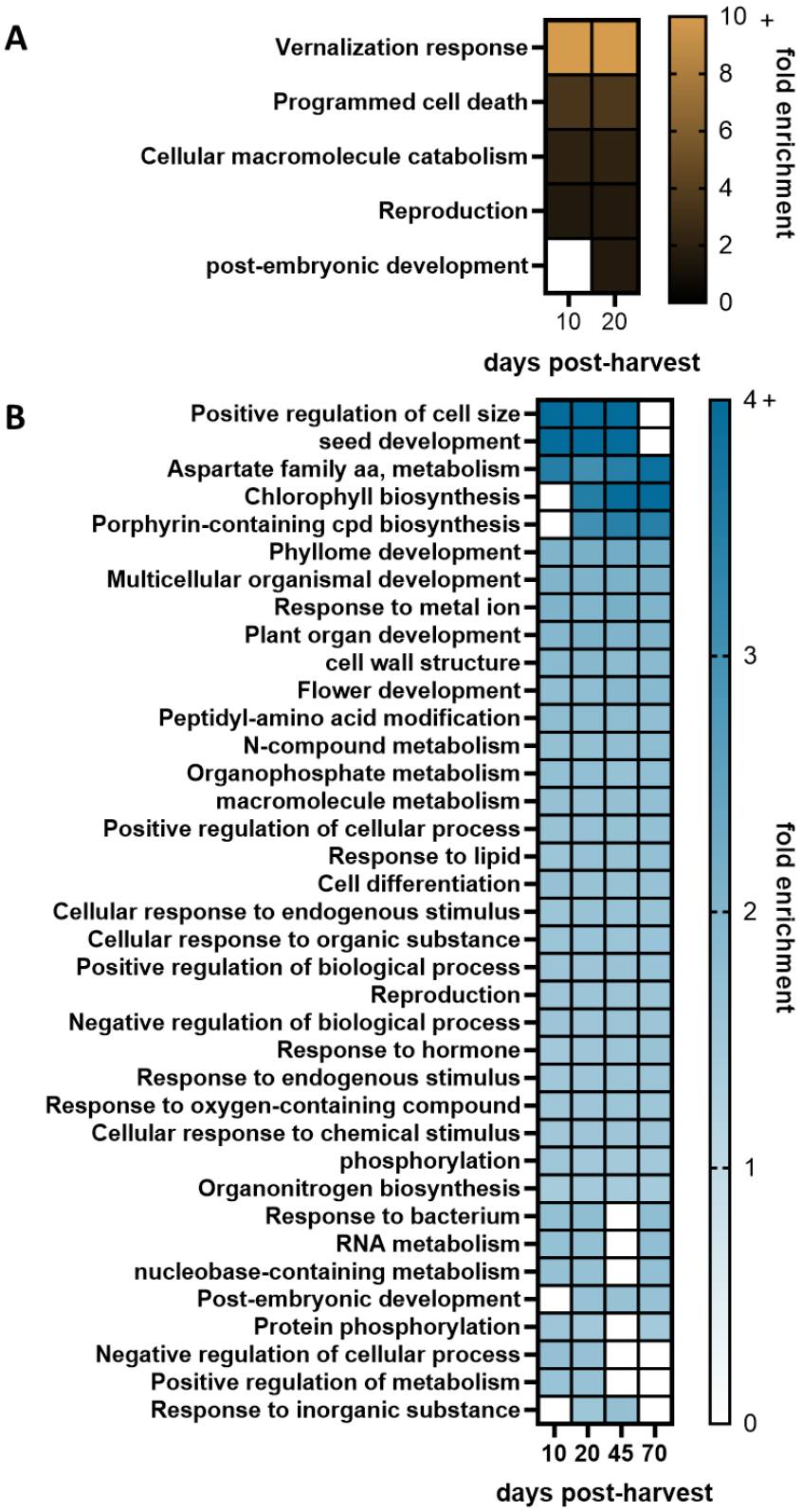


Figure 4.7: Enriched GO terms in post-harvest cabbage GO BP terms significantly enriched in **(A)** upregulated and **(B)** downregulated genes from each timepoint of the post-harvest cabbage RNA-seq (FDR<0.05; white squares indicate no significant enrichment at that timepoint).

enriched GO BP term (~25-fold enriched) related to the vernalisation response (Figure 4.7A) . Vernalisation is the process by which biennial brassicas, like cabbage, use sustained cold temperature as a signal to switch from their vegetative to reproductive life stage through this interim dormant stage which renders them competent to flower when temperatures increase (Woodhouse et al., 2021). Indeed, both the 10 and 20 dph timepoints were enriched for genes related to reproduction. This suggests that the harvest process or the transfer of cabbages to 4°C may have upregulated the vernalisation response at an early stage of post-harvest storage, pushing the cabbage towards reproductive competence. GO terms were significantly enriched at all post-harvest timepoints for downregulated genes (Figure 4.7B). These also included developmental processes, such as leaf, flower and seed development. This suggests that the onward reproductive step out of vernalisation and into flowering was downregulated during cold storage. Metabolism of RNA, macromolecules, nitrogen-containing compounds and organophosphates were also amongst the enriched GO terms in downregulated genes throughout the post-harvest period (Figure 4.7B). This suggests that harvested tissues downregulated energy-expensive cellular processes during cold storage.

A more detailed dissection of expression profiles could provide additional insight into the transcriptomic changes taking place during post-harvest storage. To that end, I grouped DEGs with similar expression patterns using Ward's algorithm into 14 clusters (distance <20) (Figure 4.8, Appendix F). To identify the common features within these clusters,

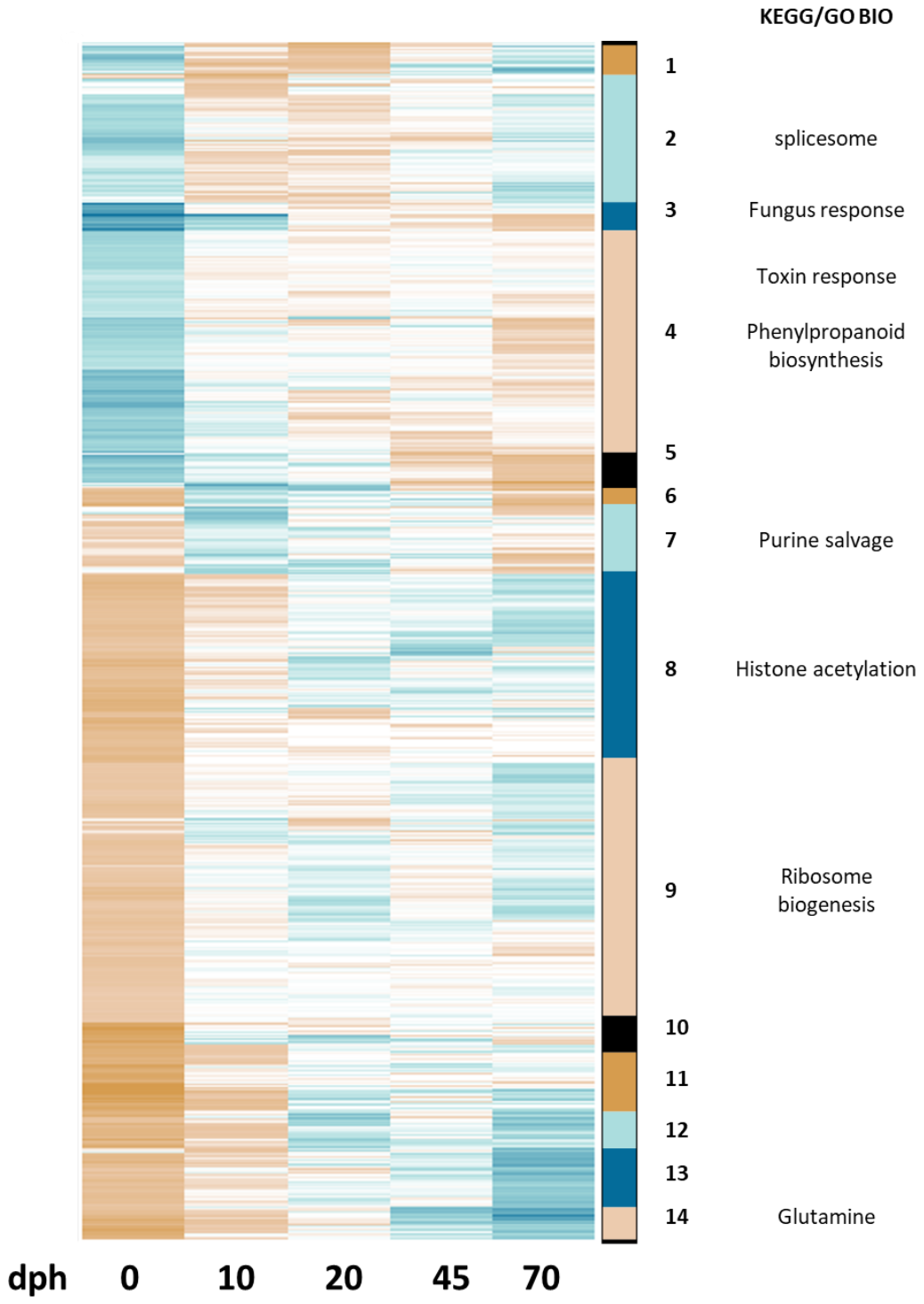
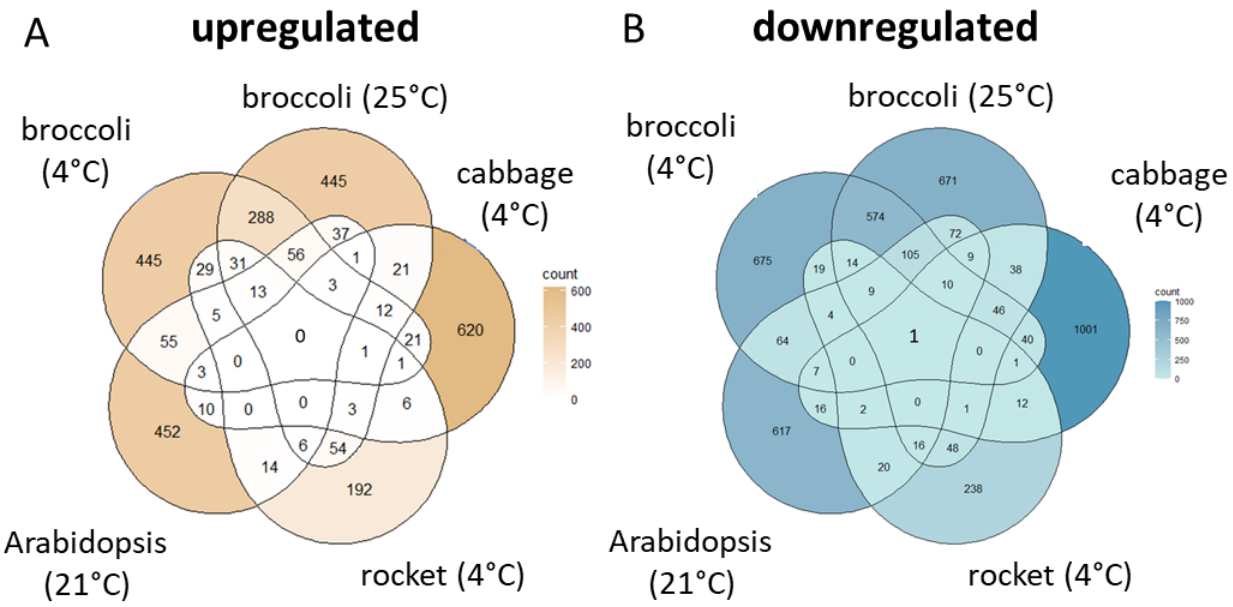


Figure 4.8: **Post-harvest clustering of cabbage DEGs:** A heat map of all DEGs in the post-harvest cabbage RNA-seq dataset clustered using Ward's algorithm. Where significantly enriched (FDR<0.05) GO BP or KEGG terms were found in genes from a cluster, they are listed beside to the right of the relevant cluster number.

I analysed the GO BP and KEGG annotations using Shiny GO 0.77. There were no significantly enriched annotations for half of the clusters. However, the cluster of genes (#7) that showed downregulation in early harvest and later upregulation, was enriched for genes annotated with 'purine salvage'. This process helps to recycle amino acids, is required when nutrient levels are low, and is associated with low growth (Ashihara et al., 2018). Cluster 8, which showed consistent post-harvest downregulation, was most enriched for GO BP term annotation related to histone acetylation. Histone acetylation has been associated with gene activation (Clayton et al., 2006). Downregulation of histone acetylation in post-harvest cabbage may be indicative of reduced gene activation. What is more, the largest cluster of genes (#9) that also showed consistent post-harvest downregulation, albeit it from a lower initial peak, was significantly enriched in genes annotated with GO BP terms related to protein synthesis (Figure 4.8). This suggests that there was a general post-harvest downregulation in protein translation to accompany the reduced gene transcription.

Taken together, these data demonstrate that in the early stages of post-harvest storage, the cold-stored cabbage shifted its developmental transcriptome away from vegetative growth and began the pre-flowering vernalisation stage. Energy-expensive processes were generally downregulated.



C

	Arabidopsis (21°C)	broccoli (4°C)	broccoli (25°C)	salad rocket (4°C)	cabbage (4°C)
Arabidopsis (21°C)		response to ABA & osmotic stress			
broccoli (4°C)	cell wall biosynthesis	upregulation / downregulation		catabolism	response to hypoxia & nitrogen
broccoli (25°C)				small mol., osmotic stress cold response	
salad rocket (4°C)	photosynthesis				
cabbage (4°C)		cell cycle	cell wall biosynthesis		

Figure 4.9 Shared genes and enriched GO terms in post-harvest brassicas: Shared **(A)** upregulated and **(B)** downregulated Arabidopsis homologue DEGs at 3 dph, except cabbage at 10 dph; **(C)** key enriched GO BP terms in Arabidopsis homologues shared between two datasets (upregulated terms in orange (enrichment FDR<0.05); downregulated in blue; black squares denote no comparison made within the species/variety; white squares show where significant GO BP terms could not be identified owing to low gene number (<20), or no enrichment).

4.2.3 Brassicas with shorter shelf lives may exhibit similar post-harvest stress responses

Having looked at the post-harvest transcriptional changes in Arabidopsis and pointed cabbage, I then wanted to identify whether either of these transcriptomes were representative of other leafy brassica crops. Consequently, I utilised published post-harvest leafy brassica transcriptomic datasets for broccoli (Ahlawat et al., 2022) and salad rocket (Bell et al., 2020). In order to make direct comparisons between the DEGs in each dataset, the closest Arabidopsis gene orthologues identified in each dataset were used. Genes common to all of the post-harvest brassicas could shed light on conserved processes across the species and varieties. To identify common genes expressed at the most similar post-harvest timepoint, the Arabidopsis orthologues of up- or down-regulated DEGs were compared at 3 dph for rocket, broccoli and Arabidopsis, and the earliest timepoint for cabbage (10 dph). There were no common upregulated genes between all varieties (Figure 4.9A), and only one common downregulated gene, *AT3G18050* (Figure 4.9B), a chloroplast-related gene involved in response to cold and regulation of the cell cycle; it is otherwise uncharacterised. This suggests that there are no common post-harvest transcriptome changes across all leafy brassicas tested.

To identify if there were interesting similarities between species/varieties, I carried out pairwise comparisons of the Arabidopsis orthologues of DEGs between the datasets at 3 dph (and cabbage at 10 dph). Where more than 20 DEGs were shared between datasets, I identified enriched GO BP terms (Figure 4.9C) for both upregulated

and downregulated genes. Interestingly, the upregulated genes shared between the Arabidopsis model system and the brassicas with shorter shelf-life, i.e., broccoli and rocket, were enriched in annotations for ABA and osmotic stress responses. This suggests that the brassicas with shorter shelf-lives had a common response to water deprivation. In terms of downregulated genes, rocket shared genes with broccoli and Arabidopsis enriched for photosynthesis. Cabbage and cold broccoli (4°C) shared downregulated genes related to the cell cycle. Broccoli shared downregulated genes with Arabidopsis and cabbage enriched for GO BP annotation related to cell wall biosynthesis. Taken together, these data suggest an overall downregulation of growth-related activities in all the harvested brassicas.

Analysis of GO terms enriched in individual datasets before cross-comparison between varieties/species provides a larger dataset to explore. As such, I analysed the enriched GO BP terms (FDR<0.05) from each 3 dph dataset individually, and compared the results. I charted common GO BP terms found in the broccoli, rocket and Arabidopsis datasets (cabbage did not have significantly enriched GO terms at 10 dph (Figure 4.10)). The most enriched GO BP terms in upregulated genes related to various stress responses: response to hypoxia, dehydration, cold, salt, ABA, heat and nutrient levels. This suggests that the post-harvest brassicas with shorter shelf-life were experiencing similar abiotic stress responses. There were multiple GO BP terms enriched in the downregulated genes (Figure 4.11) in broccoli, rocket, and Arabidopsis, including photosynthesis and cell cycle processes.

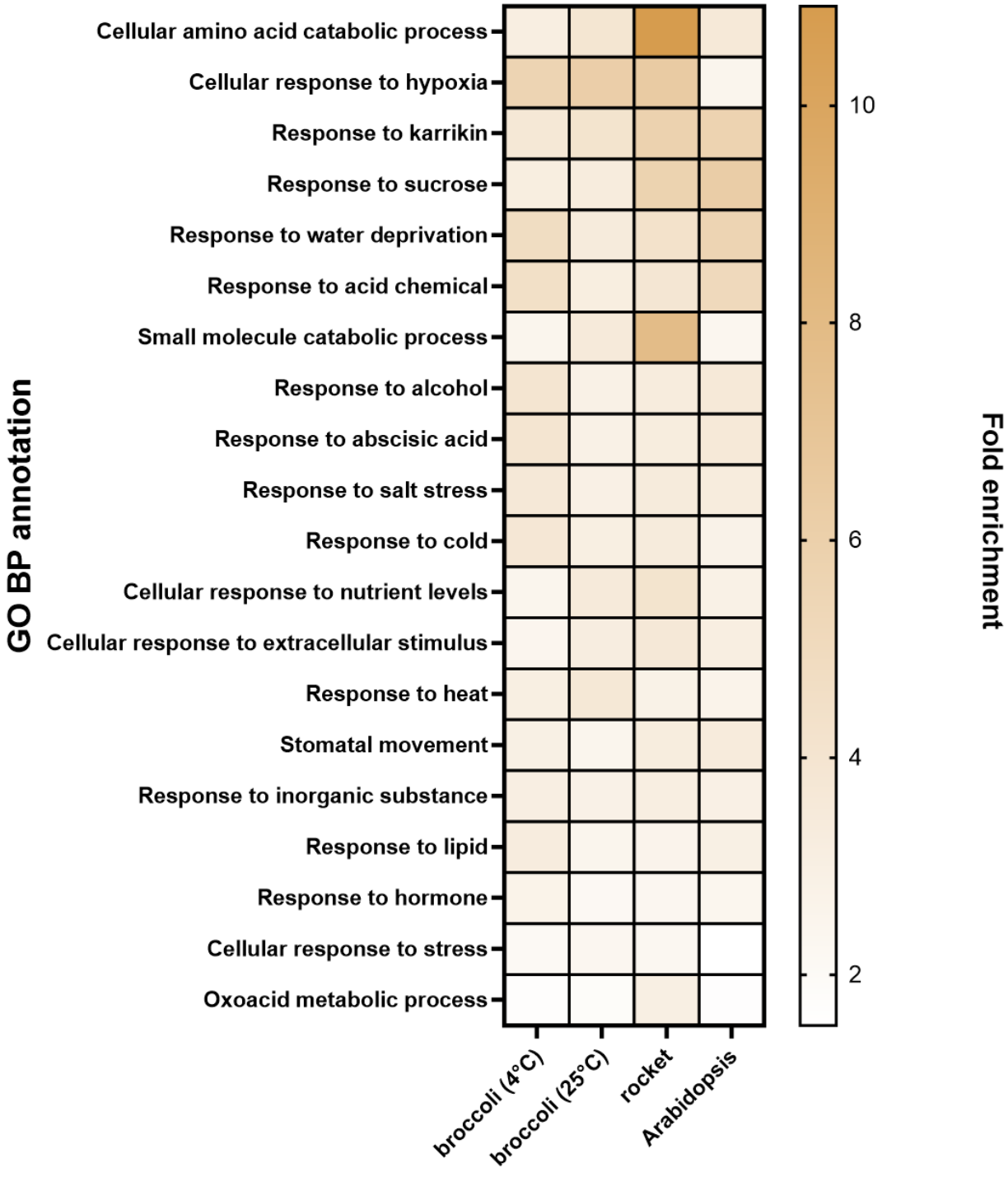


Figure 4.10: Common upregulated biological processes in post-harvest leafy brassicas
Enriched GO BP terms in broccoli, rocket and Arabidopsis genes that were upregulated 3 days after harvest.

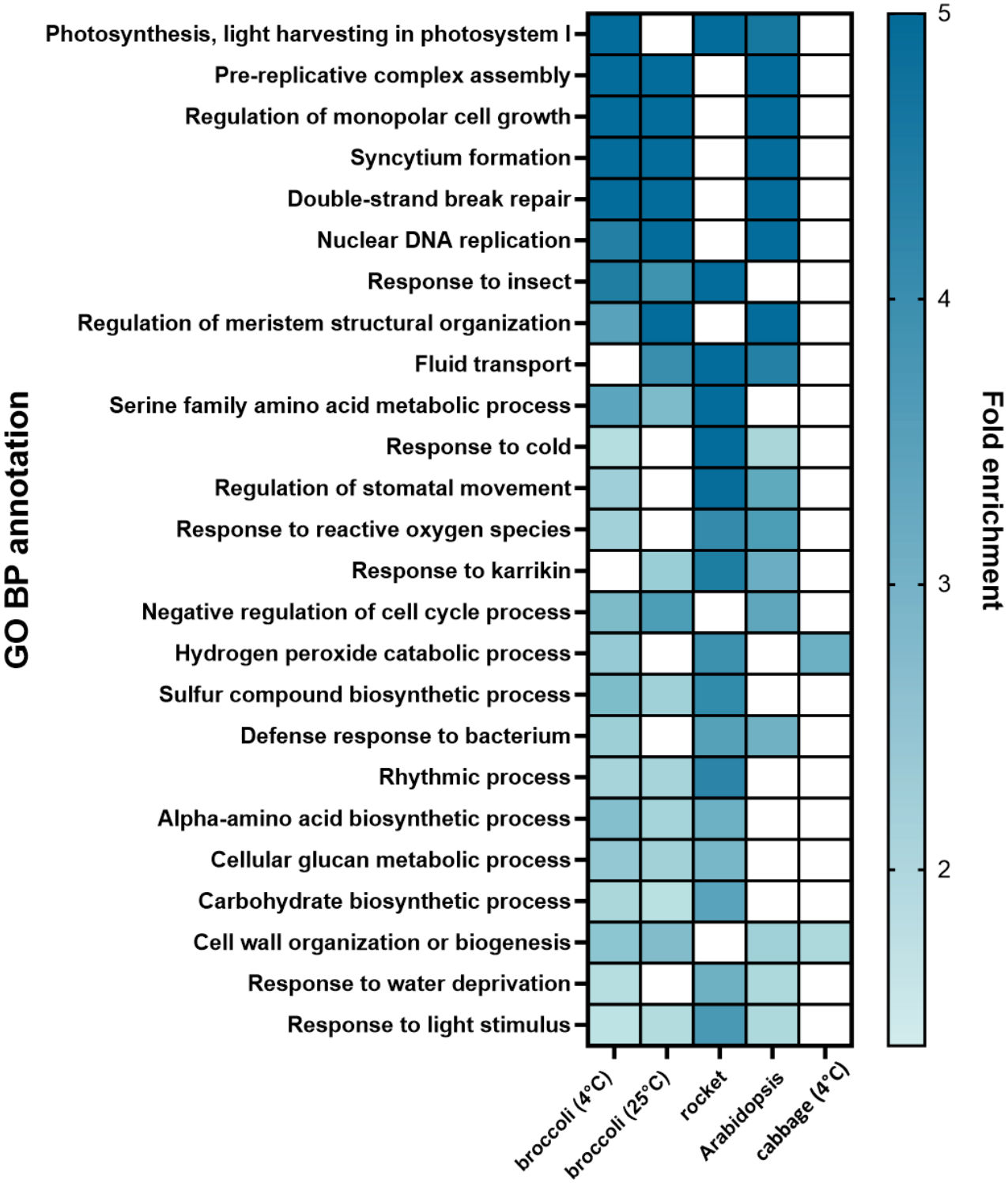


Figure 4.11: Common downregulated biological processes in post-harvest leafy brassicas Enriched GO BP terms in broccoli, rocket and Arabidopsis genes that were upregulated 3 days after harvest, and cabbage 10 days after harvest (white squares indicate no enrichment).

Cabbage also shared downregulation of cell wall construction and H_2O_2 catabolism with some of the other datasets, but largely did not share GO BP terms of its downregulated genes.

Overall, the cross-comparison of enriched GO terms provided more shared abiotic processes between the post-harvest datasets of shorter-shelf-life brassicas, and underlined that post-harvest cabbage is transcriptionally very different from its fellow leafy brassicas.

4.2.4 Harvest changes immune hormone responses in cabbage

Our transcriptomic comparisons of leafy brassicas have highlighted the differences between plants with a shorter shelf-life like Arabidopsis, broccoli and rocket, and longer-term storage plants like cabbage. In the Arabidopsis model system, the impact of these multiple harvest stresses resulted in a change in immune hormone pathways: jasmonic acid (JA)-responsive gene expression was enhanced, whereas salicylic acid (SA)-responsive gene expression was repressed (Chapter 3). In order to identify whether harvest had a similar impact on a brassica with a very different post-harvest stress response, changes in immune hormone pathways in post-harvest cabbage were tested. Plants grown on soil to the ten-leaf stage (Figure 4.12A) had their roots removed and were kept on damp filter paper in covered trays for two days. In order to identify the impact of harvest on the expression of genes in the JA pathway, the harvested and on-soil cabbage were sprayed with 0.1 mM MeJA, and the levels of JA-responsive gene expression was measured 4 hours post spraying (hps).

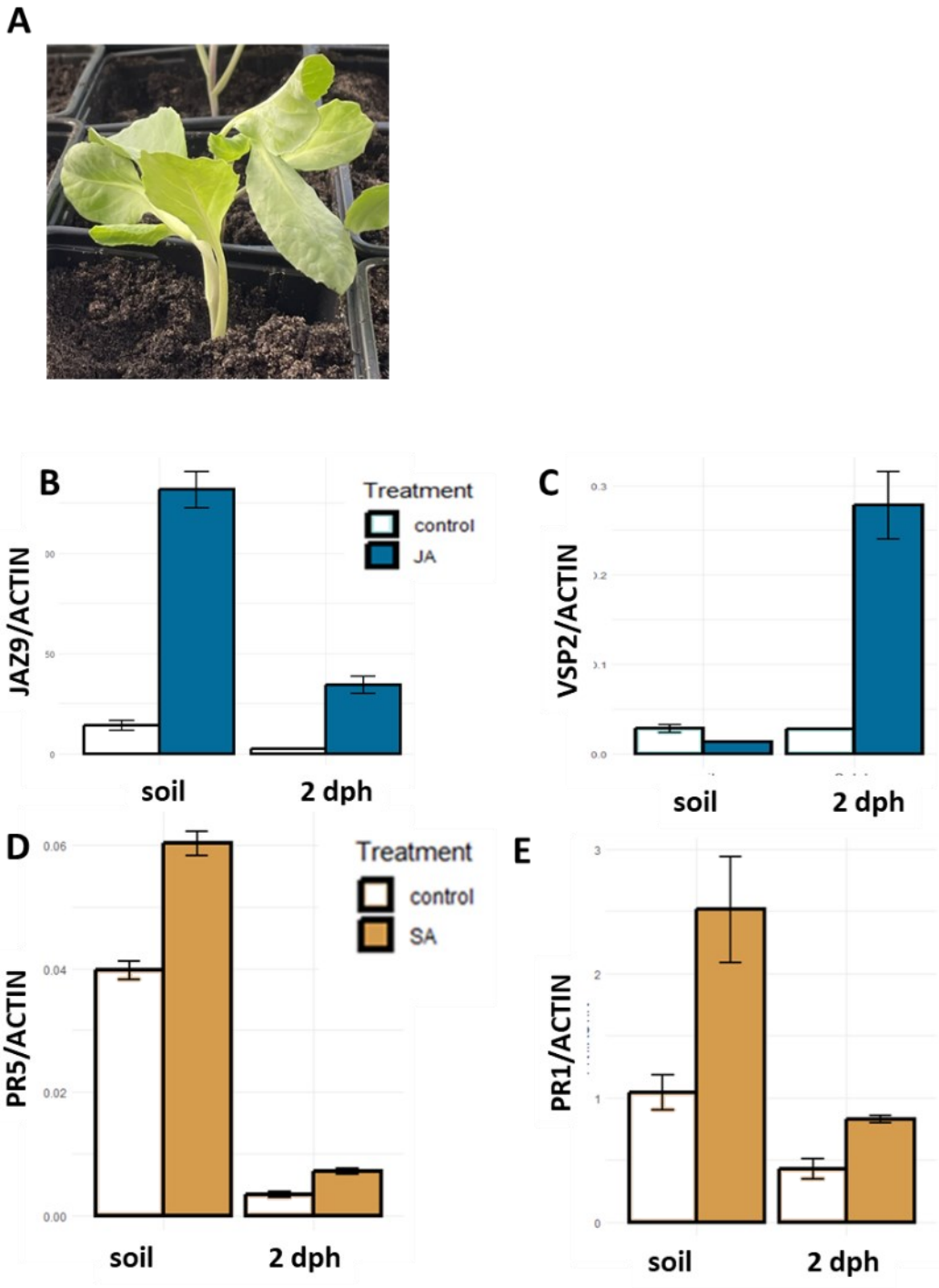


Figure 4.12: Immune hormone responses in harvested cabbage (A) Young pointed cabbage plants grown on soil were **(B-C)** sprayed with 0.1 mM MeJA, and mRNA levels of JA responsive genes **(B)** *JAZ9* and **(C)** *VSP2* were measured*;* **(D-E)** cabbage was sprayed with 0.5 mM SA and mRNA levels of SA-responsive genes **(D)** *PR5* and **(E)** *PR1* were measured. (Gene expression is shown relative to housekeeping gene *ACTIN*, mRNA levels measured by qRT-PCR, graphs are representative of N=2, error bars show 95% confidence interval of technical error.)

The JA-responsive genes *JAZ9* and *VSP2* showed opposite responses to harvest: *JAZ9* was less induced post-harvest, whereas *VSP2* was more induced (Figure 4.12B-C). This suggests that JA-responsive gene expression is affected by harvest, but perhaps not in a unidirectional manner. In order to identify changes in the SA-responsive pathway in harvested cabbage, I sprayed soil-growing and harvested cabbage with 0.5 mM SA, and measured mRNA levels 4 hps. The SA-responsive genes *PR1* and *PR5* showed less upregulation in harvested leaves relative to those on soil (Figure 4.12D-E). This suggests that SA-responsive gene expression was attenuated in post-harvest cabbage.

4.3 Discussion

This study examined the transcriptome of a model Arabidopsis post-harvest system for leafy brassicas, and compared it to the post-harvest transcriptomes of the brassica crops pointed cabbage, broccoli and rocket. The brassicas with shorter shelf-lives, i.e., broccoli and rocket, shared multiple stress responses with the Arabidopsis model: the ABA pathway and osmotic stress response were upregulated, and growth was downregulated. Cabbage, however, showed a remarkably different post-harvest transcriptome, having no significantly upregulated osmotic or nutrient stress responses. Rather, in cabbage, the vernalisation response was upregulated, and general metabolic processes were downregulated. Cabbage did, however, share some of the post-harvest changes in immune hormone signalling seen in the Arabidopsis model system, with a reduction of SA-induced gene expression, and some increased JA-induced gene expression.

The Arabidopsis model system demonstrated a significant transcriptomic shift in post-harvest tissues. It was surprising that extensive gene upregulation occurred throughout the post-harvest period despite the lack of nutrient and water inputs. According to GO BP analysis, these upregulated genes were enriched for terms related to the osmotic stress response, anthocyanin biosynthesis and the ABA pathway. The effect of water deprivation and anthocyanin accumulation were visible as reduced turgor and increased pigmentation of the harvested rosettes. Osmotic stress is known to trigger accumulation of ABA (Yamaguchi-Shinozaki and Shinozaki, 2006), and ABA is thought to work in concert with JA and sucrose signalling to increase anthocyanin levels (Shi et al., 2022) when tissues are exposed to light (Wu et al., 2017). It would, therefore, be interesting to study the impact of storing post-harvest Arabidopsis without light to examine if anthocyanin still accumulates.

On balance, post-harvest Arabidopsis exhibited more down- than up-regulated genes, notably those related to growth, targets of cell-cycle TFs, and a transient reduction of protein synthesis-related genes. Reduced growth is expected with reduced nutrient availability in harvested tissues (Shimotohno et al., 2021). The downregulation of immune-related genes became evident after 2 dph. In Chapter 3 of this study, both pattern-triggered immunity (PTI) and the SA signalling pathway were suppressed in harvested tissue. The upregulated ABA pathway could have a role in suppression of immune gene expression (Berens et al., 2019).

The Arabidopsis model system shared differentially regulated processes with brassica crops with shorter storage life, such as broccoli and rocket. Although there

was little overlap between the specific differentially-expressed Arabidopsis orthologues, all three species showed abiotic stress response upregulation, including the ABA response, and downregulation of photosynthetic processes. By contrast, the cabbage post-harvest transcriptome had little in common even with broccoli, which is the same species. What is more, the cold broccoli, rocket and cabbage were all stored at 4°C and in the dark, so more similarity in responses would be expected relative to the Arabidopsis maintained in day-night cycles at 20°C. However, there were no enriched, upregulated abiotic stress pathways in the post-harvest cabbage samples. Instead, vernalisation was the key annotation for upregulated genes in cabbage, while development and cellular processes were the main downregulated pathways. Cabbage leaves, which comprise the dormant, over-wintering storage organ, seemed to be reducing investment in energy expensive processes, such as abiotic defence. Rather, they reduced their metabolic activity, and promoted nutrient recycling, which is likely to contribute to their longer-term storage potential. It may be that important, transient transcriptional changes occurred in cabbage between the 0 and 10 dph timepoints. A more granular post-harvest time-course in the early stages of harvest could identify any transient abiotic stress responses, such as those seen in the Arabidopsis model.

Despite differences in the post-harvest transcriptomes of Arabidopsis and cabbage, they showed similar post-harvest changes in responses to immune hormones (seen in Chapter 3), with down-regulated SA-responsive genes, and some upregulated JA-responsive genes. The cabbage plants used for the hormone assays were at the

ten-leaf rosette stage, not the compact heads of leaves used in the RNA-seq experiment. These earlier leaves do not have the same thickness, turgor or primary and secondary metabolite content as the inner, heading leaves (Zhao et al., 2020), so the experiment would ideally be repeated on the more mature leaves of the harvested cabbage head to see whether the maturity of the crop impacts post-harvest immunity. However, common changes in immune signalling in Arabidopsis and cabbage identified here and in Chapter 3 suggest that these changes may be applicable to other brassica crops.

In order to make use of the bioinformatic resources and annotations available in Arabidopsis, that are not currently available in *Brassica oleracea* and rocket, comparisons between brassica crops and the Arabidopsis model were useful for exploring cellular processes. However, these brassica crops have many more gene duplications or multiplications compared to their Arabidopsis homologues, and these duplicated genes often have diversified functionality (Lysak et al., 2005). Consequently, using Arabidopsis homologues as a common language between the brassicas may lose some interesting information specific to the individual species/varieties . As new tools and datasets become available in brassica crop genomics, this will facilitate future transcriptomic analyses, and enable use of bioinformatic techniques that aid cross-species analysis, such as OrthoClust (Lee et al., 2019).

In conclusion, this study demonstrates the usefulness of an Arabidopsis post-harvest system in modelling changes in transcription in short-storage leafy brassica

crops, such as broccoli and rocket. The post-harvest transcriptomes of leafy brassicas with short-shelf-lives shared multiple abiotic stress responses, which made them markedly different from cabbage. Cabbage has a longer-shelf-life, and its post-harvest transcriptome was characterised by downregulation of energy-rich activities, which likely contributes to its post-harvest longevity.

Chapter 5: Engineering harvest-inducible traits in brassicas

5.1 Introduction

Post-harvest food loss and waste contributes significantly to global food insecurity: ~9% of the world population is undernourished, and yet >13% of food produced never gets consumed (FAO et al., 2023; FAO, 2021). Food loss not only impacts human health and nutrition directly, but also represents a significant waste of resources used to grow, distribute and dispose of food, including water, soil, labour and fossil fuels. As such, food waste is estimated to account for ~6% of global greenhouse emissions (Ritchie, 2020; Poore and Nemecek, 2018). The importance of food loss to global sustainability is manifested in the ambitious UN Sustainable Development goal 12.3 to halve food waste by 2030 (UN General Assembly, 2015).

Leafy vegetables are particularly vulnerable to food loss, with up to 50% of crops discarded or unsold (FAO, 2011). In the UK, 34% of fresh vegetables and salads purchased are thrown away uneaten by consumers (Gillick and Quested, 2018), predominantly because they became inedible before they could be consumed (Quested and Luzecka, 2014). The edibility of fresh leaves is negatively affected by wilting, senescence, mechanical damage, and opportunistic pathogens, to all of which they are particularly susceptible owing to their relatively high water content and respiration rate (Kader and Saltveit, 2002). Extending the health and shelf-life of leafy vegetables by even a short period would contribute to a significant reduction in food waste.

Existing treatments to extend the shelf-life of leafy vegetables have focused on chemical treatments, controlled atmosphere and packaging (Mahajan et al., 2014). There are ever fewer effective chemical treatments available, as pathogens evolve resistance (Bradshaw et al., 2021) and restrictions on use are applied due to concerns over health and environmental safety, such as the 2018 EU ban on post-harvest use of the fungicide, iprodione, which was previously used to control *B.cinerea* in stored cabbage (European Commission, 2015). Thus, there is a need for sustainable strategies to improve post-harvest health, requiring less energy and fewer resource inputs, such as can be accomplished by bioengineering plants themselves.

Host Species	**Genetic modification**	**Reference**
Tobacco ***Nicotiana benthamiana***	*SAG12::IPT*	Gan and Amasino, 1995
Broccoli ***Brassica oleracea*** **var.*italica***	*SAG13::IPT*	Chan et al., 2009
Lettuce ***Lactuca sativa***	*35S::LsXTH16 RNAi*	Wagstaff et al., 2010
Pak choi ***Brassica campestris*** **ssp.** ***chinensis***	*Brnye1*	Wang et al., 2022

Table 5.1: Genetic engineering in leafy crops for delayed post-harvest senescence

To provide tangible benefits to farmers, the focus of most genetic improvement strategies in crops is to maximise yield and quality by improving plant health and

productivity on soil. In terms of food security, many of these on-soil yield gains are dwarfed by the extent of post-harvest losses. Only a small number of genetically engineered leafy crop varieties have been developed specifically for improved post-harvest traits, and all of these have targeted delayed senescence (Table 5.1).

Chapters Three and Four of this thesis demonstrate that biotic and abiotic responses of harvested plants are markedly different from those on soil, and thus require different strategies for bolstering and enhancement. The interconnected nature of stress response networks in plants suggests that genetic improvements for the post-harvest period could have unintended consequences for on-soil health and yield, and *vice versa* (Altmann et al., 2020). In addition, constitutive expression of defence genes generally has a negative impact on yield, owing to the growth-defence trade-off (Denancé et al., 2013). To prevent deleterious impacts to pre-harvest health and yield, a strategy to specifically optimise post-harvest traits is required.

Inducible gene expression systems have been developed using synthetic or endogenous plant promoters to drive gene expression in response to wounding (Barbosa-Mendes et al., 2009), osmotic stress (Jameel et al., 2020; Kim et al., 2021), nutrient deficiency (Belcher et al., 2020) and senescence (Gan and Amasino, 1995) (Chapter 1, Table 1.3). Ideally, in order to provide maximum protection or benefit to plants only in the post-harvest period, an optimal inducible expression system would respond rapidly and specifically to harvest, with minimal expression on soil. The development of such an inducible expression system requires knowledge of the transcription factors (TFs) and pattern of associated cis-regulatory elements (CREs),

involved in the upregulation of harvest-responsive genes (Vandepoele et al., 2009; Lieberman-Lazarovich et al., 2019; Dror et al., 2015).

In this study, harvest-induced promoters and their common CREs were identified in *Arabidopsis thaliana*, *Brassica oleracea* var. *capitata* (pointed cabbage) and *Brassica oleracea* var. *italica* (broccoli). These harvest-inducible promoters were triaged for specificity to harvest and maximal long-term upregulation. Finally, we show that harvest-responsive promoters can be used to drive transgene expression and protein accumulation specifically in the post-harvest phase. These findings demonstrate that inducible gene expression systems can be utilised to introduce new traits specifically in post-harvest tissues.

5.2 Results

5.2.1 Harvest-induced gene promoters in Arabidopsis contain G-box and ABRE cis-regulatory elements

Gene expression data can be used to infer the activity of promoters (Cooper et al., 2006). In order to identify promoters which showed increased activity in post-harvest leafy brassicas, I analysed transcriptomic data of the Arabidopsis model harvest system (Chapters 2 and 4) over the course of four days post-harvest. To obtain promoters that are active throughout the whole post-harvest period, only genes that were upregulated at every timepoint ($log_2FC>1$ relative to soil) were selected. Of 9911 differentially expressed genes (DEGs) in the entire experiment, 1354 were upregulated at all post-harvest timepoints (Figure 5.1A). To identify only those promoters that had minimal activity in the on-soil growth phase, these 1354 genes were further filtered to

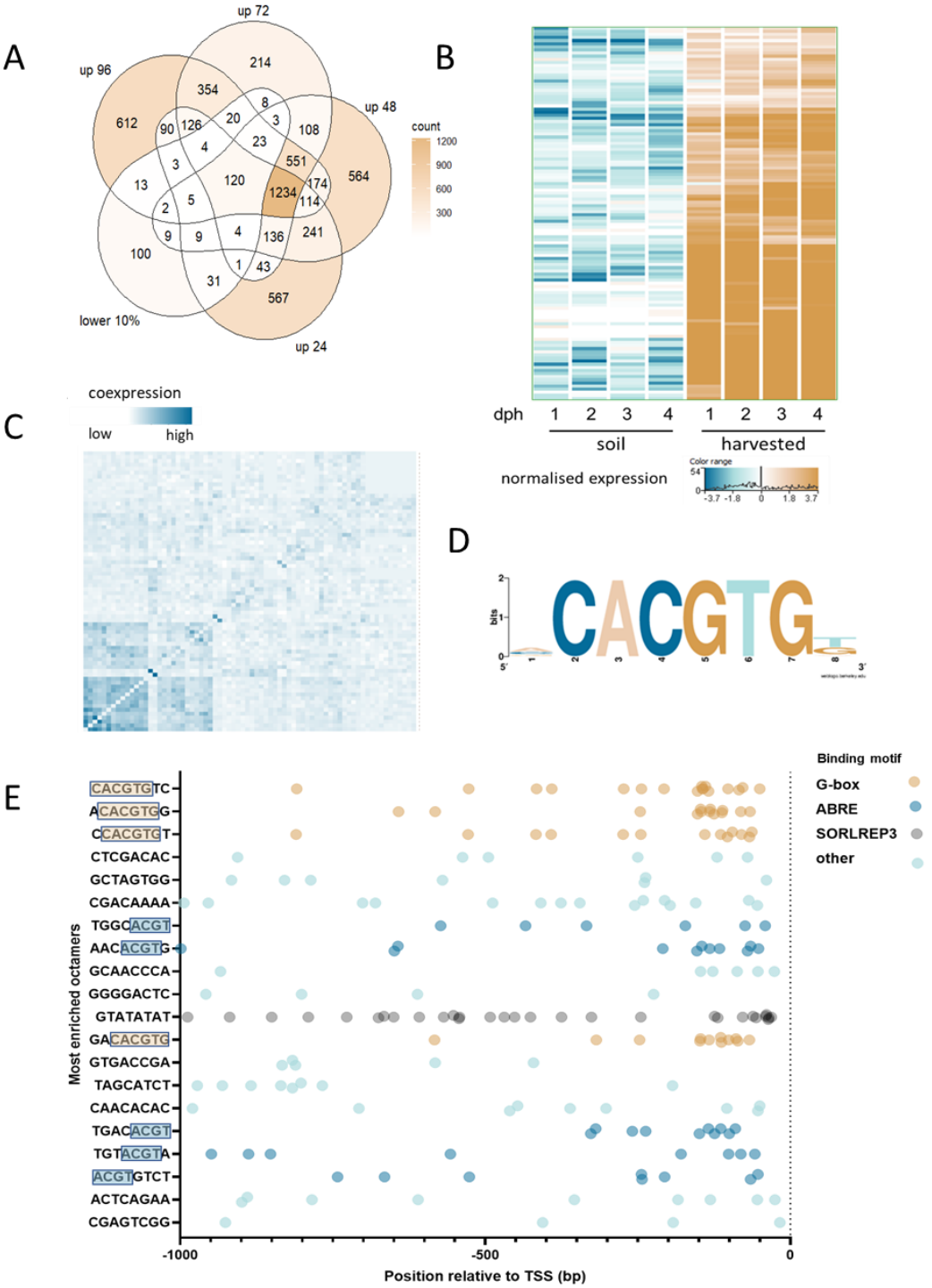
retain only those with the lowest 10% of raw RNA-seq reads on soil (Figure 5.1A). The remaining 120 genes that met these criteria were true harvest-induced genes with sustained rather than transient expression profiles (Figure 5.1A-B).

Genes that show high co-expression patterns across a range of conditions are likely to share CREs (Khan et al., 2020). In order to test if harvest-induced genes were likely to share regulatory elements, their level of co-expression in publicly available transcriptome datasets was calculated using ATTED-II software (Obayashi et al., 2022). Less than 4% of pairs of harvest-induced genes showed significant co-expression (z score > 3) (Figure 5.1C), suggesting their regulatory elements likely vary.

Nonetheless, shared features in promoters of harvest-induced genes can provide information about how their expression is controlled (Obayashi et al., 2007). To identify CREs that contribute to harvest-inducible expression, the promoters of all 120 harvest-induced genes were compared. The average promoter length in Arabidopsis has been estimated as 500 bp long (Korkuć et al., 2014), so to capture all key shared CREs in our harvest-inducible gene promoters, 1 kb upstream of the putative transcription start site (TSS) was used for analysis. Local Distribution of Short Sequences (LDSS) software (Yamamoto et al., 2007) was used to identify n-mers enriched in the 120 harvest-induced promoters relative to expected distribution in random promoter sequences. The twenty most enriched octamers (Fisher exact test [two sided] $p<0.001$) contained nine variants of the ABSCISIC ACID RESPONSE ELEMENT (ABRE), with a core ACGT sequence motif, and the three most enriched octamers contained the full canonical CACGTG G-box motif (Figure 5.1D-E). It is worth

emphasising that the ABRE element is contained within the G-box. The only other known, enriched motif was that of light-responsive SORLREP3: TGTATATAT (Hudson and Quail, 2003). These data suggest that harvest-induced gene expression may be controlled by transcriptional regulators that interact with the G-box and/or ABRE motifs, such as bHLHs and basic leucine zipper (bZIP) TFs (Ezer et al., 2017).

The copy number of CREs in a promoter and their location relative to the TSS can impact their role in regulating gene expression (Mehrotra et al., 2005). Generally, CREs within the closest 200 bp upstream of the TSS have the strongest impact on gene expression (Zou et al., 2011). To identify which CREs were most likely to control the expression of the harvest-induced genes, the position of the 20 most enriched octamers within each promoter was mapped relative to the putative TSS. On average, two thirds of the octamers containing the full G-box motif occurred in the 200 bp upstream of the TSS, compared to less than one third of those with ABRE motifs (Figure 5.1E). This suggests that the G-box in particular has an active role in post-harvest gene regulation. Copy number of an individual CRE in a promoter generally increases its gene regulatory activity (Rushton et al., 2002). In particular, two or more ABREs are thought to be required for ABA-responsive gene expression (Gómez-Porras et al., 2007), whereas one copy of the G-box in combination with other CREs can be sufficient (Liu et al., 2016).


A
up 72
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up 96
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8
612
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3
up 48
108
4
23
551
3
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13
120
1234
174
114
2
5
9
9
4
136
241
100
1
43
31
567
lower 10%
up 24
B
count
1200
900
600
300
dph 1 2 3 4 1 2 3 4
soil
harvested
normalised expression
Color range
C
coexpression
low
high
D
bits
E
Binding motif
G-box
ABRE
SORLREP3
other
Most enriched octamers
CACGTGTC
ACACGTGG
CCACGTGT
CTCGACAC
GCTAGTGG
CGACAAAA
TGGCACGT
AACACGTG
GCAACCCA
GGGGACTC
GTATATAT
GACACGTG
GTGACCGA
TAGCATCT
CAACACAC
TGACACGT
TGTACGTA
ACGTGTCT
ACTCAGAA
CGAGTCGG
-1000
-500
0
Position relative to TSS (bp)
F
#promoters
frequency of G-box motif
G
#promoters
frequency of ABRE motif

Figure 5.1 (previous page) Identification of harvest-inducible genes and their common promoter motifs (A) Upregulated DEGs from each timepoint (hours post-harvest) of the post-harvest RNA-seq experiment ($log_2FC>1$ harvest/soil at same timepoint, one way ANOVA, Benjamini Hochberg correction for multiple hypothesis testing, $p<0.05$, $log_2FC>1$); and those among the lowest 10% of expression on soil; numbers represent the number of genes in each overlapping category. **(B)** Normalised expression profiles of the 120 harvest-inducible genes from RNA-seq data according to the days post-harvest (dph); orange and blue represent relatively high and low expression, respectively . **(C)** Heatmap of co-expression analysis (ATTED-II) of harvest-inducible genes with significant co-expression (z-score>3) across publicly available RNA-seq datasets . **(D)** The G-box motif within the most enriched octamers (two-sided Fisher Exact Test p value <0.001); size of letter represents prevalence of the nucleotide in the motif **(E)** The 20 most enriched octamers in harvest-inducible gene promoters (in descending order of enrichment) and their position within 1 kb upstream of the TSS of all 120 harvest-inducible genes; orange circles show octamers containing the full G-box motif CACGTG; dark blue circles show octamers containing the ABRE ACGT, but not the full G-box; light blue circles show octamers without an ACGT element.. **(F)** Frequency of the ABRE AGCT motif and **(G)** G-box motif in the promoters of harvest-inducible genes.

To identify the potential role of CRE copy number in harvest-induced expression, the frequency of the G-box and ABRE motifs was calculated within individual harvest-induced promoters. Of the 34% of harvest-induced promoters containing the full G-box motif, over two-thirds only had one copy of the CRE (Figure 5.1F). By contrast, the 42% of harvest-induced promoters harbouring ABRE motifs had a median number of 3 copies, with some even containing up to ten copies (Figure 5.1G). Thus, the frequency of G-box and ABRE motifs in harvest-induced promoters corresponded to the expected numbers of CREs needed for effective regulation of gene expression.

Approximately one third of all Arabidopsis genes have a G-box or ABRE motif 500 bp upstream of their TSS (Ezer et al., 2017), but less than 0.5% of these are induced by harvest (Figure 5.1A). To identify additional factors that could be

responsible for regulating harvest-responsive gene expression, the GO Shiny 0.77 program was used to investigate if harvest-inducible promoters were enriched in target datasets of particular TFs (Table 5.2). Harvest-induced genes were enriched in targets of five TFs, the most significant of which was LEAFY COTYLEDON1 (LEC1), which has the potential to regulate the expression of ~20% of harvest-induced genes (Table 5.2). Overall, however, less than half of harvest-induced promoters were known targets of these five TFs, all of which are known to bind to G-box or ABRE-related motifs (Table 5.2; Figure 5.2A). This suggests that multiple TFs were involved in harvest-responsive gene expression, some of which will not have been captured by our analysis.

Transcription factor	#genes	Enrichment FDR	Database	Binding motif	Reference
BZIP28	21	0.000101	Plant.GSAD	cACGTG	Kim et al., 2018
BETA-AMYLASE 8 (BAM8)	23	0.000371	Plant.GSAD	gCACGTG	Reinhold et al., 2011
NUCLEAR FACTOR Y-BINDING 9 (NFBY9)/LEAFY COTYLEDON 1 (LEC1)	27	1.20E-06	GTRD	cACGTGtc	Pelletier et al., 2017
ABSCISIC ACID RESPONSE ELEMENT BINDING FACTOR 4 (ABF4)	23	5.86E-05	GTRD	c/gACGTGGC	Uno et al., 2000
ABSCISIC ACID RESPONSE ELEMENT BINDING FACTOR 1 (ABF1)	19	5.86E-05	GTRD	cACGTGgC	Mathelier et al., 2016

Table 5.2: Harvest-induced genes (#genes) enriched in transcription factor (TF) target datasets identified in GO Shiny 0.77 searches (Database). The TF's predicted binding motif and the reference citing the identification of each motif are shown.

The upregulated expression of TFs themselves, particularly in response to stress, can correlate with activation of target genes (Zaborowski and Walther, 2020). Thus, I examined expression of the five putative regulatory TFs (Table 5.2) of harvest-induced genes in the post-harvest RNA-seq dataset. Of these, only *ABSCISIC ACID RESPONSE ELEMENT BINDING FACTOR 1/4* (*ABF1* and *ABF4*) showed upregulation (~1.5-2 log2FC harvest/soil) in harvested samples (Figure 5.2B). These data support the potential role of ABF1 and ABF4 in regulating the expression of a subset of the harvest-inducible genes.

Transcriptomic time courses can be used in combination with ChIP-seq data to identify TFs that might be responsible for divergence of gene expression profiles (Ernst et al., 2007). I used the DREM 2.0 program to cluster the four-day post-harvest Arabidopsis RNA-seq time course into discrete expression profiles and identify TFs significantly associated with each branch (Figure 5.2C; $p<0.05$) (Schulz et al., 2012). The only TF significantly associated with strong upregulation of expression after harvest ($log_2FC>4$ by 24 hph) was the light-responsive PHYTOCHROME INTERACTING FACTOR3 (PIF3), which can bind to the G-box motif (Mathelier et al., 2016). Notably, *PIF3* itself was significantly upregulated in post-harvest tissues ($log_2FC>2$) from 24 h onwards (Figure 5.2B). These analyses suggest that PIF3 could be involved in upregulation of a subset of harvest-responsive genes.

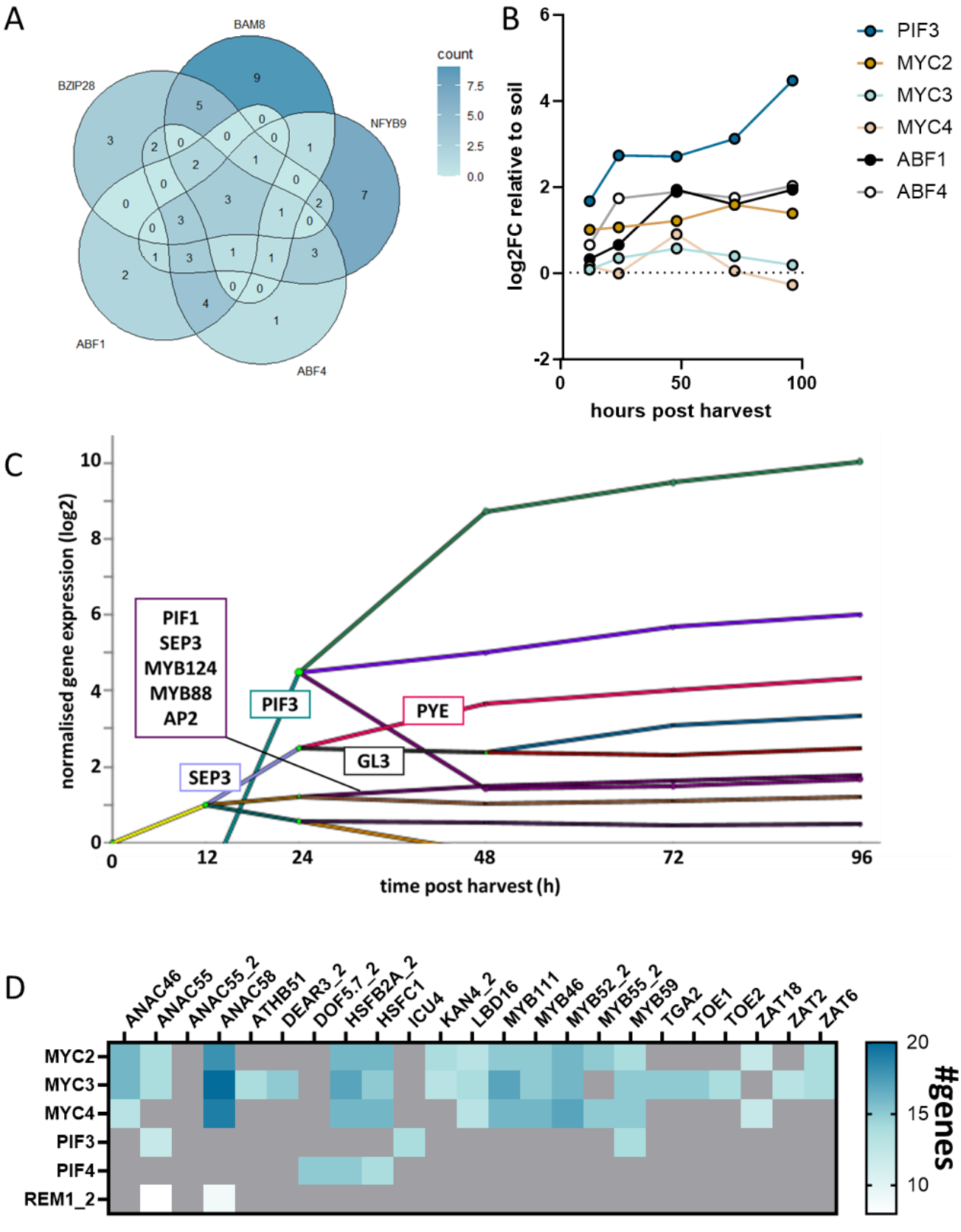

A
BAM8
BZIP28
NFYB9
ABF1
ABF4
count
7.5
5.0
2.5
0.0
B
log2FC relative to soil
hours post harvest
PIF3
MYC2
MYC3
MYC4
ABF1
ABF4
C
normalised gene expression (log2)
time post harvest (h)
PIF1
SEP3
MYB124
MYB88
AP2
PIF3
PYE
GL3
SEP3
D
ANAC46
ANAC55
ANAC55_2
ANAC58
ATHB51
DEAR3_2
DOF5.7_2
HSFB2A_2
HSFC1
ICU4
KAN4_2
LBD16
MYB111
MYB46
MYB52_2
MYB55_2
MYB59
TGA2
TOE1
TOE2
ZAT18
ZAT2
ZAT6
MYC2
MYC3
MYC4
PIF3
PIF4
REM1_2
#genes

Figure 5.2 (previous page) Analysis of potential transcriptional regulators of harvest-inducible genes: (A) The five transcription factors with the most harvest-inducible genes amongst their known targets (Enrichment FDR <0.05). **(B)** Relative expression of possible harvest-regulatory TFs (log_2FC/soil) in Arabidopsis RNA-seq. **(C)** Transcription factors whose targets are significantly enriched (p<0.05) in genes showing the expression profiles of the relevant branch. Transcription factors are overlapped (where space allows) with, and share a border colour with, the branch that they relate to. Figure was created in DREM 2.0, with transcription factor target information from Agris (agris-knowledgebase.org). **(D)** Predicted TF binding site combinations in harvest-inducible genes (analysed in PMET, Benjamini-Hochberg corrected p value<0.01); blue scale shows #genes from the harvest-inducible gene list which have the putative regulation; dark grey shows no significant TF pairing.

Specific combinations of different TFs can control gene expression and provide additional specificity (Rich-Griffin et al., 2020). In order to assess if specific TFs work in concert to upregulate harvest-inducible genes, enrichment for combinations of TF binding sites was assessed using the PMET program (Rich-Griffin et al., 2020). The most significantly enriched combinations of motifs were for the jasmonic acid (JA)-responsive MYC2/3/4 transcription factors together with ANAC58, which was found in 12% of the harvest-inducible genes (Figure 5.2D). MYC2/3/4 are basic helix-loop-helix (bHLH) TFs that associate with the G-box motif (Boter et al., 2004; Fernández-Calvo et al., 2011), while ANAC58 has not been extensively characterised, but is predicted to regulate genes involved in chlorophyll catabolism and was shown to bind to an extended ABRE motif (O'Malley et al., 2016). Taken together, these collective analyses suggest that a variety of TFs may be responsible for upregulating harvest-inducible genes. The G-box and ABRE element emerge as key CREs associated with sustained harvest-inducible gene expression. However, no consistent TF combinations or

patterns were identified that explain why harvest-inducible genes exhibit high and sustained expression profiles in post-harvest tissues.

5.2.2 Identification of five promoters with highly specific responsiveness to harvest

Synthetic promoters of multiple G-box and ABRE motifs have been characterised (Liu et al., 2016; Shen and Ho, 1995): although strength of upregulation increases with copy number of CREs, the background level of expression also increases (Rushton et al., 2002). Our harvest-inducible system requires high expression throughout the post-harvest period but also minimal expression on soil. Therefore, selecting the most suitable endogenous promoters from our harvest-induced gene set would ensure that CREs responsible for on-soil repression as well as post-harvest upregulation would be included.

The post-harvest RNA-seq dataset used to identify our original set of harvest-inducible genes captured mRNA levels in the leaves of adult plants with no additional abiotic nor biotic stress applied. Therefore, a number of different transcriptomic datasets were consulted to eliminate from our 120 harvest-inducible candidate genes those with potential undesirable upregulation on soil or downregulation post-harvest. Harvested plants are subject to pathogen infection in the post-harvest period; a desirable harvest-inducible gene should not show down-regulation in response to pathogen infection. The shortlist of harvest-inducible gene candidates was therefore filtered to remove 66 genes whose expression was reduced post-harvest by *Botrytis cinerea* infection (See Chapter 3, Figure 3.10C) (Figure 5.3A). In order to eliminate

genes with unwanted on-soil upregulation, expression patterns across >350 expression datasets were visualised using ePlant (Fucile et al., 2011). Candidate genes were eliminated if expression was $\log_2$FC>2 in developmental stages pre-harvest, such as in the hypocotyl and rosette leaf 1 (Figure 5.3B-C), or in roots.

Genes were further filtered to remove those strongly ($\log_2$FC>2) upregulated by multiple abiotic stresses that could be experienced by plants on-soil, such as cold, heat and drought (Figure 5.3C). Overall, these screening methods resulted in a short list of seven harvest-inducible candidate genes.

The levels of mRNA in a cell are not only dictated by the promoter-driven transcription of a gene, but also by the stability of the mRNA (Narsai et al., 2007). The mRNA stability can be affected by expression of trans-acting small interfering RNAs (tasi-RNAs), which are short RNA sequences that bind to other mRNA transcripts and facilitate their degradation (Rajagopalan et al., 2006). One gene from the shortlist of harvest-inducible genes, *PAP2/MYB90* (*AT1G6630*), is negatively regulated by *TRANS-ACTING SIGNALLING RNA4* (*TAS4*) (Yang et al., 2013). Thus, the *PAP2* promoter is unlikely to provide harvest-specificity, so this gene was removed from the candidate list.

After screening, the 1 kb promoter regions of the remaining six harvest-inducible genes were examined. *NADP-ME1* (*AT2G19900*) was eliminated owing to its short upstream intergenic sequence (<50 bp), which made it difficult to predict the location of CREs.

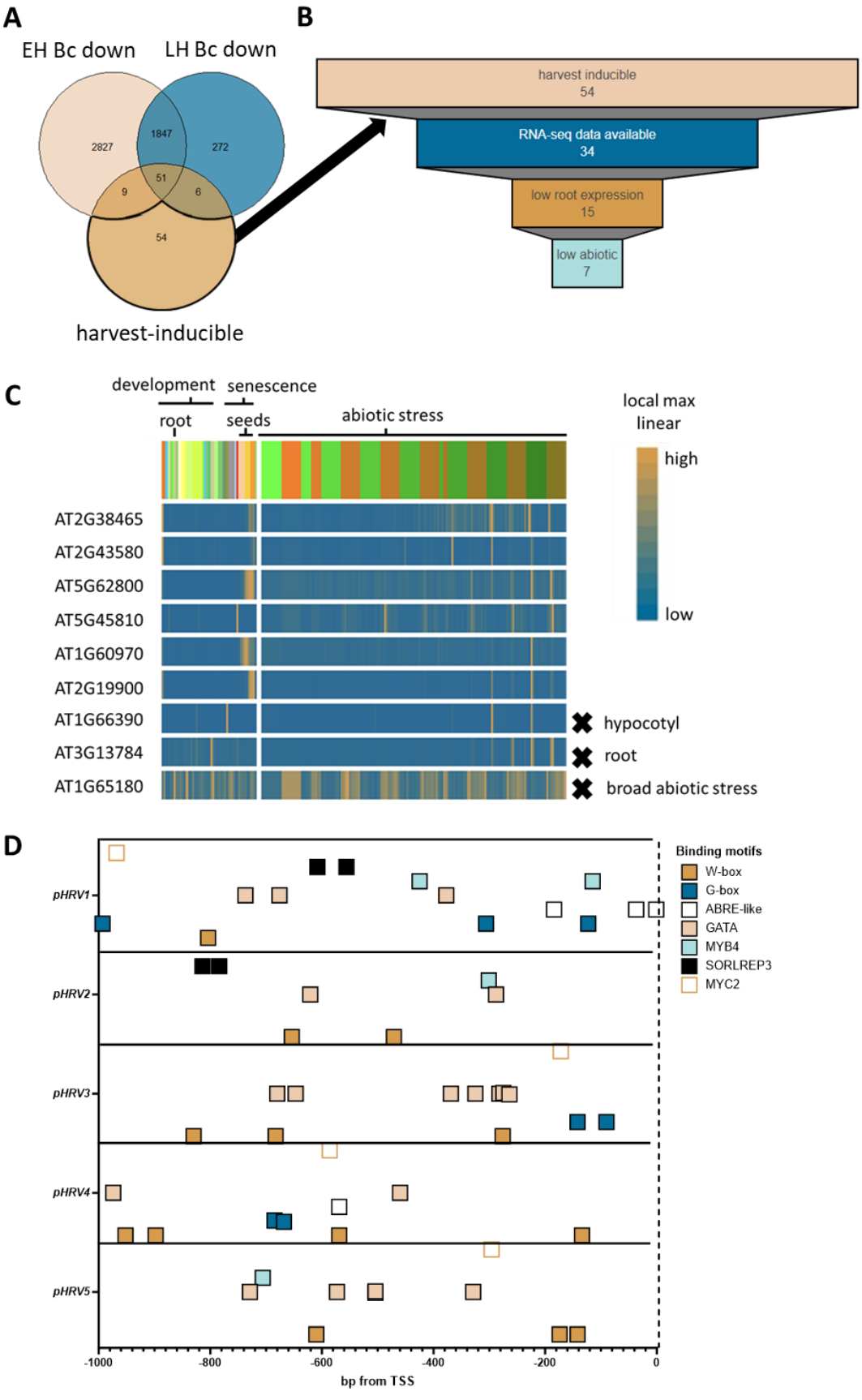

A
EH Bc down
LH Bc down
2827
1847
272
51
9
6
54
harvest-inducible
B
harvest inducible
54
RNA-seq data available
34
low root expression
15
low abiotic
7
C
development
senescence
root
seeds
abiotic stress
local max
linear
high
low
AT2G38465
AT2G43580
AT5G62800
AT5G45810
AT1G60970
AT2G19900
AT1G66390
AT3G13784
AT1G65180
hypocotyl
root
broad abiotic stress
D
Binding motifs
W-box
G-box
ABRE-like
GATA
MYB4
SORLREP3
MYC2
pHRV1
pHRV2
pHRV3
pHRV4
pHRV5
-1000
-800
-600
-400
-200
0
bp from TSS

Figure 5.3 (previous page): Filtering endogenous harvest-inducible genes for highly-specific harvest-responsive promoters: (A) Elimination of 66/120 candidate harvest-inducible genes that are downregulated in harvested Arabidopsis 2 days post-infection with *Botrytis cinerea* (EH and LH are pre- and post-harvest infection respectively) ($log_2FC<-1$, one way ANOVA $p< 0.05$, Benjamini-Hochberg multiple hypothesis testing correction). **(B)** Filtering process of the remaining 54 candidate harvest-inducible genes to identify those with known low expression in roots and developing shoots, and low broad-spectrum abiotic stress responses using data from ePlant (https://bar.utoronto.ca/eplant/). **(C)** Expression of candidate harvest-inducible genes in known RNA-seq datasets for development and abiotic stress, adapted from ePlant; the heatmap shows the genes which passed this screening stage (top 7) and three examples of eliminated genes (black cross), along with their associated upregulation, e.g. high expression in roots, or upregulation by multiple abiotic stresses. **(D)** Potential regulatory motifs in the 1 kb promoter sequences upstream of the putative TSS of the five top candidate harvest-inducible genes. Boxes show the start location of the sequences as identified in AgrisDB (Yilmaz et al., 2011); promoter sequence based on Araport 11 annotation, TAIR10 release (Lamesch et al., 2012).

promoter	Locus (TAIR10)	name	Annotation	predicted TSS
pHRV1	AT2G38465	-	unannotated	Chr2: 16106935 (-)
pHRV2	AT2G43580	-	Chitinase	Chr2: 18080094 (-)
pHRV3	AT5G62800	-	E3 ligase	Chr5: 25218634 (+)
pHRV4	AT5G45810	*CIPK-19*	CBL-interacting serine/threonine-protein kinase 19	Chr5: 18584676 (+)
pHRV5	AT1G60970	-	SNARE-like family protein	Chr1: 22449536 (-)

Table 5.3: Features of the harvest-inducible promoters used in this study (+) and (-) denote directionality of the sequence. Full sequences of promoters in Appendix G.

The remaining five harvest-inducible gene promoters were labelled *pHARVEST 1-5* (*pHRV1-5*) (Table 5.3). None of these showed significant (z-score>3) co-expression with each other (Figure 5.1C). The main predicted regulatory motifs in these 1 kb promoter sequences were G-boxes, WRKY-TF binding W-boxes, and GATA-binding motifs (Figure 5.3D), but no distinctive pattern of CREs was evident.

The specificity and activity of promoter sequences can be tested *in planta* by utilising them to drive a reporter gene (Hou et al., 2012). In order to characterise the harvest-inducible promoters, 1 kb fragments upstream of the putative TSS of *pHRV1-5* were fused to the *sGFP* coding sequence in the Gateway destination vector *pGWB4* (Nakagawa et al., 2007). *pHRV4* could not be amplified, so cloning continued with *pHRV1-3,5*. Subsequently, *Agrobacterium tumefaciens* strain GV3101 was used for transient and stable expression of the *pHRVx::sGFP* transgenes in Arabidopsis.

5.2.3 Harvest-inducible promoters drive post-harvest accumulation of GFP reporter

To assess the activity of the harvest-inducible promoters independent of chromatin context, Agrobacterium containing the cloned *pHRVx::sGFP* plasmids were infiltrated into leaves of *efr* mutant plants. These mutants are more amenable to transient expression assays than WT plants (Zipfel et al., 2006). As the number of transformed cells varies between infiltrations, mRNA levels of *GFP* were measured relative to the expression of the kanamycin resistance gene (*KANR*) - which resides adjacent to *GFP* in the *pGWB4* vector - or the *UBQ5* housekeeping gene. Preliminary data suggests that *GFP* expression driven by *pHRV3* showed the largest fold change (~4.8x) relative to the on-soil control, while *pHRV1 and pHRV5* drove 3.7 and 2.6-fold upregulation, respectively (Figure 5.4A-B). These data demonstrate that harvest-inducible promoters can drive gene expression upon harvest even when expressed transiently.

Next, stably transformed plants were generated for *pHRV1-3::sGFP* constructs. Preliminary data for *pHRV1::sGFP* revealed that two out of three independent transformants exhibited harvest-inducible *GFP* expression (Figure 5.4C). Although there was some variation between independent transformants likely due to positional insertion effects, these experiments further corroborate that *pHRVx* promoters drive harvest-induced gene expression.

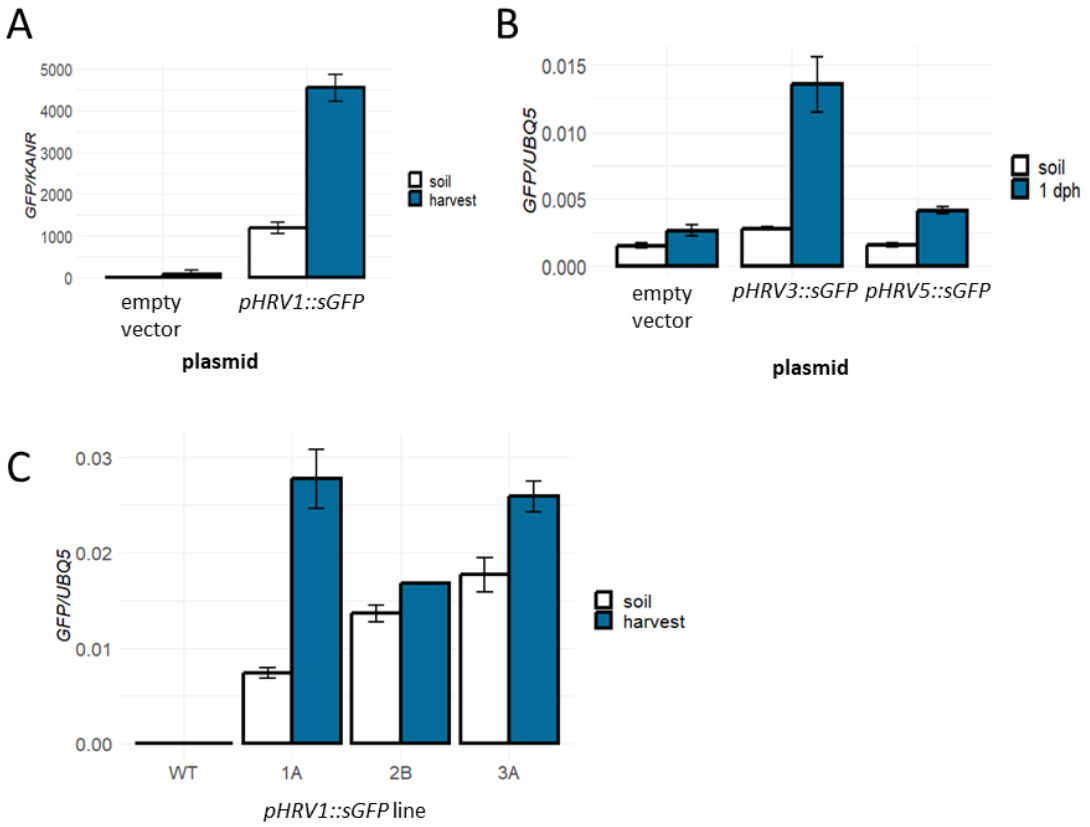


Figure 5.4: Harvest-inducible promoters drive upregulation of expression in post-harvest tissues. qRT-PCR data of transiently expressed **(A)** *pHRV1::GFP, GFP* mRNA relative to *KANR* (to control for the extent of transient expression) [N=1] and **(B)** *pHRV3::sGFP* and *pHRV5::sGFP* [N=2] relative to *UBQ5* housekeeping gene [NB. *KANR* levels were too low in these samples to use as a control]. Plasmids were transiently expressed in Arabidopsis *efr* mutant (error bars show 95% confidence interval of technical error), with plants harvested one day after infiltration, and one day prior to sample collection. **(C**) mRNA levels of *GFP* relative to the *UBQ5* housekeeping gene in *pHRV1::sGFP* independently transformed lines 1A, 2B and 3A on soil or 2 dph (N=1).

To examine if *pHRVx* driven expression of *GFP* also results in substantial GFP protein accumulation, protein was extracted from *pHRVx::sGFP* lines and visualised by western blotting. One of the *pHRV1::sGFP* lines showed greater accumulation of protein in the harvested tissue compared to on-soil plants (Figure 5.5A). All three *pHRV2::sGFP* lines had accumulation of GFP protein post-harvest relative to soil (Figures 5.5A-E), but the increase between harvest and soil was not striking. The *pHRV3::sGFP* lines showed low levels of GFP protein on soil, and a high level of protein after harvest (Figures 5.5B-E). Taken together, these data show that endogenous harvest-inducible promoters can be used to drive protein accumulation in harvested tissues, and that *pHRV3* shows the most promise for future bioengineering applications.

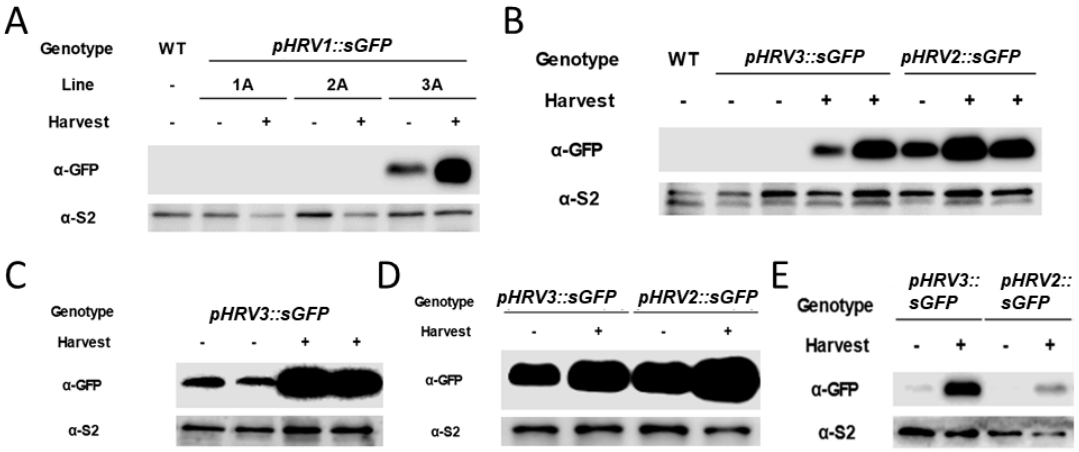


Figure 5.5 Harvest-inducible promoters drive GFP protein accumulation. Levels of sGFP protein in three stably transformed lines of **(A)** *pHRV1::sGFP*, (**B-E**) *pHRV2::sGFP* and *pHRV3::sGFP*; α-S2 was used as a loading control.

5.2.4 Harvest-inducible promoter candidates in brassica crops

Having demonstrated in the Arabidopsis model system that suitable promoters can be identified from transcriptomic datasets to drive protein accumulation in a harvest-responsive manner, the same strategy was applied to brassica crops. Although Arabidopsis and brassica crops are closely related, it is unlikely that the direct use of Arabidopsis *pHRV1-5* or their brassica orthologues would be optimal as indicated by diverse crop-specific post-harvest transcriptomes (Chapter 4). In addition, many Arabidopsis homologues exist as duplicates or triplicates in the *Brassica oleracea* genome, with considerable sub-functionalisation (Town et al., 2006). Consequently, here I used variety-specific transcriptomic datasets to identify harvest-responsive promoter candidates.

Pointed cabbage (*Brassica oleracea* var. *capitata*) has a shelf-life of a few weeks, but in optimal storage conditions can be stored for months (Janssens et al., 2022). In order to identify genes that are upregulated throughout the potentially long storage life of cabbage, I sought harvest-inducible genes in the cabbage RNA-seq dataset covering 70 days post-harvest (Chapter 4). I identified 93 genes with low expression on soil (normalised gene expression<5 at t=0) that were also upregulated at all post-harvest timepoints ($log_2FC>1/t=0$) (Figure 5.6A). Because our cabbage post-harvest dataset does not contain information on earlier points of development, I used published transcriptomic datasets of cabbage at different developmental stages to filter out genes with undesirable expression during on-soil growth (Kim et al., 2014, interrogated through NCBI). Genes with high gene expression (raw read count >100)

at the 9-day-old seedling stage were eliminated. Subsequently, in order to select genes that showed rapid strong upregulation after harvest, I selected the ten harvest-inducible genes with the largest fold-change 10 dph compared to 0 dph (one way ANOVA, $p<0.05$) (Table 5.4). To identify potential CREs involved in the harvest-inducibility of these shortlisted cabbage genes, I analysed their promoter regions up to 1 kb upstream of the putative TSS. The most frequent, known CREs found in these promoter regions were W-boxes for WRKY TF binding, T-box motifs (ACTTTG), and ABRE-like binding sites. The *AtRAF7* homologue had six copies of the binding site for cell cycle-related TF E2F (de Veylder et al., 2007), which is otherwise absent in the other nine candidate promoters (Figure 5.6B). These analyses suggest that even over the course of longer post-harvest storage, harvest-inducible candidate promoters can be identified in pointed cabbage, and that WRKY TFs may be particularly involved in their regulation.

***Brassica oleracea* id**	**Arabidopsis orthologue**	**At gene name**	**Notes**
XP_013601487.1	none		
XP_013618501.1	AT5G13170	*SAG29*	sugar transporter, senescence-associated gene
XP_013619795.1	AT1G68500		
XP_013597029.1	AT4G33450	*MYB69*	MYB-like TF
XP_013624140.1	AT3G06620	*RAF7*	probable serine/threonine-protein kinase
XP_013627317.1	AT3G20960	*CYP705A33*	cytochrome P450 family 705
XP_013585144.1	AT1G07645	*DSI-1VOC*	Mb0911c-like
XP_013614176.1	AT3G52560	*UEV1D*	VQ motif-containing protein 25-like
XP_013614737.1	none		
XP_013604026.1	AT1G62800	*ASP4*	*ASPARTARE AMINOSTRANSFERASE 4*

Table 5.4: Top candidate harvest-inducible genes in cabbage (*Brassica oleracea* var. *capitata*), whose promoters have the potential to drive harvest-inducible expression of transgenes.

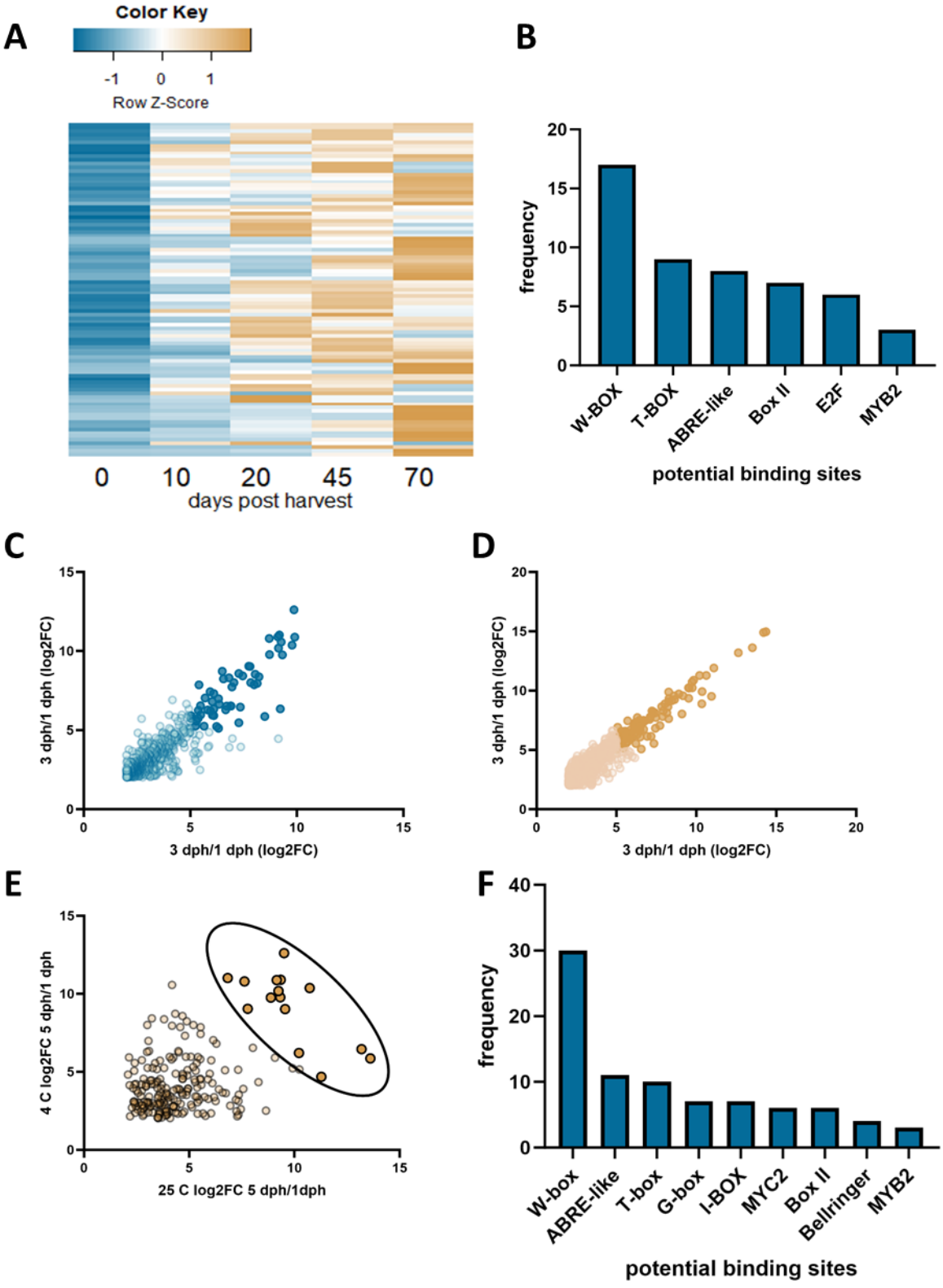
Broccoli (*Brassica oleracea* var. *italica*) has a shorter shelf-life than cabbage, and exhibited a similar post-harvest stress response to Arabidopsis (Chapter 4). As such, I investigated if harvest-inducible promoter candidates in broccoli were similar to their Arabidopsis homologues. For this purpose, I took advantage of a published post-harvest transcriptomic dataset of broccoli kept at room temperature (25°C) or in cold storage (4°C) over the course of five days (Figure 5.6C-D) (Ahlawat et al., 2022). In order to identify harvest-inducible genes that were activated regardless of the storage temperature, I selected genes that were strongly upregulated ($log_2FC>5$ relative to 1 dph, $p<0.05$)) at 3 and 5 dph, and at both 4°C and 25°C (Figure 5.6C-D).

***Brassica oleracea* id**	**Arabidopsis orthologue**	**At gene name**	**Notes**
XP_013626600.1	AT3G15500	*NAC03*	TF that binds to CATGTG motif; induced by salinity, drought, ABA and JA, but not cold.
XP_013593922.1	AT3G51750	-	unknown protein
XP_013590858.1	AT5G44430	*PDF1.2C*	predicted anti-fungal peptide (Wang et al., 2019)
XP_013599792.1	AT1G15040	*GAT1_2.1*	Class I glutamine amidotransferase-like superfamily protein
XP_013622119.1	AT3G09390	*MT2A*	-
XP_013597029.1	AT4G33450	*MYB69*	myb domain protein 69 (*MYB69*); TF
XP_013627597.1	AT4G09600	*GASA3*	GAST1 protein homolog 3 (*GASA3*)
XP_013609988.1	AT5G06760	*LEA46*	*LATE EMBRYOGENESIS ABUNDANT 4-5* (*LEA4-5*)
XP_013618822.1	AT1G80160	-	-
XP_013600848.1	AT1G27110	-	Tetratricopeptide repeat (TPR)-like superfamily protein

Table 5.5 Top harvest-inducible candidate genes in broccoli (*Brassica oleracea* var. *italica*), whose promoters have the potential to drive harvest-inducible expression of transgenes.

Of this subset of genes, I selected 15 genes with the highest combined upregulation at 4°C and 25°C (Figure 5.6D). To further eliminate genes that would be expressed earlier during growth on soil, I interrogated a published broccoli RNA-seq developmental dataset (Gao et al., 2014). Consequently, a further five genes that were upregulated in the early seedling stage (11 days post-germination $log_2FC>2$) were removed. This resulted in a list of ten broccoli genes whose promoters are candidates for harvest-inducible gene upregulation (Table 5.5). To identify potential CREs involved in the harvest-inducibility of the ten shortlisted broccoli genes, I analysed their promoter regions 1 kb upstream of the putative TSS. Similar to cabbage, the most frequent, known CREs in the promoter regions were W-boxes (C/TTGACT/C) for WRKY TF binding, ABRE-like binding sites and T-box motifs (Figure 5.6F). These data suggest that WRKY TFs may play an important role in both broccoli and cabbage post-harvest gene regulation.

Although broccoli and pointed cabbage have different stress responses to harvest (Chapter 4) and different shelf-lives, they belong to the same plant species, and are therefore closely related. To identify any potential shared harvest-inducible promoters between the two varieties, I compared the lists of candidate promoters from both crops (Table 5.4 & 5.5). Intriguingly, the orthologue of *MYB DOMAIN PROTEIN69* (*MYB69*) was a harvest-inducible candidate in both varieties. This suggests there may be scope for using similar harvest-inducible promoters between different crop varieties.


A
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T-BOX
ABRE-like
Box II
E2F
MYB2
potential binding sites
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3 dph/1 dph (log2FC)
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10
0
W-box
ABRE-like
T-box
G-box
I-BOX
MYC2
Box II
Bellringer
MYB2
potential binding sites

Figure 5.6 (previous page) Identification of candidate harvest-inducible promoters in brassica crops (**A**) Genes from the *Brassica oleracea* var. *capitata* (pointed cabbage) RNA-seq dataset that show log_2FC>2 at all post-harvest timepoints relative to t=-0, with normalised expression at t=0 < 5; data is scaled for each row. **(B)** The most common TF binding sites in cabbage (*Brassica oleracea* var. capitata) harvest-inducible candidate gene promoters within 1 kb upstream of the putative TSS; T-box motif is ACTTTG; Box II motif is GGTTAA; E2F motif is TTTCCCGC; and MYB2 motif is TAACTGGTT (from Agris.org) (**C-E**) Upregulated genes in broccoli (*Brassica oleracea* var. *italica*) log_2FC >2 at 3 and 5 dph relative to 1 dph at 4°C **(C)** and at 25°C **(D)** (Ahlawat et al., 2022) (solid-filled points show genes with log_2FC>5 at both timepoints). **(E)** Broccoli genes with the highest log_2FC at 5 dph for both 4°C and 25°C were selected (inside black oval). **(E)** The most common TF binding sites in broccoli gene promoters within 1 kb upstream of the putative TSS; I-BOX motif is GATAAG; Bellringer is AAATTAAA.

5.3 DISCUSSION

The aim of this study was to identify harvest-inducible genes in leafy brassicas that could drive post-harvest accumulation of a protein of choice, using both the model brassica, *Arabidopsis thaliana*, as a proof of concept, as well as agriculturally relevant brassica crops. In order to understand the CREs controlling post-harvest expression, transcriptomic data was used to identify genes in Arabidopsis that have high expression throughout the post-harvest period, and a low expression on soil (Figure 5.1B). The promoter sequences of these genes were enriched in the G-box (CACGTG) and ABRE (ACGT) motifs (Figure 5.1D-G), as well as a variety of ABA-responsive TFs associated with regulation of the harvest-induced genes (Figure 5.2). In this chapter I show that harvest-inducible promoters are able to drive the expression and protein accumulation of a *GFP* reporter gene (Figures 5.4 & 5.5). Moreover, a similar harvest-inducible promoter identification strategy was applied to transcriptomic datasets

derived from pointed cabbage and broccoli (Figure 5.6), enabling the identification of candidate promoters for future post-harvest crop improvement strategies.

Analysis of the promoters of harvest-inducible genes in Arabidopsis suggested that a variety of TFs were involved in controlling the harvest response. Multiple methods of CRE analysis highlighted the likely role of ABREs and G-box motifs (Figure 5.1D-G). These motifs are known to be bound by bHLH, bZIP and NAM/ATAF1/CUC2 (NAC) TFs, which make up 9.5%, 4.8% and 5.3% of all Arabidopsis TFs, respectively (Toledo-Ortiz et al., 2003; Jakoby et al., 2002; Qu and Zhu, 2006). Indeed, TFs with targets enriched in the harvest-inducible gene dataset all bind directly or indirectly to the ABRE and G-box motifs; these include PIF3, LEC1, ABF1/4 and the MYC2-ANAC058 combination (Figure 5.2 & Table 5.2). PIF3 is a light-responsive TF that controls expression of genes related to anthocyanin biosynthesis and binds to G-box motifs in their promoters (Shin et al., 2007). LEC1, MYC2 and ABF1/4 all regulate expression in response to changes in cellular ABA levels and interact directly or indirectly with ABREs (Yamamoto et al., 2009; Choi et al., 2000). No specific TFs were found to regulate more than 20% of the harvest-inducible genes (Figure 5.2A), and co-expression was generally low (Figure 5.1C). It is therefore likely that there is no single pathway responsible for the regulation of harvest-inducible genes in Arabidopsis.

Previous studies that created synthetic inducible promoters consisting of ABRE and G-box motifs - 6 x ABRE (Wu et al., 2018) or 3 x WRKY + G-box motifs (Liu et al., 2016) - have seen strong upregulation on induction, but also residual expression in control conditions, suggesting that it would be difficult to prevent mis-expression on

soil using a synthetic promoter consisting solely of G-box/ABRE motifs without further regulatory elements. New emerging technologies and bioinformatic tools continue to reveal the entire functional cistrome of Arabidopsis, such as sequential extraction assisted-active TF identification (sea-ATI) (Wen et al., 2023) and integrative Regulatory Network (iRegNet) (Shim et al., 2021), which could allow future development of a synthetic harvest-inducible promoter. Currently, target binding motifs have only been characterised for ~30% of known transcription factors (O'Malley et al., 2016), so it is possible that additional new TFs involved in harvest-inducible expression could be identified as more datasets become available. However, with the current data available, utilising an endogenous promoter has the greatest potential to provide specific harvest-inducible gene expression.

Endogenous promoters were selected from 1 kb upstream of the predicted TSS of the harvest-upregulated genes from the Arabidopsis RNA-seq dataset. Most CREs are predicted to lie in the first 500 bp upstream of the TSS (Korkuć et al., 2014), but there is growing evidence of cis-regulatory elements outwith the upstream sequence: e.g., some exceptional enhancers were identified >1.5 Mb upstream of the TSS (Wang et al., 2017). Thus, by using 1 kb upstream of the TSS, the majority of relevant regulatory motifs and enhancers should have been included in harvest-responsive candidate promoters.

Indeed, generation of *pHRVx::sGFP* constructs demonstrated that the selected harvest-responsive promoters can drive harvest-inducible gene expression and protein accumulation in leaf tissues (Figure 5.4 & 5.5). Further mRNA data is needed to

confirm harvest-induced changes in gene expression in the stably transformed *pHRVx::sGFP* lines, and time courses over extended post-harvest periods would provide further useful data on the transcriptional dynamics. Transgenic lines should also be tested in combination with abiotic and biotic factors that plants are likely to experience either on soil, such as pathogen infection and high UV, or post-harvest, such as dark, cold and high humidity.

Gene	Post-harvest benefit	Reference of transgenic lines
isopentenyl transferase (IPT) **)(Agrobacterium)**	Reduced senescence; increased resistance to *Botrytis cinerea*	Gan and Amasino, 1995; Swartzberg et al., 2008
MYB15	Increased disease resistance (through lignin deposition)	Kim et al., 2020
MYB75	Improved nutritional content (flavonoids)	Kreynes et al., 2020
WRKY33	Increased early pathogen defence and resistance to necrotrophs	Zheng et al., 2006
PR1	Increased defence against biotrophic pathogens	Fang et al., 2019

Table 5.6 Candidate genes for harvest-inducible expression.

While most *pHRV::sGFP* lines showed increased protein accumulation after harvest, some lines also showed undesirable levels of GFP protein whilst growing on soil (Figure 5.5). Repressive complexes are rarely able to maintain complete binding to promoter elements, and this is often the cause of leaky expression in inducible systems (Anthony et al., 2004). To reduce this on-soil expression, multiple *pHRV*s could be used

in combination in synthetic gene circuits (Lloyd et al., 2022), requiring two or more active *pHRV*s to control expression of the target gene. The low co-expression between *HRV1-5* genes may allow for increased specificity in a harvest-responsive gene circuit, and reduce the likelihood of developmental or stress-related expression on soil. The utility of these harvest-inducible promoters should also be tested by driving target genes with quantifiable and beneficial post-harvest phenotypes, such as reduced senescence or increased disease resistance (Table 5.6).

In addition to Arabidopsis, this study identified potential harvest-inducible promoters in the leafy brassica crops, broccoli (*Brassica oleracea* var. *italica*) and cabbage (*Brassica oleracea* var. *capitata*) (Figure 5.6, Tables 5.4 & 5.5). Even over the course of 70 days post-harvest in cabbage, there were multiple genes that were upregulated at all post-harvest timepoints relative to those on soil (Figure 5.6A). The known CREs most upregulated in the shortlist of broccoli and cabbage candidate harvest-inducible genes were binding sites for WRKY TFs, and ABRE and T-box motifs (Figures 5.6B & 5.6F). It is difficult to assess whether they are significantly enriched due to the lack of genome-wide information about frequency of CREs in *Brassica oleracea* promoters. There are ABRE motifs found in harvest-induced gene promoters in both brassica crops and Arabidopsis; however, the G-box motif, which was the most enriched CRE in the selected Arabidopsis promoters, was not commonly found in the brassica crop promoters. Equally, WRKY TF binding sites were the most common motif in the top harvest-induced genes for cabbage and broccoli, but were not significantly enriched in the longlist of candidate Arabidopsis promoters (Figure 5.1E), although

multiple copies were present in the promoters of *pHRV1-5* (Figure 5.3D). WRKY TFs are broadly associated with regulating immunity, growth and development (Song et al., 2023). There are estimated to be 150 WRKY TFs in cabbage (Yang et al., 2022), very few of which have been characterised, although some are responsive to ABA and drought stress (Yang et al., 2022; Rushton et al., 2011). Some WRKY TFs in broccoli are upregulated in advance of post-harvest degreening (Luo et al., 2019). It would be interesting to identify which WRKY TFs are specifically involved in post-harvest upregulation in brassica crops.

There are fewer transcriptomic datasets and resources available for *B. oleracea* compared to Arabidopsis, making filtering of candidate genes and analysis of promoters more challenging. The ever-increasing number of genomic resources for brassica crops will hopefully make it possible to identify CREs and TFs responsible for harvest-responsive upregulation. The benefits of post-harvest gene induction in brassicas are evident from the transgenic lines expressing cytokinin biosynthesis enzyme *isopentenyltransferase* (*IPT)* driven by senescence gene promoters (Gan and Amasino, 1995; Chan et al., 2009). However, harvest-inducible promoters have the potential to upregulate genes more quickly post-harvest relative to senescence promoters.

This study demonstrates that robust endogenous gene promoters can be used to drive harvest-inducible gene expression and protein accumulation in Arabidopsis. There is considerable scope for future bio-engineering work to develop harvest-

inducible circuits that drive desirable traits in brassica crops to improve their post-harvest health and longevity.

Chapter 6 Discussion and Conclusion

Harvested rosettes are living tissues capable of interacting with their environment. It is remarkable that, despite the removal of the roots that supply nutrients and water, leafy brassicas, once harvested, can be stored for weeks and even months. Nevertheless, leafy vegetables face the risk of being discarded by retailers or consumers if crop quality deteriorates post-harvest as a result of disease, wilting or senescence-related chlorosis and odours (Barrett et al., 2010). Food loss and waste are particularly prevalent in crops that are harvested during their actively growing stage, such as broccoli and rocket, as their storage life is relatively short. Even small increases in their storability could significantly reduce the volume of food discarded. Consequently, the overarching aim of this study was to identify specific processes and pathways unique to harvested leafy brassica tissues that could be beneficially upregulated, and to use bioengineering principles to design a novel strategy to induce traits in harvested crops to improve post-harvest quality and reduce food waste.

In order to identify target traits for improving post-harvest quality in leafy brassicas, in Chapter 3 and 4 I used *Arabidopsis thaliana* to identify changes in the transcriptome and immune responses of harvested tissues. I combined these data with new and existing datasets in agriculturally-relevant leafy brassicas (pointed cabbage, broccoli and rocket) to identify whether there was one common or multiple distinct leafy brassica post-harvest transcriptomes. The transcriptomic and immunity-related analyses highlighted pathways that would benefit from post-harvest

enhancement. I further used the post-harvest transcriptomic datasets in Chapter 5 to identify candidate harvest-inducible promoters to drive post-harvest upregulation of desirable traits, and tested harvest-inducible expression in the model system.

6.1 Identifying target harvest-inducible traits in leafy brassicas

Harvested leafy brassicas are affected by both biotrophic and necrotrophic pathogens in post-harvest storage, which leads to a reduction in quality or saleable quantity of the crop. Despite this, research in leafy crops has largely focused on abiotic stress responses and storage conditions, and limited information was available regarding their post-harvest immune pathways. The challenges of transforming brassica crop plants, and their long generation times led me to develop a novel model harvest system (Chapter 2.3), which could take advantage of the many transgenic lines and databases already available in Arabidopsis.

I had originally hypothesised that harvested tissues would be more susceptible to the necrotrophic fungus *Botrytis cinerea*, because host plant stress and senescence were known to trigger *B.cinerea* to cause necrotic damage (Shaw et al., 2016). Indeed, in Chapter 4 of this study, the harvested brassicas with shorter shelf-lives displayed these potential triggers: they had upregulated abiotic stress responses, particularly to osmotic stress and nutrient starvation, and downregulated growth and photosynthetic genes, which are common features of senescing tissues (Watanabe et al., 2010) (Figures 4.10-11). In addition, repression of ABA levels is needed for early-stage

resistance to *B.cinerea* infection (Windram et al., 2012; Liu et al., 2015), and ABA levels are expected to be elevated post-harvest. Some strains of *B.cinerea* produce ABA, but its role in virulence has not been elucidated (Lievens et al., 2017). However, the post-harvest ABA, stress and senescence in Arabidopsis did not lead to the expected higher levels of necrosis and fungal growth in the leaf tissues (Figure 3.10). On the contrary, the fungal growth was reduced (Figure 3.10B), and it was the hemi-biotrophic pathogen, *Pseudomonas syringae,* that showed greater replication in harvested tissues (Figure 3.6B). In order to better understand the post-harvest changes in immune pathways underlying these changes in disease resistance, and to identify targets for harvest-inducible enhancement, I used the Arabidopsis model system to interrogate the components of pattern-triggered immunity (PTI) and immune hormone pathways. I found that harvest impacted multiple aspects of immunity: downstream PTI responses and salicylic acid (SA) pathway activity were suppressed (Figure 3.2B; Figure 3.5A-B), whereas activity of the jasmonic acid (JA) pathway was enhanced (Figure 3.8) (Figure 6.1). The NPR1 transcriptional activator of the SA pathway did not accumulate in response to SA in harvested plants (Figure 3.4B). However, even when *NPR1* was overexpressed using *35S::NPR1-GFP* transgenic plants, and NPR1 protein levels were high in harvested plants, SA-responsive genes were still not induced by SA treatment in harvested rosettes (Figure 3.5C), and preliminary data suggests binding to the *PR1* promoter was reduced (Figure 3.4F). This suggests that NPR1 activity was also reduced.

There is abundant scope for further research in determining post-harvest changes in post-translational control of NPR1 and the SA pathway, particularly the potential regulatory role of abscisic acid (ABA). Further ChIP qRT-PCR analysis of the *35S::NPR1-GFP* (*npr1-1*) transgenic Arabidopsis line could be used to identify whether the NPR1 promoter is preferentially bound to repressive sites in SA-responsive gene promoters in harvested plants. Equally, this *NPR1-GFP* line could be used to compare SUMOylation levels of pulled down NPR1 protein in harvested and unharvested tissues to identify whether SUMOylation could be involved in regulating the post-harvest binding of NPR1 to its target promoters (Saleh et al., 2015). Testing levels of transgenic NPR1-GFP protein and SA-responsive gene expression in harvested ABA biosynthesis mutants, such as *aba3* (as used in Ding et al., 2016), with or without the addition of exogenous ABA, would clarify the role of ABA in the attenuation of the SA signalling pathway.

It was surprising, given how prominent ethylene (ET) is in post-harvest research and crop management (Martínez-Romero et al., 2007), that the ET-responsive pathway was little affected by harvest (Figure 3.9), and was not identified as a key differentially regulated pathway in the analysis of post-harvest leafy brassicas (Chapter 4). Although the quality of stored leafy brassica crops can be strongly affected by exogenous ET, such as being stored with high ET emitters like ripe tomatoes or avocados, they themselves produce little post-harvest ET endogenously (Brummel and Toivonen, 2018). One potential explanation is that harvest does not significantly alter the leaf response to ET. Alternatively, it is plausible that the difference between the response

of harvested and unharvested leaf tissue to ET only becomes apparent over a longer period than the 4-6 hours used in my assays, and may require a more sustained exposure to ET (Jing et al., 2005).

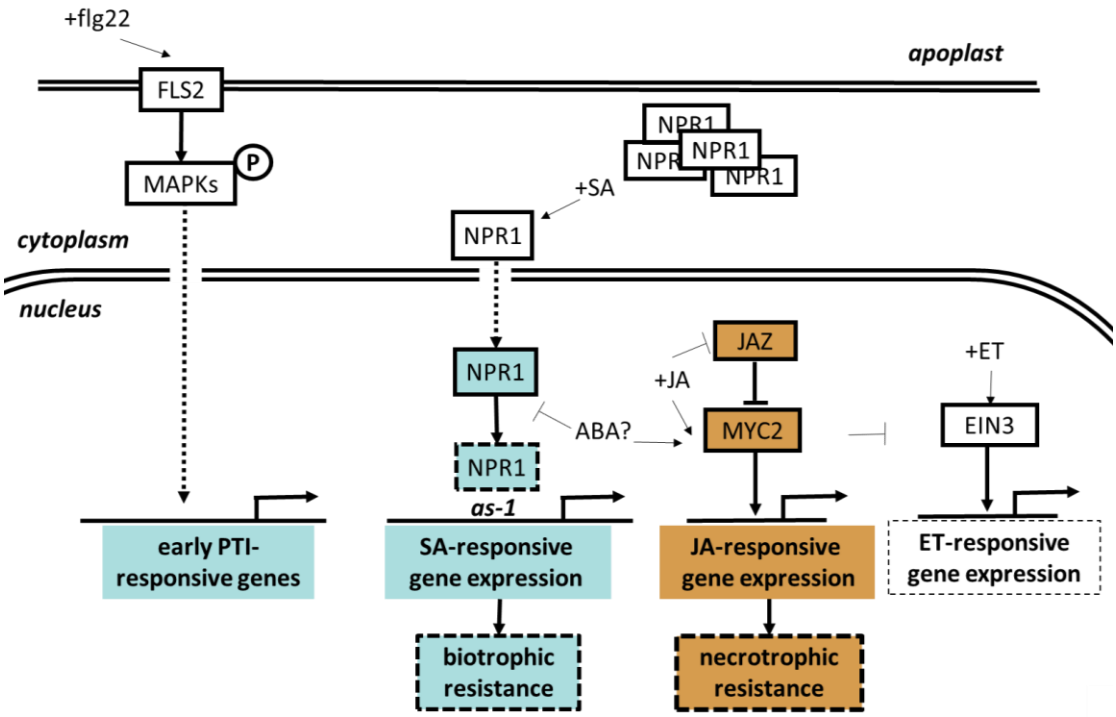

Figure 6.1: Post-harvest changes in Arabidopsis immune pathways: A summary schematic of the observed changes in Arabidopsis immunity (Chapter 3) after harvest; blue shows downregulation; orange shows upregulation; white shows no observed change; dotted line shows movement; dashed line shows preliminary data, or in the case of biotrophy and necrotrophy, evidence from one pathosystem; role of ABA is suggested.

In summary, my findings suggest that the PTI and immune hormone pathways in harvested tissues are markedly different from those on soil, and these differences are likely regulated by changes in protein activity as well as gene transcription; and bioengineering for post-harvest health would preferentially target early PTI-responsive

or NPR1-dependent genes to provide additional protection against biotrophic pathogens.

In parallel to post-harvest immunity assays and transcriptomics carried out in the model brassica *Arabidopsis thaliana*, experiments were replicated, where feasible, in pointed cabbage (Figure 4.12), and then compared with existing brassica post-harvest transcriptomes (Chapter 4).

The conditions in the Arabidopsis model system were carefully controlled for consistency between harvested and on-soil plants to identify changes caused specifically by harvest, rather than changes in temperature or light conditions. The storage in long-day conditions at 21°C is not, however, representative of most industrial post-harvest storage conditions (Tan et al., 2005), which the brassica crop datasets more closely reflect. As such, the model system should be considered an initial platform for understanding post-harvest biology of rosettes.

The combined study of post-harvest transcriptomes of both the model Arabidopsis system and leafy brassica crops highlighted the significant transcriptional changes that occur in harvested tissues. Over 30% of Arabidopsis genes and ~10% of the mapped cabbage genes were differentially expressed post-harvest (Figure 4.2 and 4.6C), emphasising the profound impact of the harvesting process. Post-harvest datasets had, on average, 50% more genes downregulated than upregulated (Figure 4.9A-B). Transcription and translation require energy and raw materials, which are limited in harvested plants, particularly those stored in the dark and unable to photosynthesise. Consequently, a general downregulation of gene expression might

be expected. However, substantial gene upregulation also took place in all post-harvest datasets (Figure 4.9A). Arabidopsis had upregulated genes related to osmotic stress, anthocyanin production and ABA pathways, and downregulation related to photosynthesis and growth (Figures 4.4A, Figure 6.2). These transcriptional changes were similar to those observed in nutrient-depletion-induced senescence (NuDIS) (Watanabe et al., 2010).

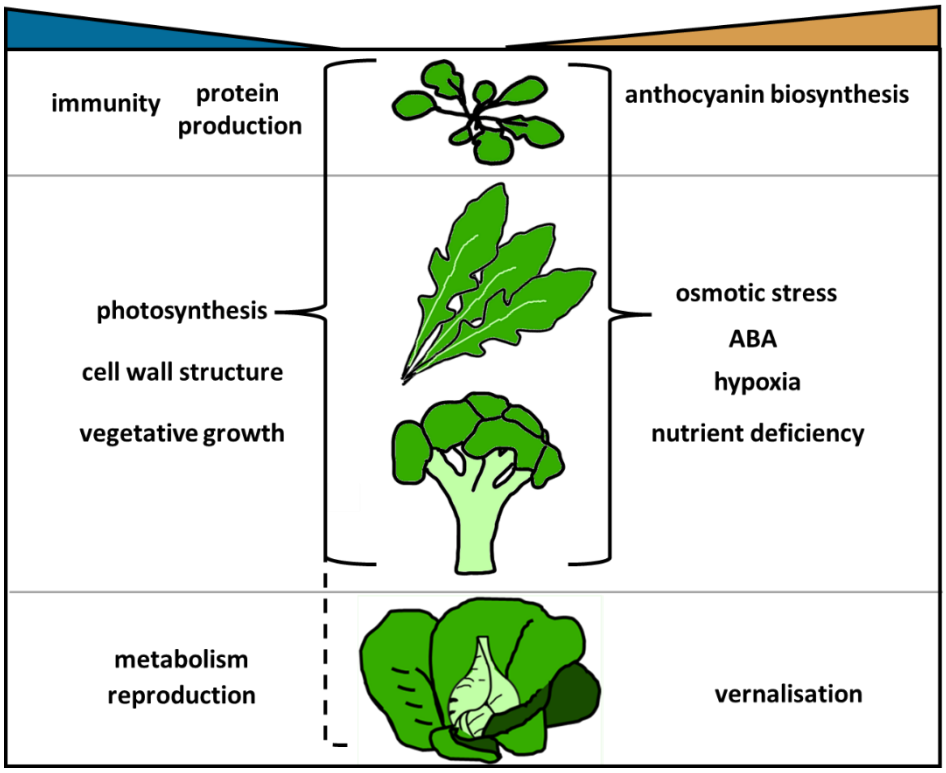

Figure 6.2: Post harvest responses in leafy brassicas: A summary of the post-harvest processes identified in Chapter 4 for (from top) Arabidopsis, rocket, broccoli and cabbage. Processes to the left are downregulated, and to the right are upregulated. The dotted line from cabbage shows the weaker association of cabbage with the shared processes.

Compared to Arabidopsis, brassica crops characterised by a short storage-life, such as broccoli and rocket, had similarly differentially regulated post-harvest processes (Figure 4.10-11), despite little similarity in expression of specific orthologues. This suggests that Arabidopsis has further value as a model for testing post-harvest responses in crops like broccoli and rocket.

There was a stark contrast between the post-harvest gene expression of short-storage leafy brassicas and that of longer-storage pointed cabbage. Abiotic stress responses were not significantly upregulated in the post-harvest cabbage transcriptome, which changed little over the course of 70 days (Figure 4.6D): metabolism and growth were downregulated, and vernalisation-related genes were upregulated (Figure 4.7). These results support the known changes in cabbage during dormancy and vernalisation, when respiration is significantly reduced, and development shifts away from vegetative growth (Kader and Saltveit, 2002; Schiessl et al., 2019; Woodhouse et al., 2021). The cabbages used in the post-harvest RNA-seq for this study were stored in the dark at 4°C; at higher temperatures, post-harvest cabbage has been observed to increase expression of senescence-associated genes (Ahlawat and Liu, 2021). Comparison of transcriptomic data from this study to the transcriptomes of cabbages maintained post-harvest in the light, or at higher temperatures, might show that the cold and dark storage in my study are suppressing senescence-associated gene expression and anthocyanin accumulation respectively.

Currently, the post-harvest transcriptome of Arabidopsis ecotype Col-0 shares little similarity with that of cabbage. One feature of Col-0 that makes it suitable for lab

work is its rapid cycling of generations, because it is a summer annual from central Europe with a short flowering time. It would be interesting to test the model harvest system using an Arabidopsis ecotype with a long flowering time, such as an overwintering Swedish accession (Stewart et al., 2015) that requires extended exposure to cold before flowering. It is possible that, after harvest, an overwintering accession would, like cabbage, upregulate vernalisation-related genes, and not abiotic stress-responsive genes. The thicker leaves of overwintering accessions (Stewart et al., 2015) may also reduce water loss during the post-harvest period, and extend post-harvest storability. In these ways, an overwintering accession could provide a closer Arabidopsis model system for longer storage leafy brassicas like cabbage, and expedite research into cabbage post-harvest health.

In conclusion, this study demonstrates that the act of harvesting exerts a profound effect on post-harvest gene expression and immunity. What is more, harvested tissues are differently susceptible to pathogens compared to their soil counterparts. Therefore, harvested plants require novel strategies tailored specifically to this shift in requirements, which could be induced after harvest.

6.2 Bioengineering for harvest-inducible traits in leafy brassicas

The specific needs of harvested tissues require a novel strategy to improve their health and quality that does not negatively affect yield or on-soil growth. Harvest-specific

induction of gene expression could allow for post-harvest enhancement or introduction of traits, either through use of a synthetic or endogenous harvest-responsive promoter. To design a method of inducing gene expression in a harvest-responsive way, in Chapter 5 I looked for the cis-regulatory elements (CREs) of genes that showed strong upregulation post-harvest, but low expression on soil. The G-box and ABRE motifs that were most enriched in the promoters of Arabidopsis harvest-responsive genes (Figure 5.1D-E) are often found in promoters of genes responsive to ABA, JA, abiotic stress or light (Shen and Ho, 1995; Chini et al., 2007; Jameel et al., 2020; Hudson and Quail, 2003). Synthetic promoters combining multiple copies of G-box and ABRE motifs have been shown to be highly activated by senescence, but also have high background levels in the absence of the inducing stress (Liu et al., 2016). This would make these forms of synthetic promoter unsuitable for post-harvest-specific upregulation without additional knowledge about the elements suppressing on-soil expression. To maximise the inclusion of unidentified CREs that could be suppressing expression on soil, I used endogenous promoters of genes that were strongly upregulated post-harvest. The promoters least likely to drive mis-expression on-soil were characterised using a GFP reporter (Figure 5.5 and 5.7). The results of this study indicate that harvest can be used as a signal to upregulate gene expression and protein accumulation in harvested rosettes.

The same strategy for designing and creating harvest-inducible genes could be applied to other leafy brassica crops. Analysis of the post-harvest brassica crop datasets offered multiple candidate harvest-inducible promoters in broccoli and

cabbage (Tables 5.4 and 5.5). Given the species/variety-specific post-harvest transcriptomes of leafy brassicas (Figure 4.9A-B), promoters specific to each crop are likely to be more effective in driving harvest-inducible expression. The reduced metabolic activity of harvested cabbage (Figure 4.8B) may impact on the efficiency of protein production in response to upregulated post-harvest increases in mRNA.

Residual background levels of mRNA expression and protein accumulation were found in the Arabidopsis harvest-inducible lines (Figures 5.5 and 5.7). There are a number of possible causes of this leaky expression. The harvest-inducible promoter activity may have been affected by CREs at the transgene insertion location (Anthony et al., 2004), particularly as the independent transgenic lines of each construct showed considerable variability in expression and protein levels. Additionally, studies in bacteria have shown that genes whose expression is regulated by recruitment of repressors to the promoter, rather than activator transcription factors, are more likely to have leaky gene expression in the absence of the induction signal (Siegele and Hu, 1997). Finally, post-transcriptional degradation, as proposed for *MYB90*, could have lowered the levels of on-soil mRNA in the RNA-seq dataset presented in this study, and masked the promoter-driven expression of the harvest-inducible promoters. *pHRV3* was the most-harvest specific promoter, with the least background expression on soil, and drove a strong accumulation of the GFP reporter protein (Figure 5.7B-E), so would be the strongest candidate for future harvest-inducible gene constructs. Further testing of the GFP reporter lines is required to identify additional stresses, such as

wounding, infection or high temperature, that could activate the expression of the harvest-inducible genes.

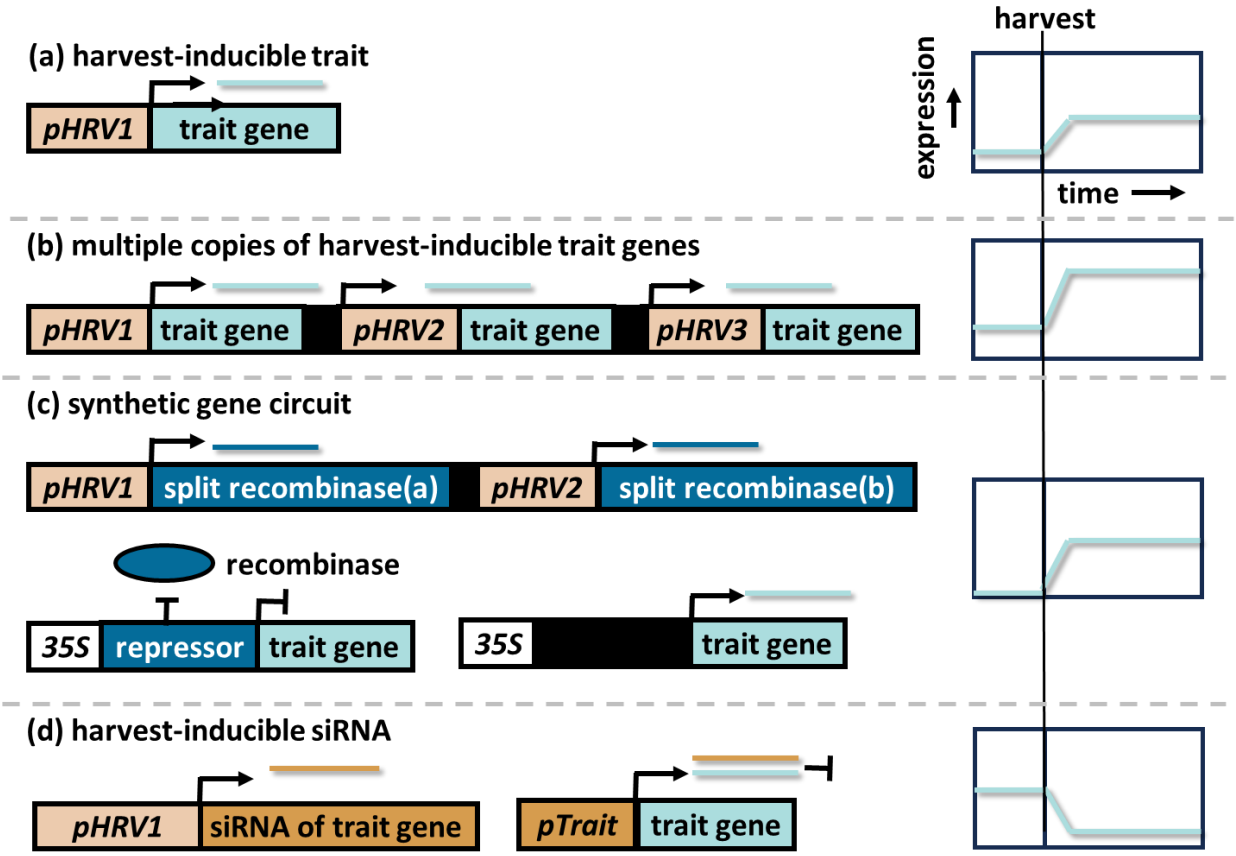

Figure 6.3: Possible future directions for harvest-inducible promoter construct development showing the theoretical constructs on the left, and the anticipated transcriptional profile in the graphs on the right. *pHRV* shows harvest-inducible promoters; *35S* is a constitutive promoter, and *pTrait* indicates the gene's native promoter.

The nature of the harvest-inducible trait will dictate whether the lowest possible expression on soil is desirable, or the highest possible post-harvest upregulation (Figure 6.3). For traits that particularly require minimal soil expression, a synthetic split-AND gene circuit combining multiple harvest-inducible promoters could be used (Lloyd et al., 2022), which would only switch-on gene expression when both promoters

were simultaneously driving transcription of a recombinase. If, however, a higher accumulation of the gene product were required, and residual expression on soil were not deleterious, then multiple copies of the trait gene could be activated by various harvest-inducible promoters in the same cell. In addition, I hypothesise that harvest-inducible promoters could also be used to drive expression of small interfering RNA (siRNA) that could downregulate expression of an undesirable trait gene after harvest (Carbonell, 2019), such as *STAY-GREEN (SGR)* which codes for a chlorophyll catabolism enzyme (Xu et al., 2019).

6.3 Improving post-harvest health and nutrition through synthetic harvest-inducible traits

This novel approach to post-harvest health has the potential to induce disease resistance traits, and it also could be used to add traits of interest related to freshness or nutrition (Table 6.1). Consumers generally favour crisp leafy vegetables with a bright green colouring and little visible pathogen damage (Barrett et al., 2010). Increased green-coloration and freshness of harvested crops has been achieved through senescence-induced expression of an *Agrobacterium* cytokinin biosynthesis gene *isopentenyl transferase* (*IPT*) (Gan and Amasino, 1995). It would, therefore, be interesting to compare the longevity of plants expressing harvest-inducible *IPT* with those expressing senescence-inducible *IPT.* Reduced pathogen damage could also be achieved by boosting post-harvest defence against biotrophic pathogens through harvest-induced upregulation of SA-responsive genes, such as *PR1*. Equally, PTI-

responsive gene expression could be boosted in harvested tissues: a MAPK-activated transcription factor, like WRKY33 could be upregulated by the harvest-inducible system. These constructs could be transiently expressed in Arabidopsis to rapidly assess the impact of their harvest-inducible expression on post-harvest senescence and immunity.

	Trait	**Harvest-inducible gene to address this**	**Reference**
addressing undesirable traits	**delay senescence**	*isopentenyl transferase (IPT)*)(Agrobacterium)	Gan and Amasino, 1995; Swartzberg et al., 2008
	reduce chlorophyll degradation	**STAY GREEN (SGR)*	Xu et al., 2019; Wang et al., 2022
	reduce wilting	**XTH*	Wagstaff et al., 2010
	boost PTI	*WRKY33*	Zheng et al., 2006
	boost biotrophic resistance	*PR1*	Fang et al., 2019
biofortification	**anthocyanin accumulation**	*MYB75*	Kreynes et al., 2020
	vitamin C accumulation	*GalUR* *DHAR*	Agius et al., 2003; Kim et al., 2022

Table 6.1: Candidate harvest-inducible genes for improving post-harvest traits. *indicates knockdown is required by antisense RNA or siRNA

The application of harvest-inducible genes need not be limited to bolstering post-harvest health and longevity, but could also be used to enhance micronutrient content, such as increasing post-harvest levels of antioxidant anthocyanins or vitamin C (Table 6.1). Post-harvest processing and storage leads to >50% loss of the vitamin C in salad leaves (Dewhirst et al., 2017) through oxidation pathways. Harvest-inducible upregulation of a reducing enzyme in the vitamin C recycling pathway, *DEHYDROASCORBATE REDUCTASE* (*DHAR*), could restore post-harvest levels of vitamin

C (Kim et al., 2022), with consequent nutritional benefits. A harvest-inducible system would be particularly useful for upregulating levels of compounds that might interfere with growth or health of the plant on soil. For example high anthocyanin accumulation during pre-harvest development in tomato can have a yield penalty (Cerqueira et al., 2023), which could be avoided if upregulated gene expression were limited to the post-harvest period.

In this study I have developed a method of identifying and testing a harvest-inducible system that could be applied to a wide variety of horticultural crops and cut ornamental flowers. Fruit crops are likely to have very different post-harvest transcriptomes compared to leafy brassicas, as the harvested organs and growth stage are different, resulting in distinct hormonal profiles (Ludford, 2002). Nonetheless, the methods used in this study could be applied to the wealth of post-harvest transcriptional data in fruiting crops, in particular, that could be explored for harvest-inducible promoter candidates: from the most widely grown vegetables, such as tomatoes (e.g. Guo et al., 2023) to more unusual, high-value fruit, such as loquats (Liu et al., 2019a). This novel approach to post-harvest has the potential to develop crops with improved storage life and nutritional value .

6.4 Conclusions

The work in this thesis identifies the effect of harvest on leafy brassicas and develops a strategy to bioengineer improved post-harvest health. This study established Arabidopsis as a model post-harvest system, and together with leafy brassica crop data, used it to identify requirements specific to harvested tissues. This study

identified marked changes in PTI and immune hormone pathways that may render harvested crops more susceptible to biotrophic pathogens. The Arabidopsis model system shared abiotic stress responses with leafy brassica crops with a short-storage life, such as broccoli and rocket, but not with cabbage, which has a longer storage-life. I designed and tested harvest-inducible promoters in the model system, which could be optimised and used as a basis for future work to boost post-harvest health, or improve the nutritional value and longevity of harvested crops. Any improvements in post-harvest health and quality of leafy brassica crops will contribute to a reduction in food loss and waste, and consequently improve the sustainability of food systems.

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Appendix A: Primers and media

Gene	±	Sequence (5' – 3')	Reference
BolFRK1	F	GTCGAACAACTGGGCCGG	-
	R	GGCGTAAAGACTCTTCGTCAC	
BolVSP2	F	GACTCCAAAACGGTGTGCAAA	Lee and Hong, 2012
	R	AGGGTCTCGTCTAGGTCAAAGA	
Bo8g18090	F	GATCATGGTGATCGTGGCG	-
	R	CATAGTTGCGCTTCCGTC	
BolACTIN	F	CGTACTACCGGTATTGTGCT	Zheng et al., 2019
	R	GAGCTGGTTTTGGAAGTCTC	
BolPR1	F	CAGCCCTTGTAGGAGCTCTTGT	Ray et al., 2016
	R	GGTTGTGAGCGTTTACATAGTCTTG	
BolPR5	F	GACGGCTACAACGTCAAGAT	Ishiga et al., 2020
	R	CCATGACACGAAGCTCGTTA	
BolLOX2	F	GATGCTACCTCCTGCTGGC	-
	R	GGAAGTGGGGATGCAACAGC	
BolJAZ9	F	CTTGGCGGTTCAGTTCCG	-
	R	ACGCTAACAGTTCCACCATAG	

Table A.1: qRT-PCR primers in cabbage

Gene name	±	Sequence (5’ – 3’)
PR1	F	CTAAGGGTTCACAACCAGGC
	R	AAGGCCCACCAGAGTGTATG
WRKY18	F	AGAAGGTACAACGCAGCGCAGA
	R	TGCGTCCCTTCGTATGTCGCTACA
WRKY38	F	CCGGTTTACCGAACCACTTA
	R	GGCTTTCCTTCTCCTGATCC
WRKY62	F	GCCTACACCAAGGACCAGAA
	R	AGAGGTGGAGGAGGAGAAGC
UBQ5	F	CCAAGCCGAAGAAGATCAAG
	R	ACTCCTTCCTCAAACGCTGA
PR2	F	CAGATTCCGGTACATCAACG
	R	AGTGGTGGTGTCAGTGGCTA
PR5	F	ACTGTGGCGGTCTAAG
	R	CGTGGGAGGACAAGTTT
NLH10	F	TTCCTGTCCGTAACCCAAAC
	R	CCCTCGTAGTAGGCATGAGC
FRK1	F	CGGTCAGATTTCAACAGTTGTC
	R	AATAGCAGGTTGGCCTGTAATC
PHI1	F	TTGGTTTAGACGGGATGGTG
	R	ACTCCAGTACAAGCCGATCC
LOX2	F	AATGAGCCTGTTATCAATGC
	R	CATACTTAACAACACCAGCTCC
PDF1.2	F	ACCCTTATCTTCGCTGCTC
	R	TCCTTCAAGGTTAATGCACTG

Gene name	±	Sequence (5’ – 3’)
NPR1-GFP	F	TCCACATCGAAATCAACCG
	R	GTCCAGCTCGACCAGGATG
HRV1	F	GGCCAGACAAAGAGATTGAC
	R	CTCTGCTTCATCGGTTGC
HRV2	F	CGAGACAGGACACTTCTGCTAC
	R	CCATGATAGTAGGAGCGGAC
HRV3	F	CTACCTTTCAGTGTGATGATGG
	R	GACCAGGGCACTTGTTACTC
HRV4	F	CGCTAAAGTCTATCTCGCACG
	R	GATCTCGCGTTTGATGTG
HRV5	F	CTAGGACCGAAGTGGAAGTG
	R	GAGTCCCTCAAGCACACTG
HYGR	F	GTACGCCCGACAGTCCCG
	R	GGTCAAGACCAATGCGGAGC
KANR	F	CATCTCACCTTGCTCCTGC
	R	GACCACCAAGCGAAACATCG
GFP	F	CACAAGTTCAGCGTGTCCGGCG
	R	TTCGGGCATGGCGGACTTG

Table A.2: qRT-PCR primers in Arabidopsis

Gene	±	Sequence (5’ – 3’)
HRV1	F	TTATACTAGGAAAGGTTCTCTCATATCTTTAAAGCAC
	R	TCTGTTTTACGTGTTAGCTGTAGAGATACC
HRV2	F	TTAACAAATGTTAGGAAATTTCGCAACGG
	R	TGGGACTGGCAATCAATTGTGATT
HRV3	F	GCATTACAAGGGATTAATGGTTAAGG
	R	GAATTGGAGAGATGGCTACACC
HRV5	F	CGTCAATTCTAGGATTTTCCAG
	R	CAAATTGGCAAATTCATATTTTCAG

Table A.3: Arabidopsis cloning primers

Gene	±	Sequence (5’ – 3’)
HRV1	F	GCCCAAAAGCCCAAAAGGCC
HRV2	F	GTGAAGGGCGGCTTTTCTTAGTTTC
HRV3	F	GCTGCATCCACTCAAATTCCATCC
HRV5	F	CCCAAATCTCGGTCCGGACT
HRVx	R	CTTGTAGTTGCCGTCGTCCTTGAA

Table A.4: Arabidopsis colony PCR primers

Gene	±	Sequence (5’ – 3’)
PR1 as1 promoter element	F	AGTGTATACAATGTCAATCGGTGATCTT
	R	GCCGCCACATCTATGACGTA

Table A.5: Arabidopsis ChIP qRT-PCR primers

Media	Component	Concentration
L Broth	bacto tryptone	10 g/L
	oxoid yeast extract	5 g/L
	sodium chloride	10 g/L
		(pH to 7.2 with NaOH)
L Broth agar	bacto tryptone	10 g/L
	oxoid yeast extract	5 g/L
	sodium chloride	10 g/L
	formedium agar	15 g/L
MS plant media	Murashige & Skoog	4.9 g/L
	sucrose	3 g/L
	bacto agar	10 g/L
		(pH to 5.7 with KOH)
SOC	bacto tryptone	20 g/L
	oxoid yeast extract	5 g/L
	sodium chloride	0.58 g/L
	potassium chloride	0.19 g/L
	magnesium sulphate	0.24 g/L
	magnesium chloride	0.20 g/L
	glucose	3.6 g/L

Table A.6: Media recipes

Appendix B: Arabidopsis RNA-seq QC

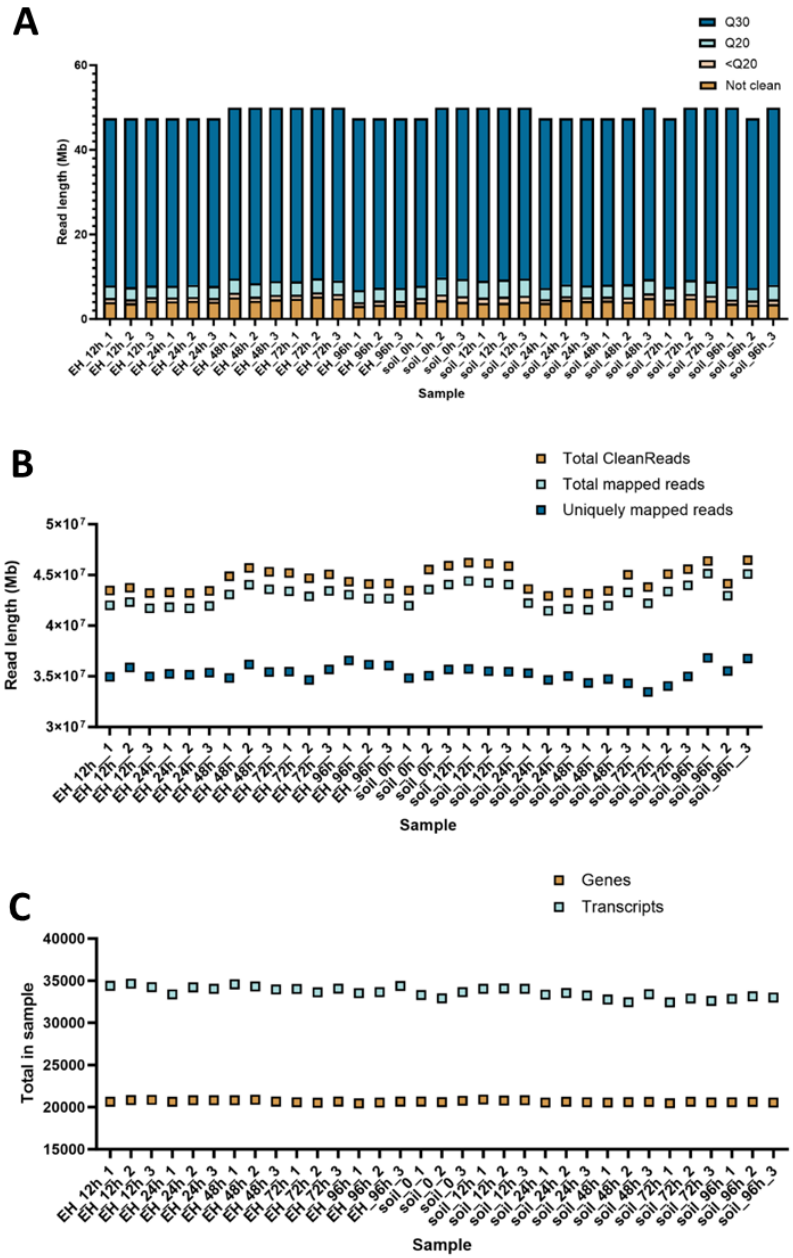


Figure B.1: Arabidopsis post-harvest RNA-seq quality control: (A) Coverage of the RNA sequencing individual samples: 'not clean' is data filtered out if >20% of the bases have <Q15; if >5% of bases are unknown; or adapter content present. **(B)** Clean reads mapped to the *Arabidopsis thaliana* genome (TAIR10); and **(C)** the number of genes and transcripts identified in each sample

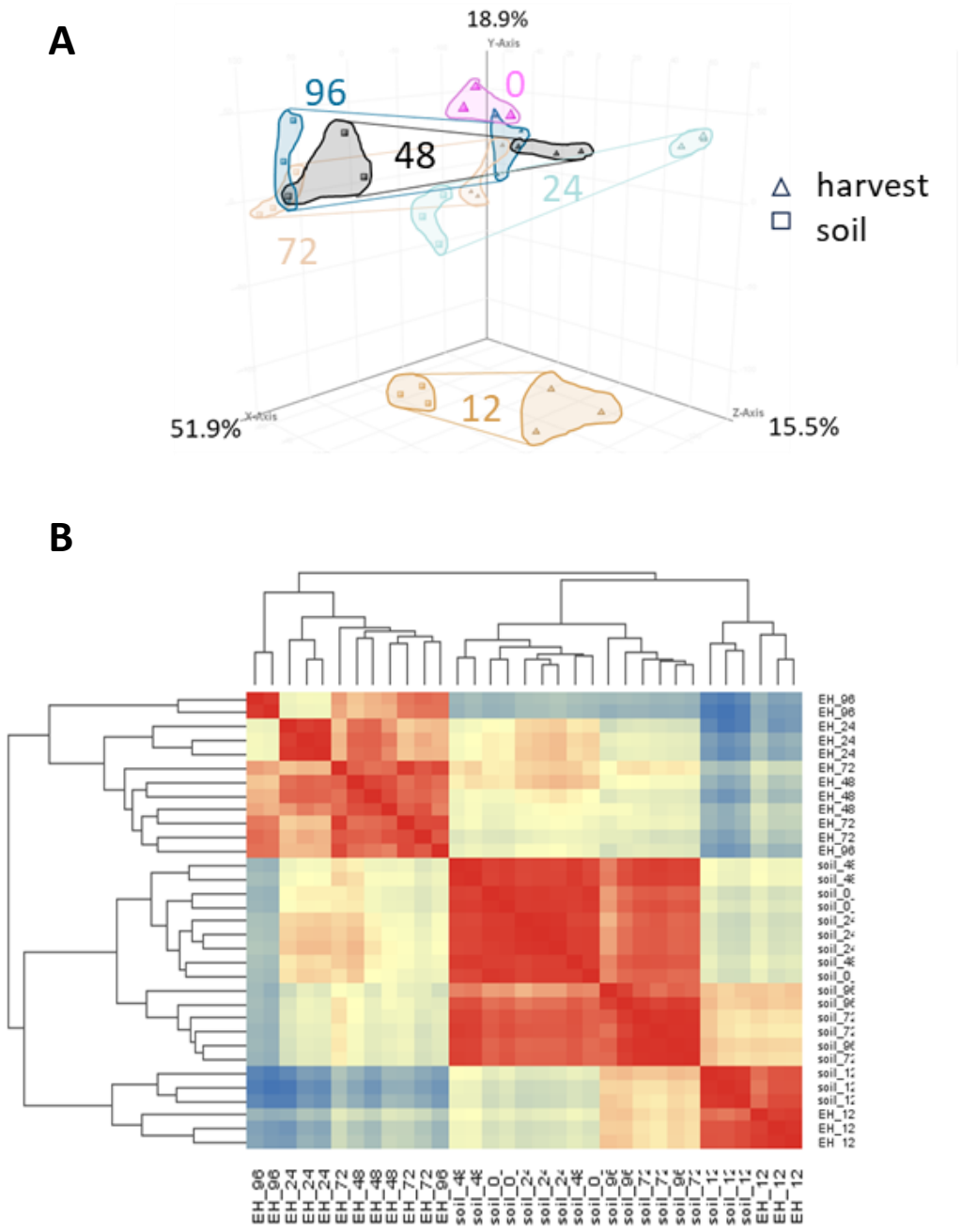
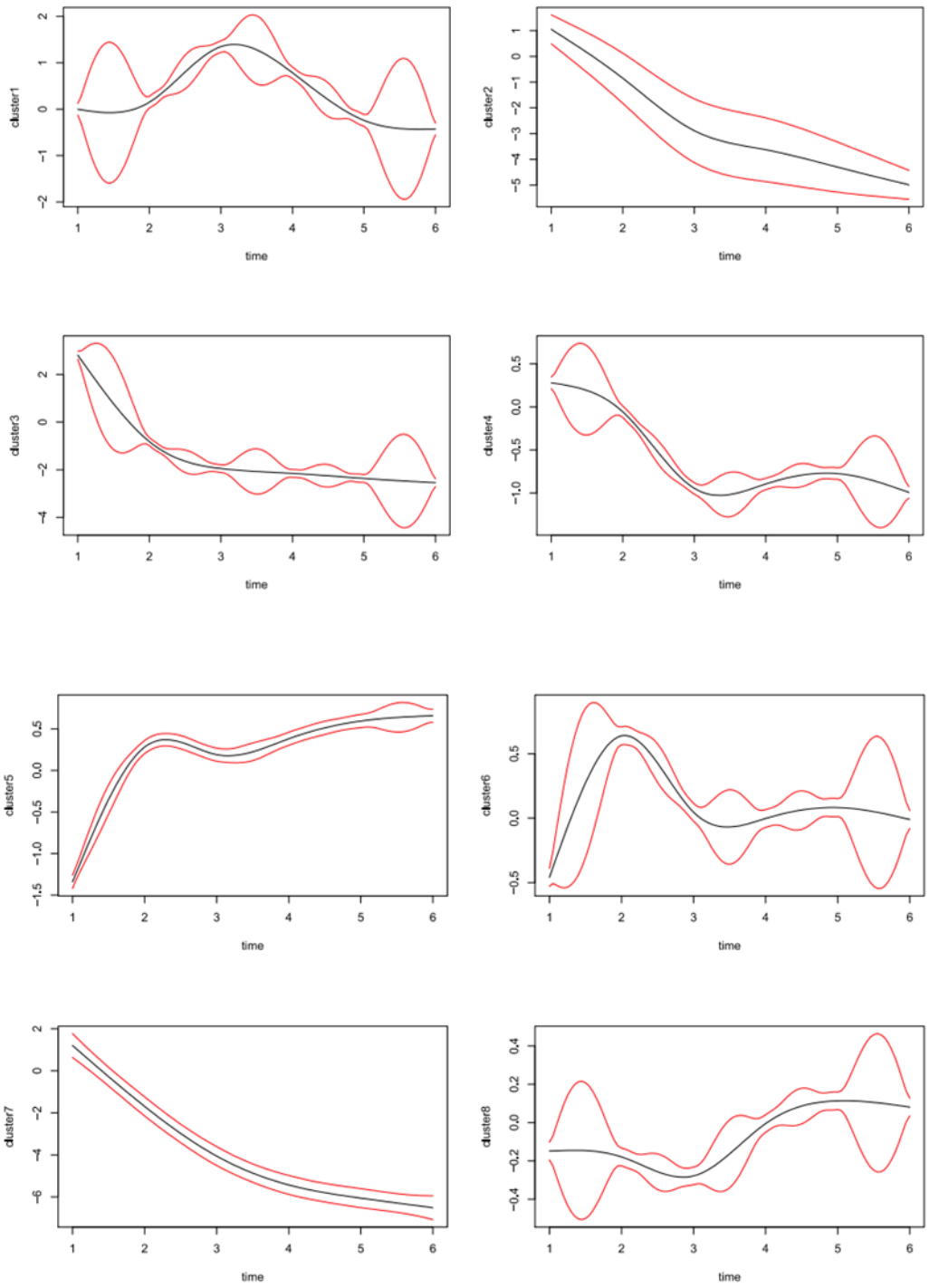
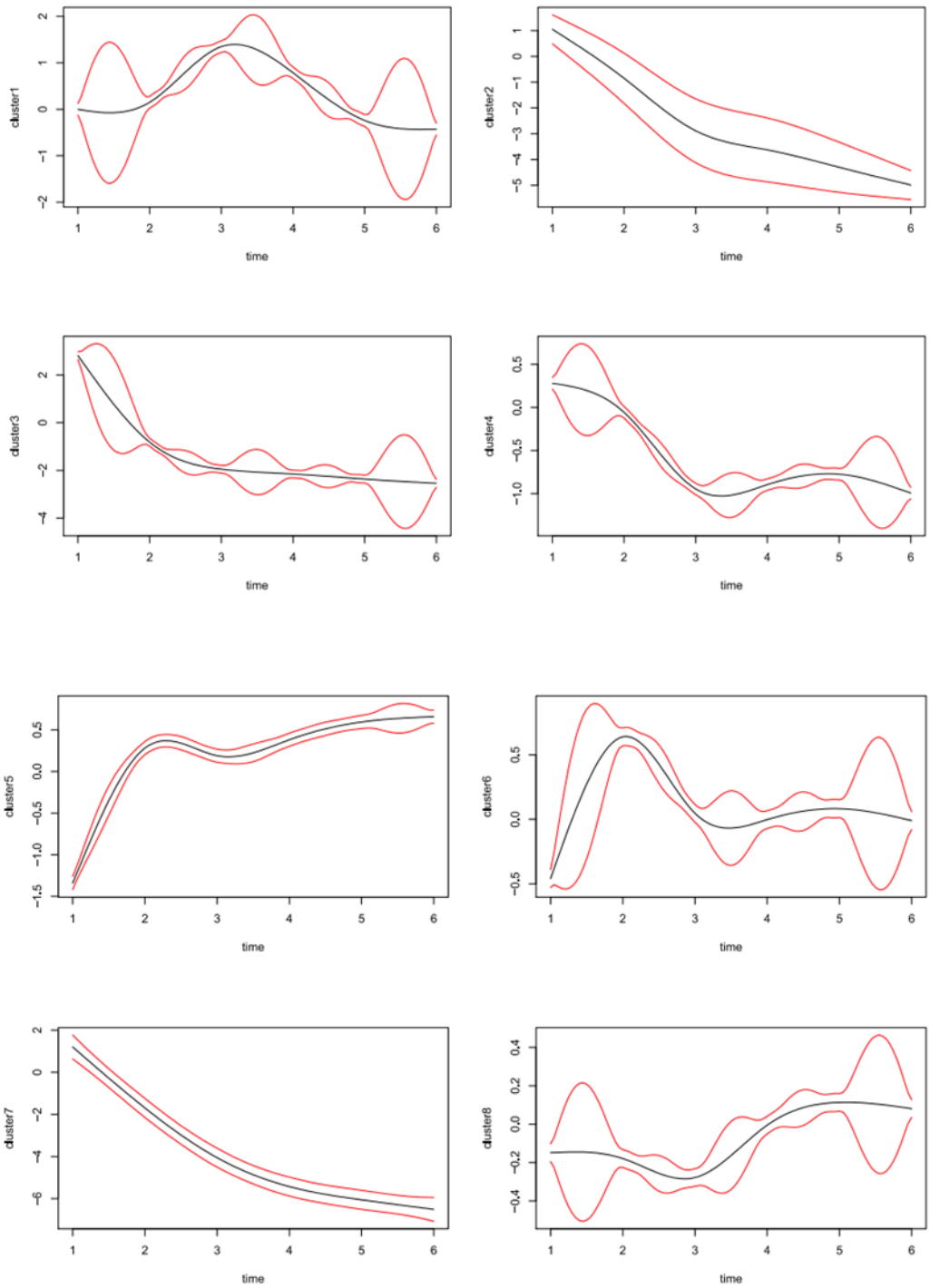


Figure B.2: Clustering of post-harvest Arabidopsis RNA-seq samples. (A) PCA analysis of the individual samples from the Arabidopsis post-harvest RNA-seq; coloured rings encircle sample replicates, numbers indicate hours post-harvest and lines join them with their harvested/soil counterparts **(B)** co-expression analysis of the replicates, where red is positive correlation, and blue negative; EH is harvested, soil is unharvested, and numbers denote hours post-harvest.

Appendix C Arabidopsis SSC RNA-seq clusters

A

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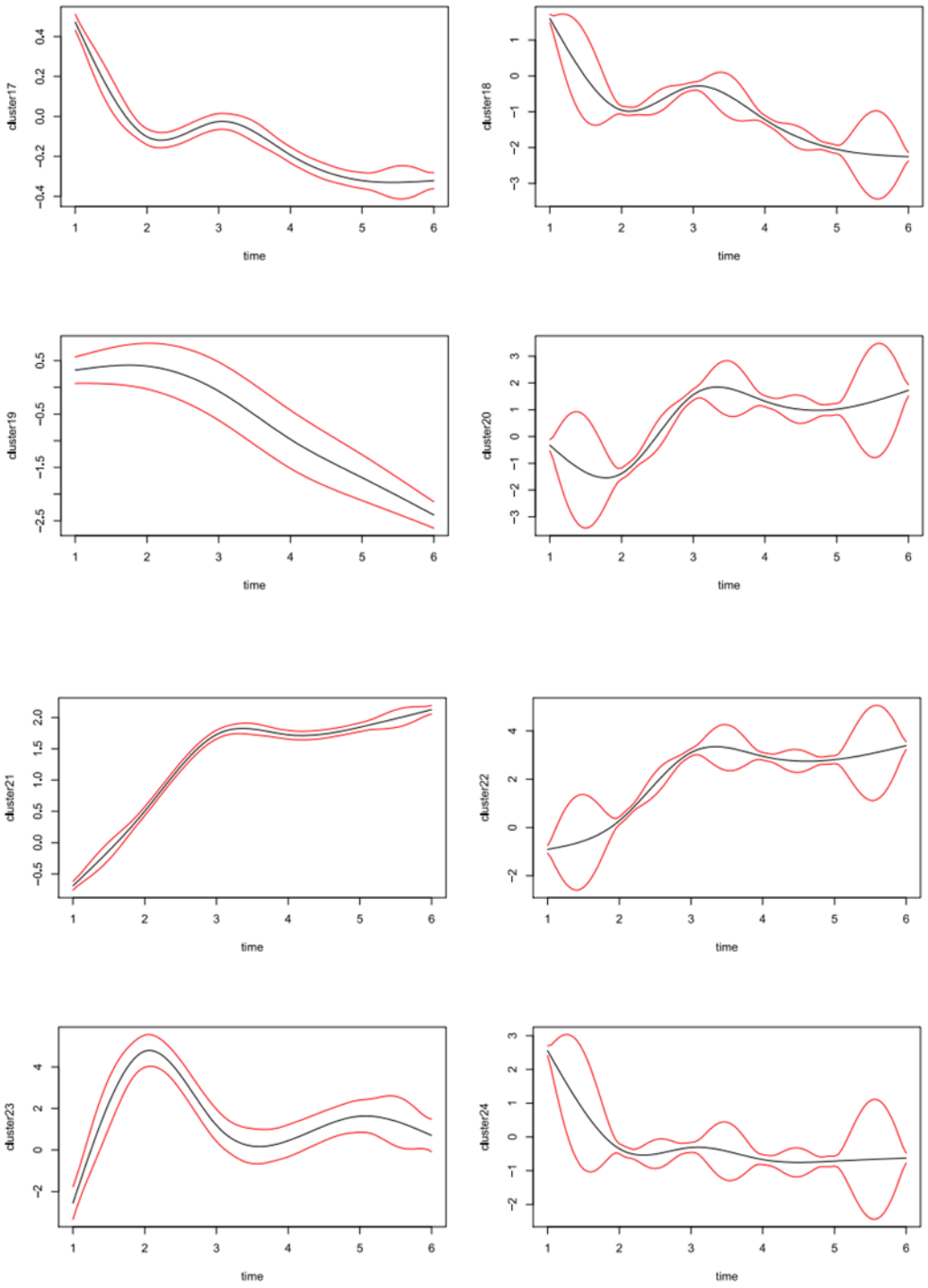
time
cluster2
time
cluster3
time
cluster4
time
cluster5
time
cluster6
time
cluster7
time
cluster8
time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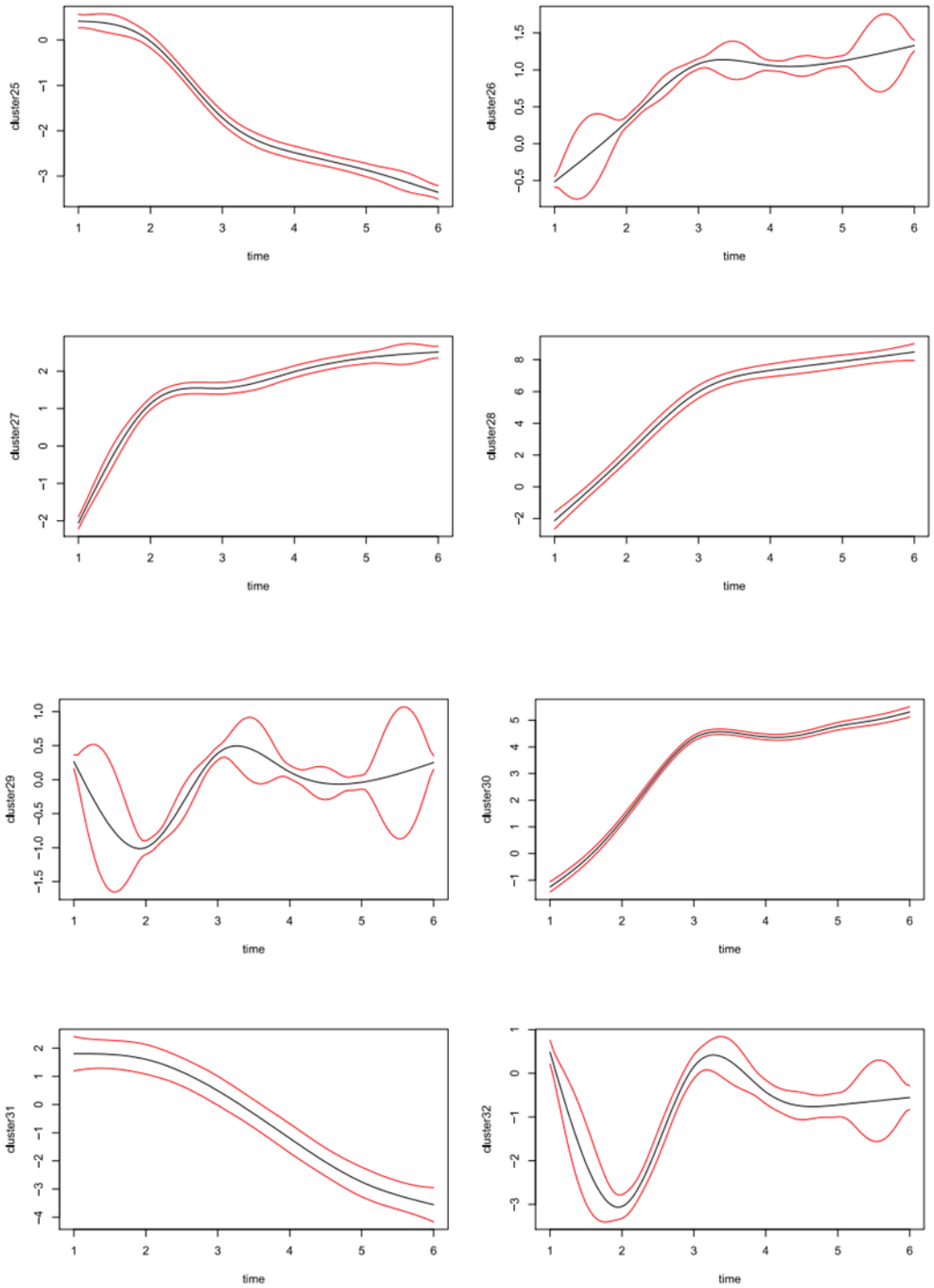
cluster18
cluster19
cluster20
cluster21
cluster22
cluster23
cluster24
time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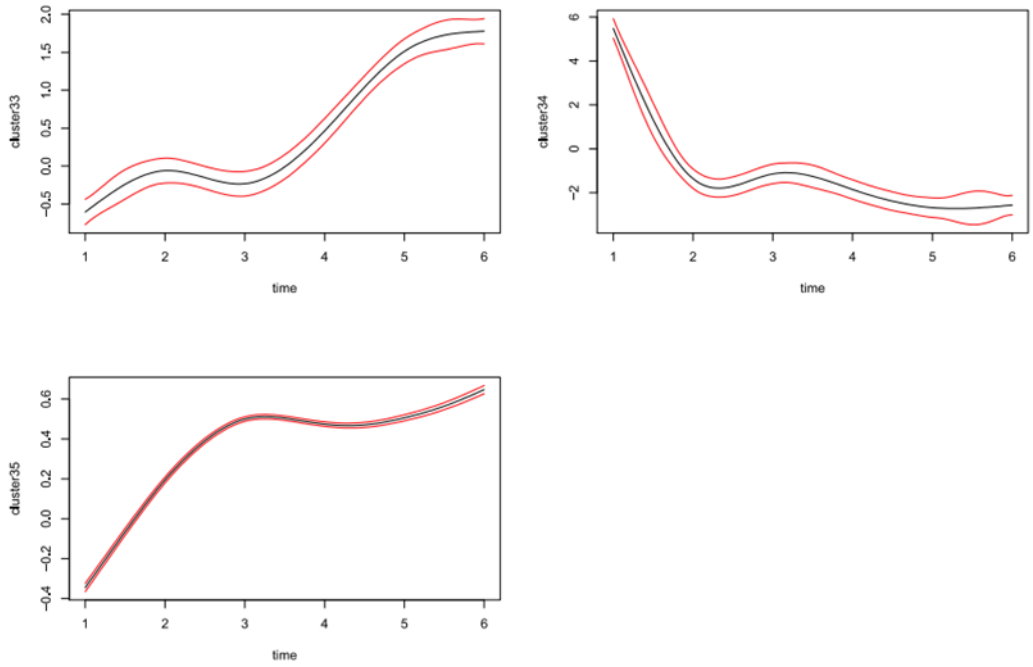
cluster27
cluster28
cluster29
cluster30
cluster31
cluster32
time



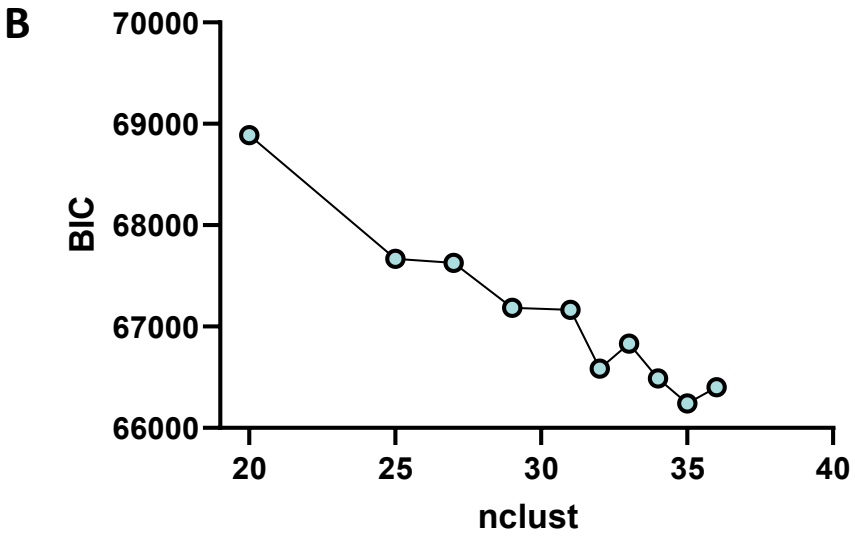


Figure C: Individual clusters from Arabidopsis RNA-seq profiles. (A) Mean profile plots of *Arabidopsis thaliana* model harvest RNA-seq generated by smoothing spline clustering (Ma et al, 2006); timepoints in hours post-harvest are (1) 0 (2) 12 (3) 24 (4) 48 (5) 72 and (6) 96 **(B)** optimal cluster number analysis: when BIC increases across >1 nclust, chain length 5, threshold = 0.1.

Appendix D SSClustR analysis of post-harvest Arabidopsis

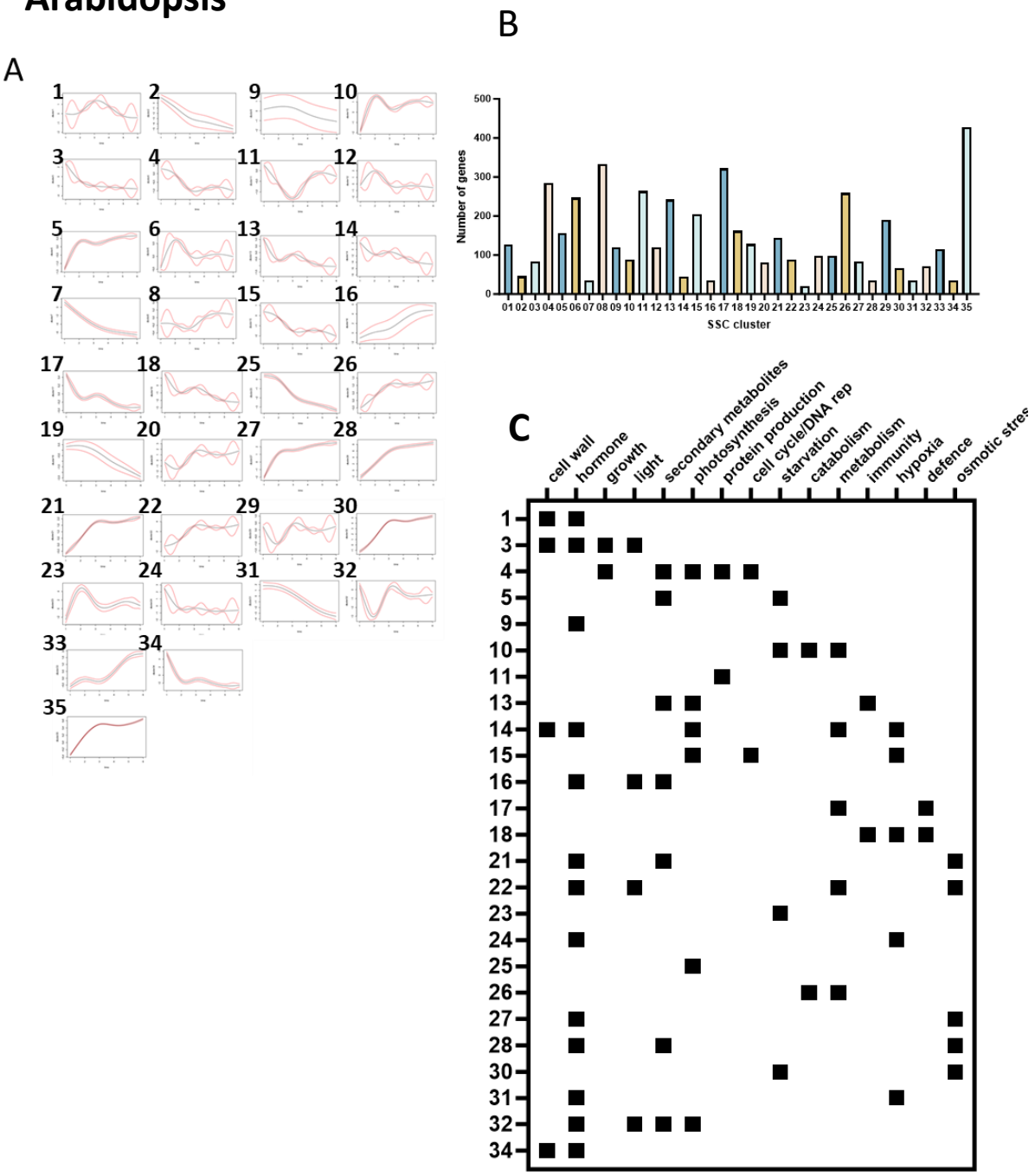
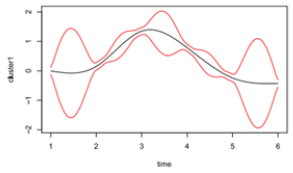
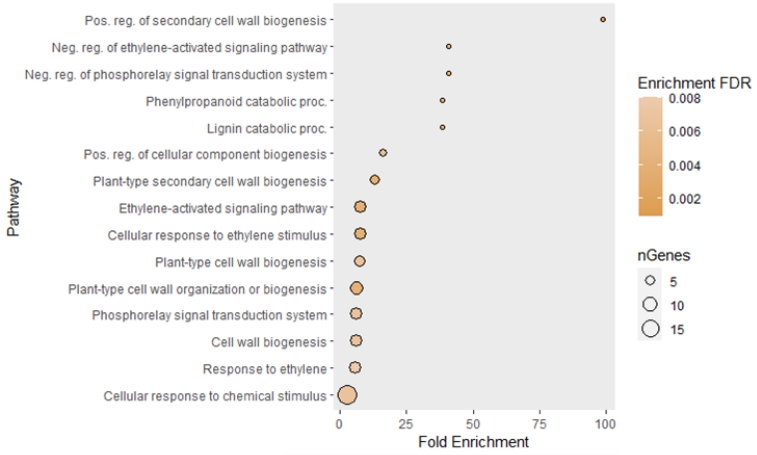


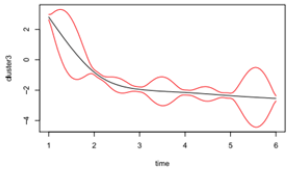
Figure D: GO terms of post-harvest Arabidopsis RNA-seq profiles (A) Full cluster profiles identified by SSClustR analysis; **(B)** the number of genes in each profile; **(C)** a summary of the enriched GO BP terms (FDR<0.05) in each cluster given in more detail overleaf (Enrichment FDR <0.05) (clusters are omitted where no enrichment was found) and **(overleaf)** full expanded profiles and most enriched GO terms for each profile.

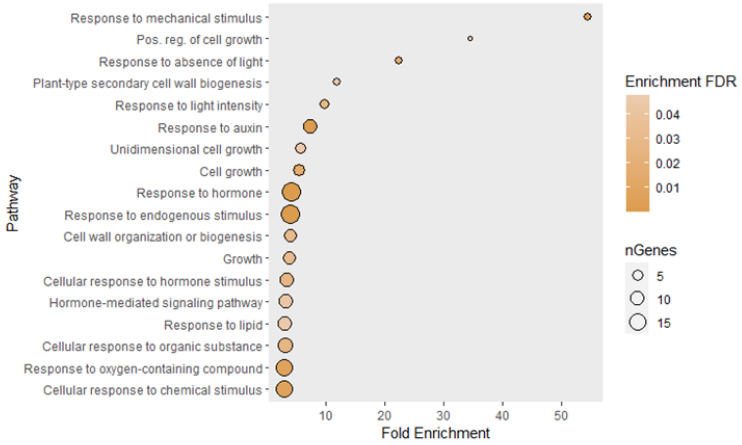
Cluster 01



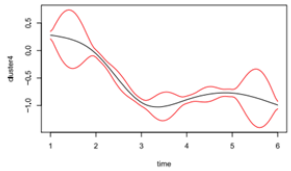


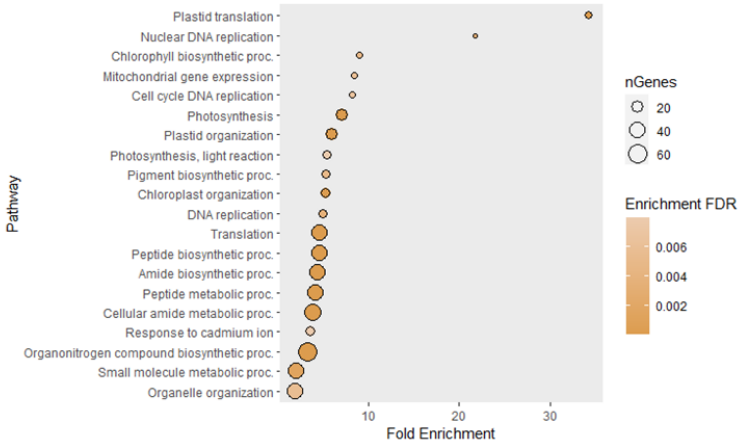
Cluster 03





Cluster 04





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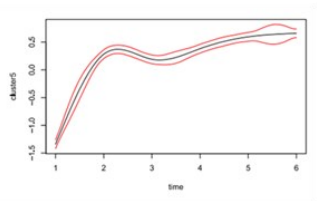
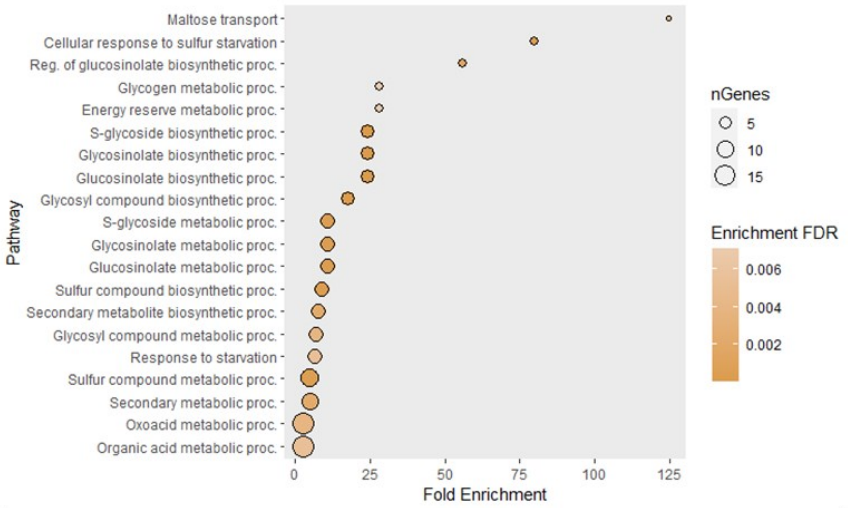
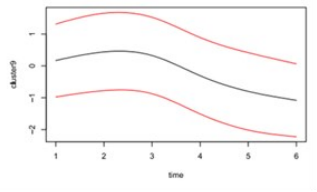
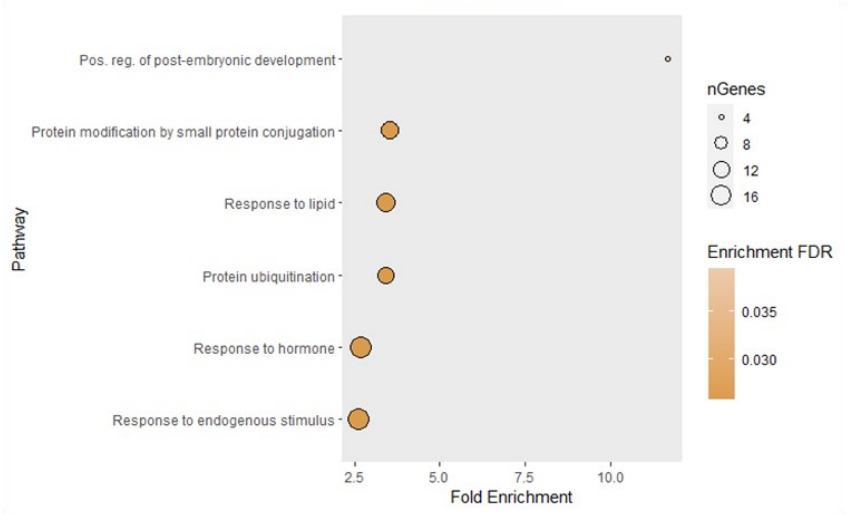


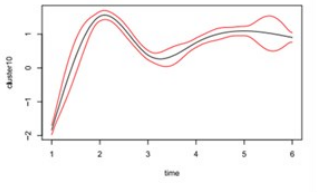


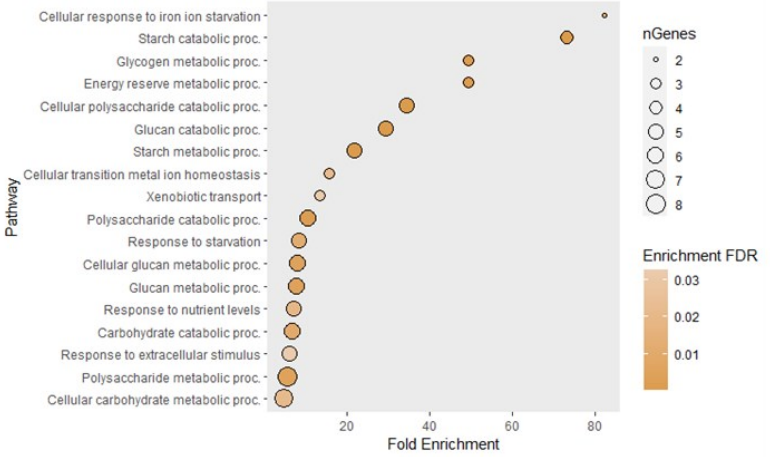
Cluster 09



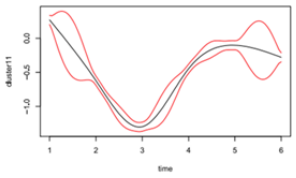


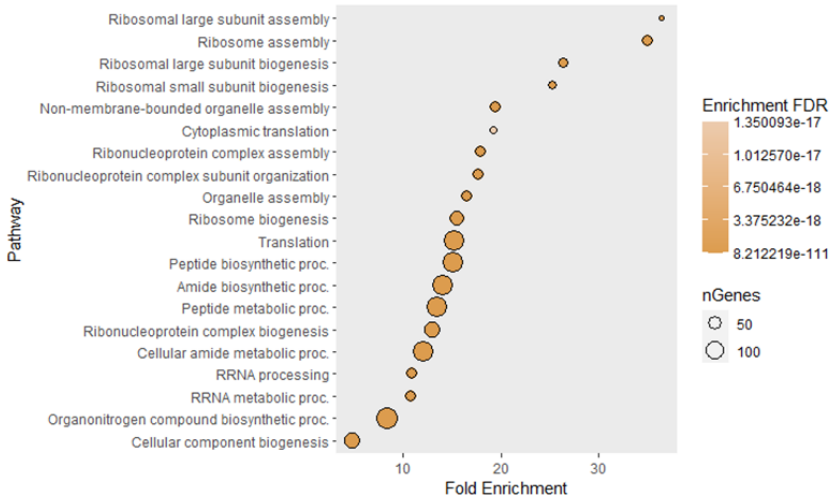
Cluster 10



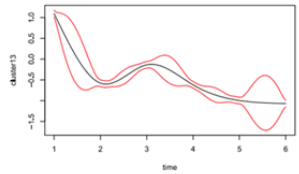


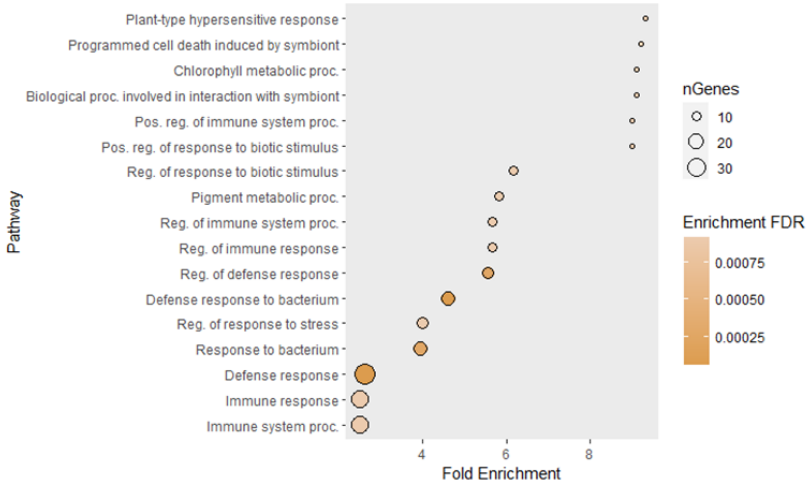
Cluster 11





Cluster 13





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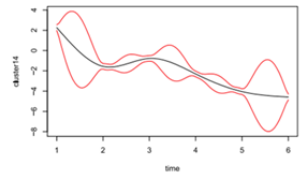

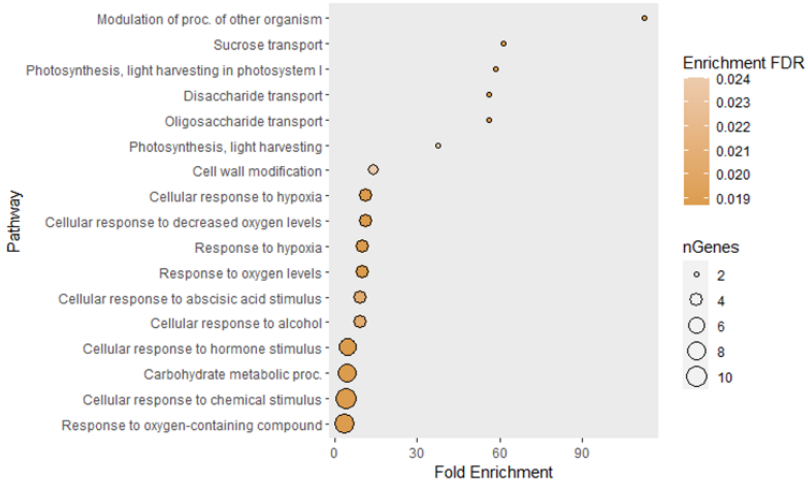

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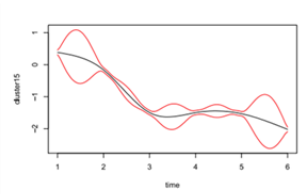
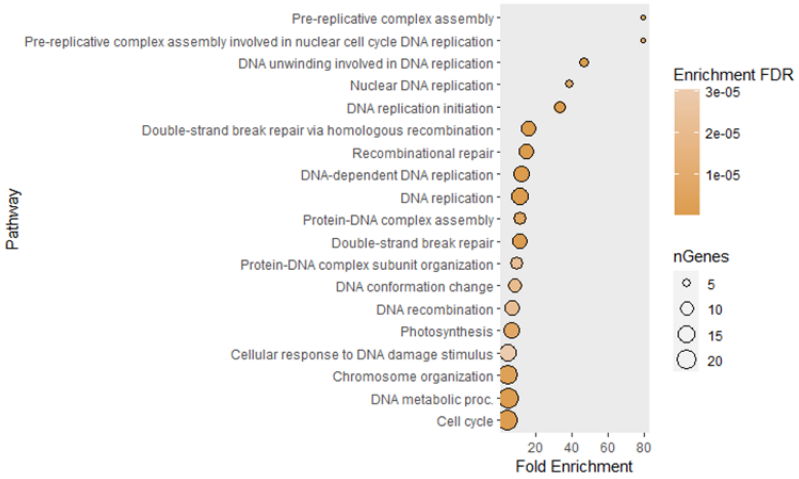
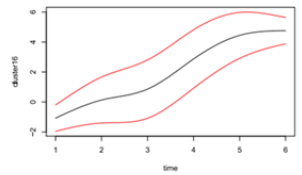
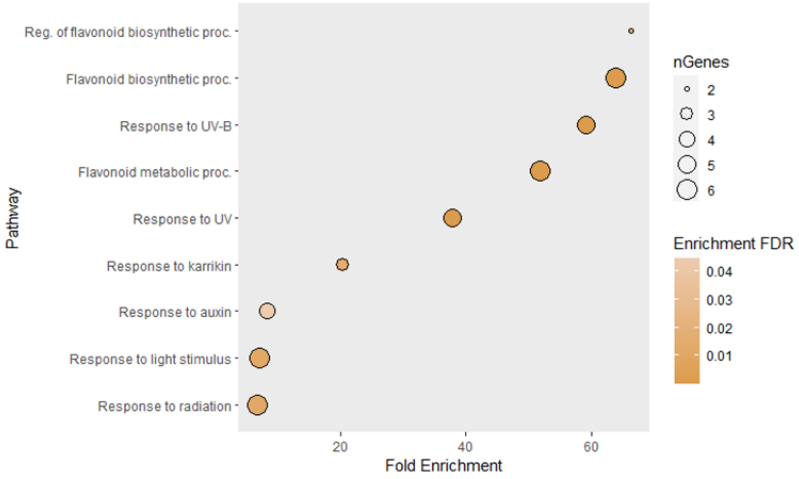




Cluster 16





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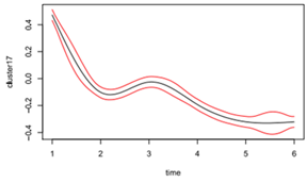

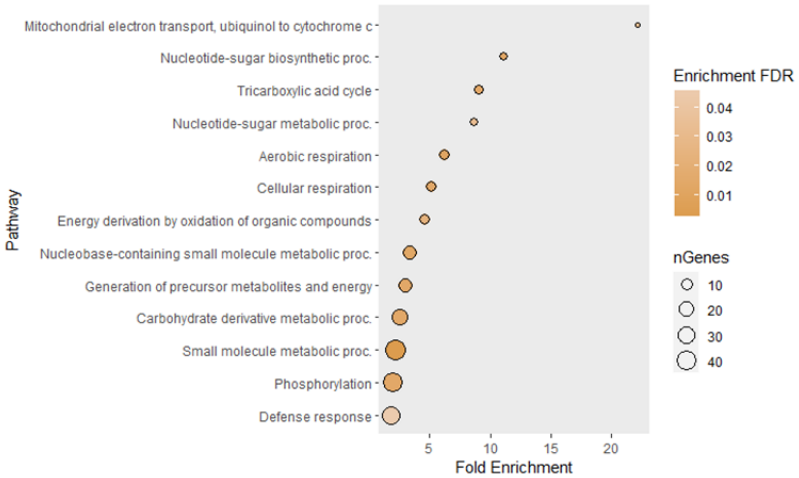

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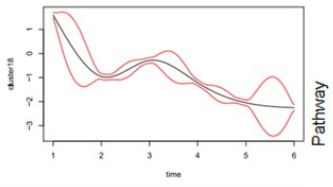
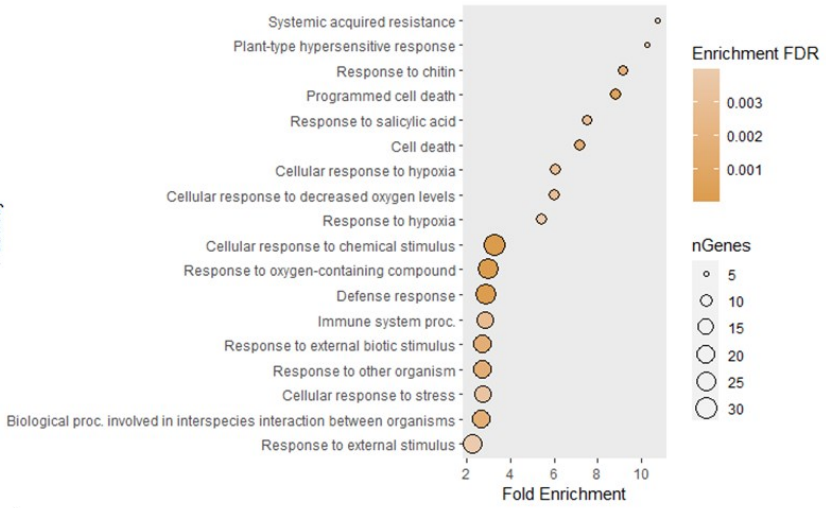
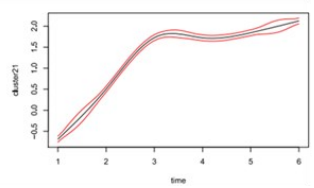
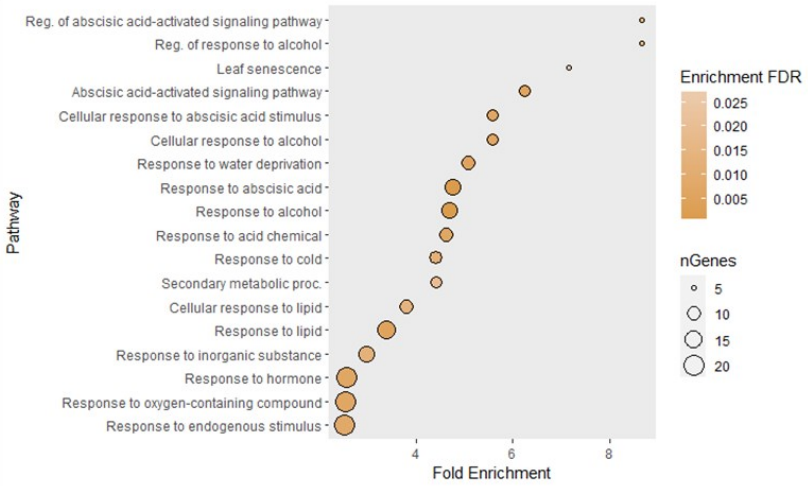




Cluster 21





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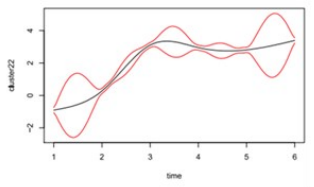

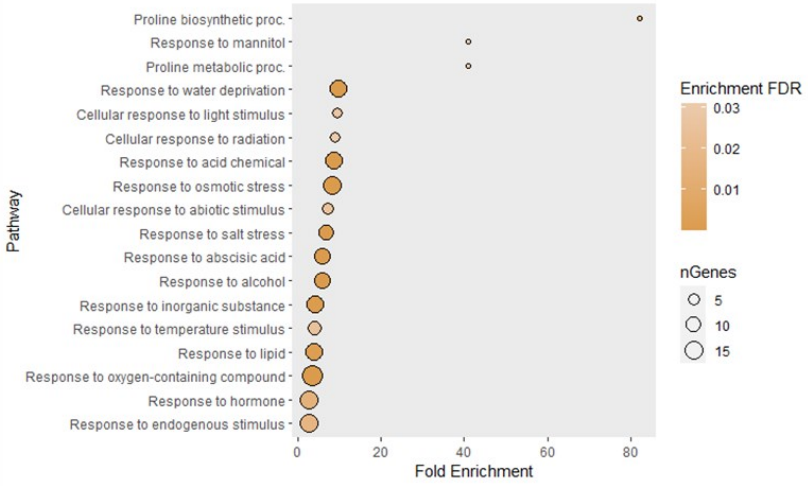

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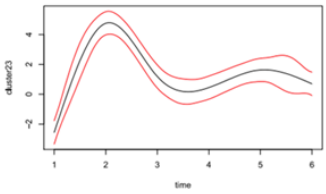

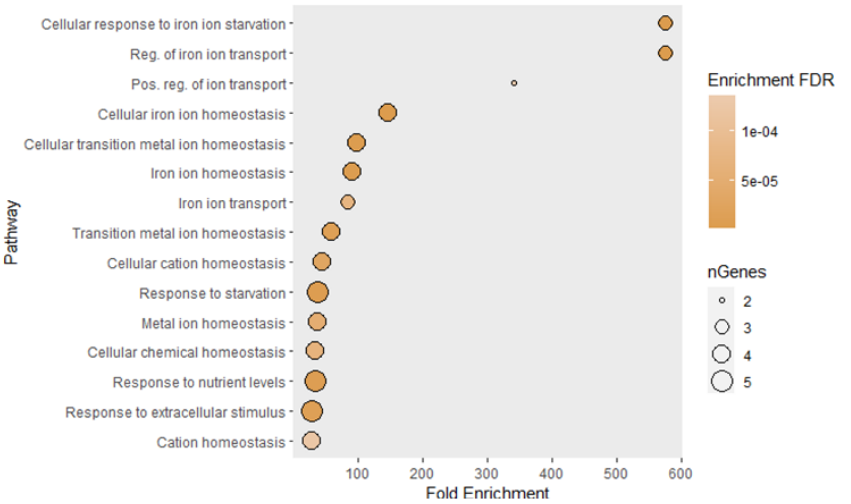

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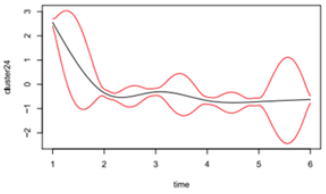

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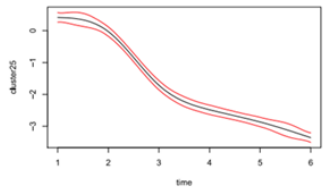

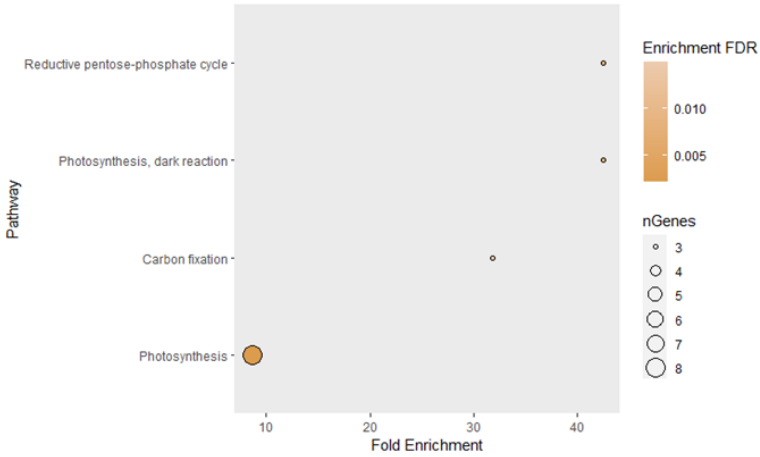

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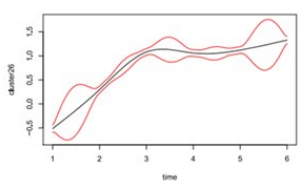

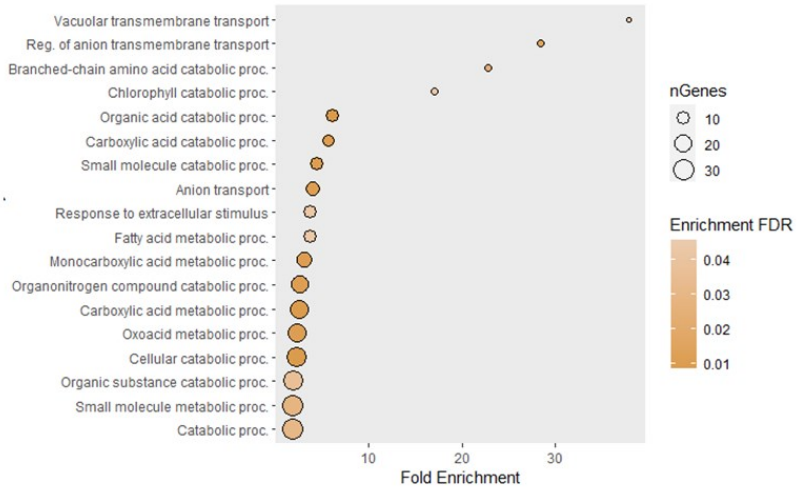

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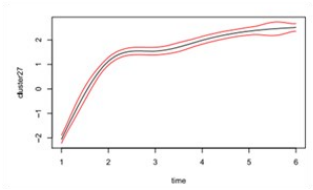
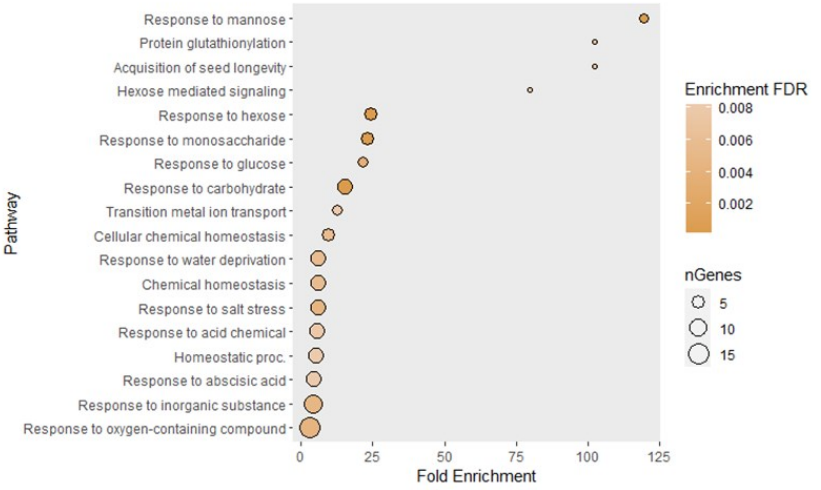
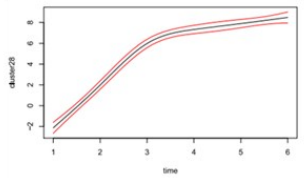
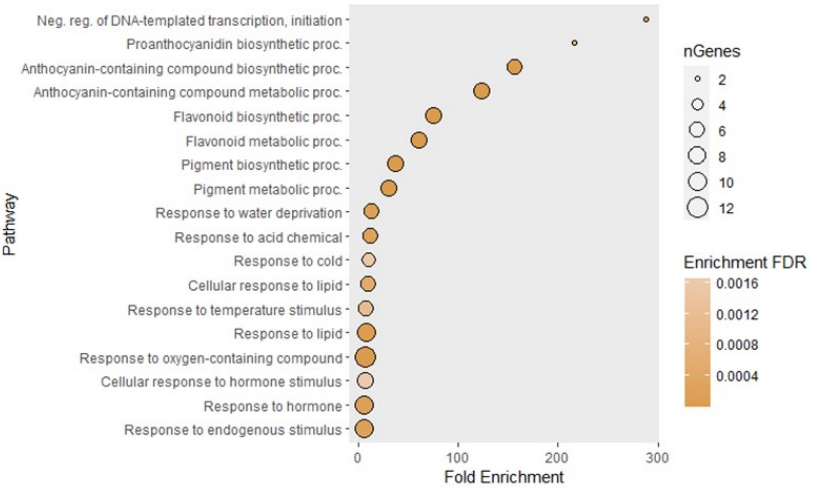


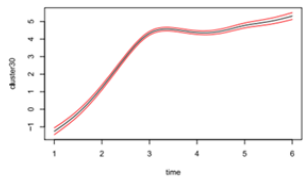


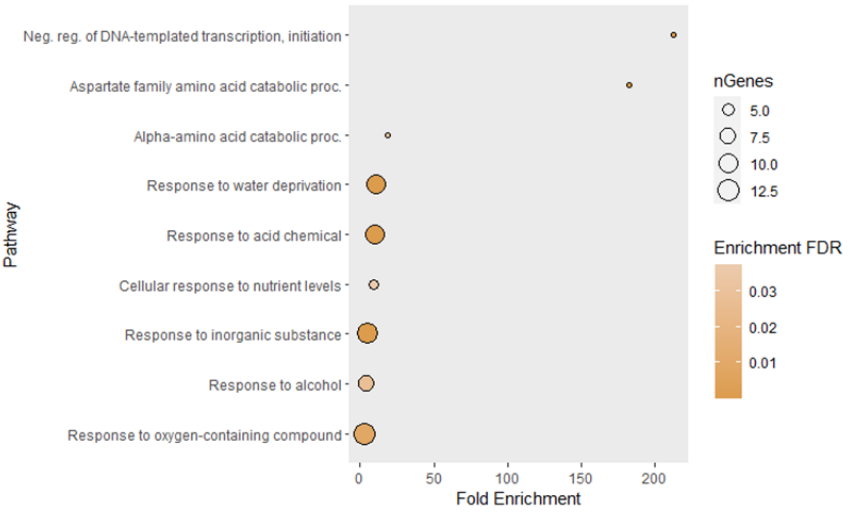
Cluster 28





Cluster 30





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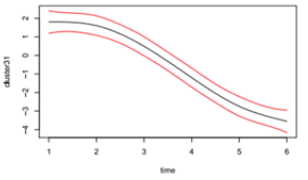
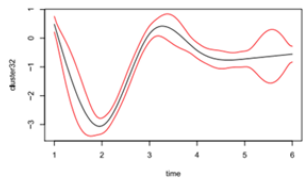
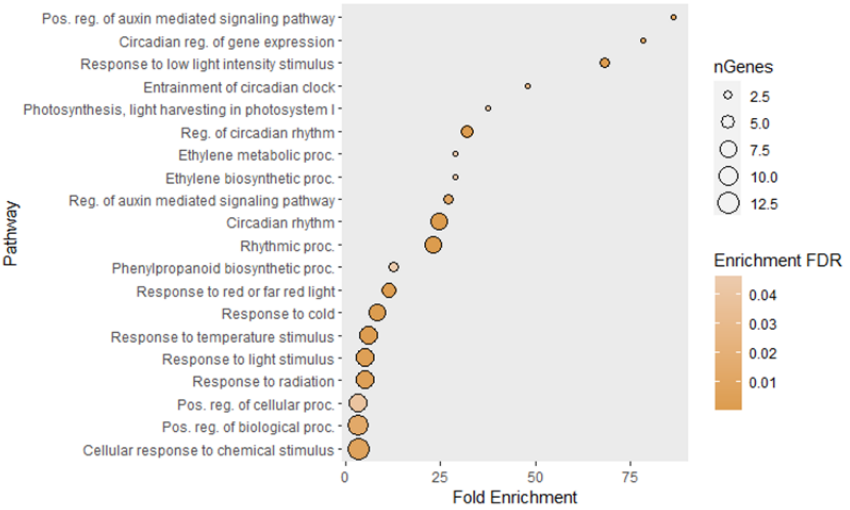
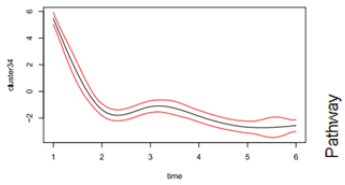


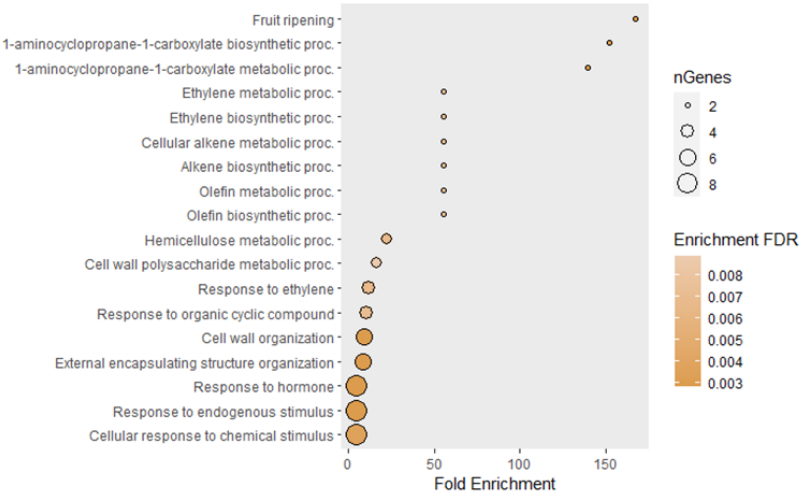
Cluster 32





Cluster 34


cluster34
time


Fruit ripening
1-aminocyclopropane-1-carboxylate biosynthetic proc.
1-aminocyclopropane-1-carboxylate metabolic proc.
Ethylene metabolic proc.
Ethylene biosynthetic proc.
Cellular alkene metabolic proc.
Alkene biosynthetic proc.
Olefin metabolic proc.
Olefin biosynthetic proc.
Hemicellulose metabolic proc.
Cell wall polysaccharide metabolic proc.
Response to ethylene
Response to organic cyclic compound
Cell wall organization
External encapsulating structure organization
Response to hormone
Response to endogenous stimulus
Cellular response to chemical stimulus
Pathway
0
50
100
150
Fold Enrichment
nGenes
2
4
6
8
Enrichment FDR
0.008
0.007
0.006
0.005
0.004
0.003

Appendix E *Brassica oleracea* var. *capitata* post-harvest RNA-seq quality control

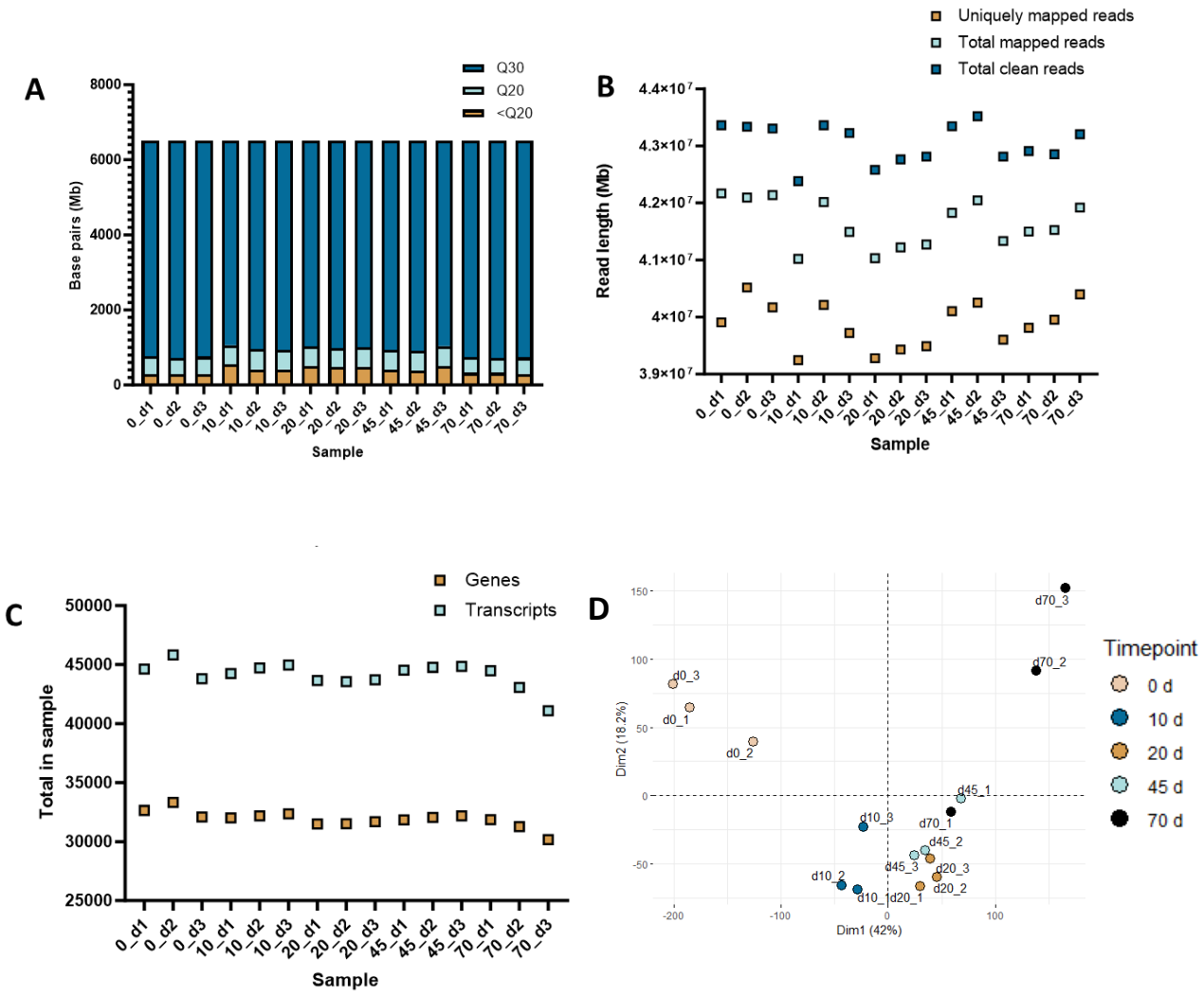


Figure E: Quality control of cabbage (*Brassica oleracea* var. *capitata*) post-harvest RNA-seq (A) Coverage of the RNA sequencing individual samples. **(B)** Clean reads (i.e. <20% bases have <Q15) mapped to the *Brassica oleracea* genome (JZS T01000); and **(C)** number of genes and transcripts identified in each sample **(D)** principal component analysis of the samples. Timepoints are days post-harvest.

Appendix F Brassica oleracea var. capitata gene expression clusters

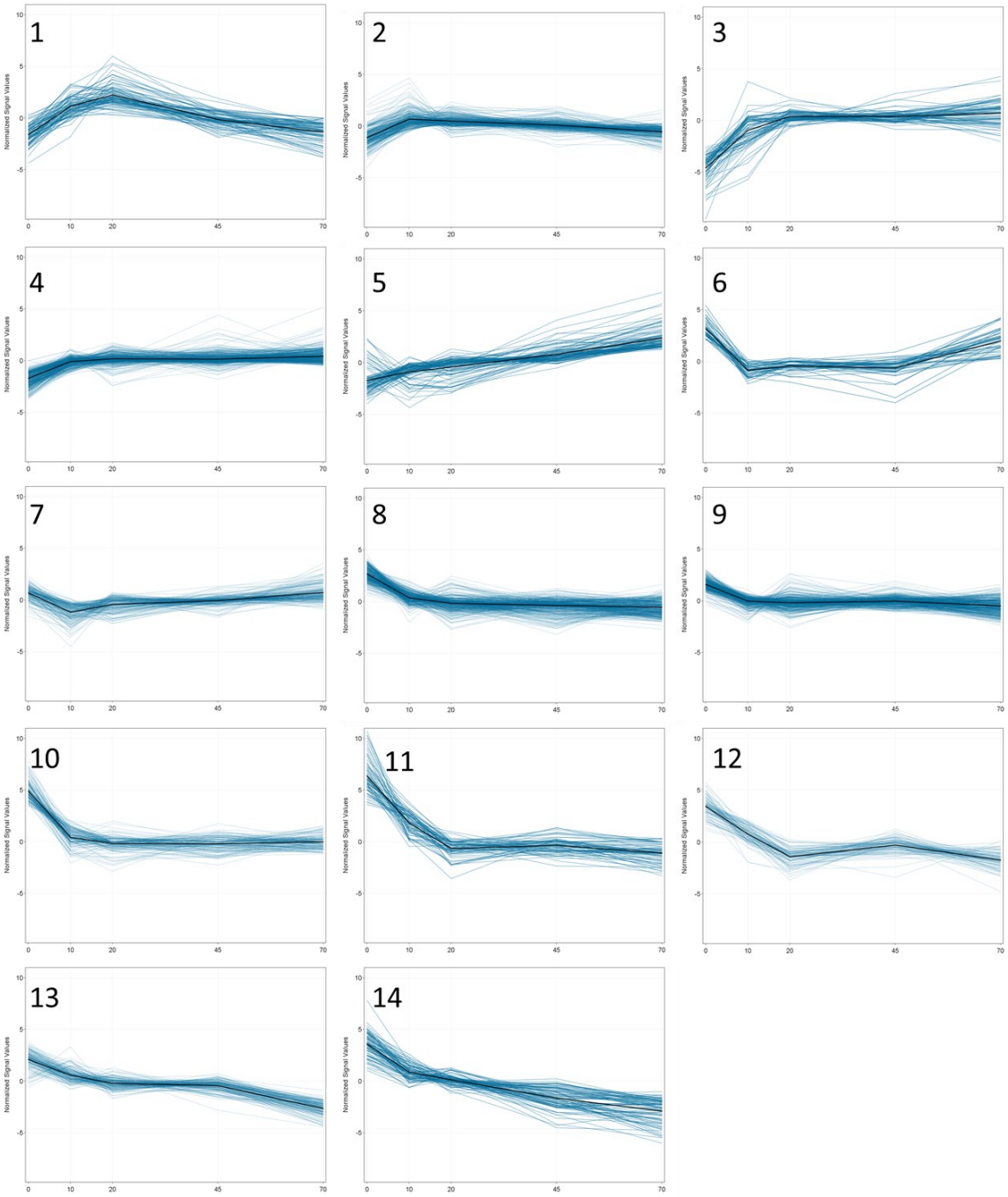


Figure F.1: Expression profiles from clustering of *Brassica oleracea* var.*capitata* RNA-seq. Gene expression profiles of pointed cabbage clustered by Ward's algorithm (distance <20), y axis is normalised signal value, x axis is days post-harvest (0-70); each line represents one gene.

Appendix G Harvest-inducible promoter sequences

>pHRV1_promoter_sequence

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AAGTGAGTTTGTGAGTATTTTACTATTTTTAGCAAAACCTCCTAACTAAAGTCGATATTAAATC
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ATTGAACACAAGATTATTCATTTAAAGAATACTAGAAACAAATATTTATTAGAATGTTAAATTC
TATGATTATCAAACTATTTTGTATCCGTATCGGTTTTGATATAGAGATAGGCTACAAATTGACC
CTAGATAACATTTTGTAGCTTCCGATCAATAAATTGGTATAACTTCTGTCGAAGGTAGGCCTTT
TTTTTTTGTATATATGTATTTGAAGTAGCCATAAATGTAATAAGAGAATTACATATTGTGTATAT
ATGTATTTGAAGTAGCCATAAATGTTCTTATTGAGATGTCAGGATGTTTGTCTTCCATCACTGC
TACCACGTCTGGCCCTCTTCTTTGGTAGTCGTCTCTGTTTTGTACATAAAACCTTACTCAGTTTG
TTGTTTCCTTAACTAGTCTTGTAATATTTGTTATTTGCTTTTTTTATCTAGACGTCCTTGTTTTATA
TAAACAGTCTATGTACGTTGTATTGTACAATGTTTAATATCAATAACAACACCCTTTGGCAAAA
AAAAAAAAATTACATATCGTGTCAAGCATAGTAGTGTAAATTGTAAATCGTTTAGGTATATAA
AAGTGATCAGTTTCTTATTGCACGATATGTGGTATGGTTCTTCACGTGGAACCTAAATTAAACG
TCATGGATTTTGTGTCGCTTTACCGTGCAAGTTGCAAACTATGCAGTGTTTGTTGATCCGATGA
CAGTTTCTTTCTTCAGCCCAAAAGCCCAAAAGGCCCAAAGTAAAATAGCCTCTTGCTTTAATAC
ACGTCGGTATCTCTACAGCTAACACGTAAAACAGA

>pHRV2_promoter_sequence

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CTATTATAGGATGTATAACTTTTTCTTTTTTTCTTTTTTAGGATGTATGACTTACTTATATATACA
AGTCATATAGGATGTATGACTTGTATATATAAAACAACATCTATGTTATAAGTTCAAAGCATTT
CATTTCGTATTTCAGATGATTTCAGTCCGCTATACGTATCTATACTCGATCTATTCATTGCAAAT
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AAAATATGTGTTGGCTTCTTTTAAGAGTCGGCTTTTAGAAAATGCTCGAGAGAAGAGGGGCAA
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CGTTTAGTAATAAGTAGTCAAATTCAATAATTTTTTCAGGTTCGATGTTCCAAGCCGTCGACAA
TTTTCTAACATCGCACTTCAAAAATTACCACATTTATATTTTTTGGTTAACATAATATTATTATTT
GTGAAGGGCGGCTTTTCTTAGTTTCATTAACATACCGACTTTAAAAAAACAAAAAAACAAACC
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ACAATTCCAATCTACCAGGTTTTTTTTTTGTTTTTAAAAGAAAATTCATTTTTTTTTTTTTTTTTT
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>pHRV3_promoter_sequence

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>pHRV5_promoter_sequence

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