Investigating the biological roles of the *HSPRO* genes in *Arabidopsis thaliana*

by

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ABSTRACT

Investigating the biological roles of the *HSPRO* genes in *Arabidopsis thaliana*

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As a consequence of an immobile lifestyle, plants have had to evolve appropriate perception mechanisms and responses to diverse environmental stresses. Stress can be the result of both biotic and abiotic agents and the *ORTHOLOG OF SUGAR BEET HS1 PRO-1* (*HSPRO1*) and *HSPRO2* genes were previously shown to be induced in response to several stresses including infection with *Pseudomonas syringae* and drought stress in *Arabidopsis thaliana*. The aim of this study was to characterise the biological role(s) played by these proteins in *Arabidopsis*. Several bioinformatics approaches provided evidence that supported function of both genes in response to both biotic and abiotic stresses and identified potential regulatory elements that may drive *HSPRO* gene expression during stress responses. Accordingly, analysis of null *hspro* mutants revealed antagonistic functions of the two proteins in PAMP-triggered immunity to *P. syringae* infections of shoot tissues and osmotic stress tolerance in plant roots. HSPRO proteins have been shown to interact with a central integrator of stress and energy signalling, SUCROSE NON-FERMENTING-1-RELATED KINASE1 (SnRK1) and microarray analysis of the null mutants suggested potential roles in carbohydrate signalling. An array of energy responsive genes including a subset of SnRK1 targets were misregulated in *hspro* mutants under standard growth conditions supporting involvement of *HSPRO* in energy signalling. Mutant phenotype and gene expression analysis revealed that *HSPRO2* may be of importance in energy perception as *hspro2* seeds were hypersensitive to exogenous glucose during germination, and that perception and/or signalling of low energy status may require HSPRO2. Although *HSPRO2* expression may be driven via perception of environmental stress cues, promoter-luciferase assays revealed a diurnal expression pattern of the gene that was driven by the circadian clock. However, phenotypic analysis did not reveal a requirement of *HSPRO2* for normal clock modulation. Since stress perception typically causes fluctuations in energy levels, it is proposed that *HSPRO* genes are important for the integration of energy and stress signalling in an effort to maintain a homeostatic balance between coping with environmental stress and normal growth and development.

LIST OF ABBREVIATIONS

µL	microlitre
µM	micromolar
7-TM GPCR	seven-transmembrane G-protein-coupled receptor
ABA	abscisic acid
ABC	ATP-binding cassette
ABF	ABRE binding factor
ABRE	ABA responsive element
ACC	1-aminocyclopropane-1-carboxylic acid
Act D	actinomycin D
AGPase	ADP-glucose pyrophosphorylase
AHK	Arabidopsis histidine kinase
AMP	adenosine 5-monophosphate
AMPK	AMP activated protein kinase
AREB	ABA responsive element binding protein
ATP	adenosine 5-triphosphate
AvrB	*P. syringae* pv *tomato* DC3000 (*avrB*)
BAR	Bio-Analytic Resource
BiFC	Bimolecular fluorescence complementation
bp	base pair
bZIP	basic region/leucine zipper
CBF	C-repeat binding factor
CBL	calcineurin B-like
CBM	carbohydrate binding motif
cDNA	complementary DNA
CDPK	calcium-dependent protein kinase
cfu	colony forming units
CIPK	CBL interacting protein kinase
CRK	cysteine-rich RLK
CRT	C-repeat
DAMP	damage associated molecular pattern
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxynucleosidetriphosphate

DRE	dehydration responsive element
DREB	dehydration responsive element binding
DTT	dithiothreitol
DW	dry weight
ECGG	expression correlated gene group
EDTA	ethylenediamine tetraacetic acid
eLRR	extracellular leucine-rich repeat
ET	ethylene
EtBr	ethidium bromide
ETI	effector triggered immunity
EtOH	ethanol
F_n	n^{th} filial generation
FatiGO	Fast Assignment and Transference of Information using Gene Ontology
FW	fresh weight
g	grams
g	gravity constant (9.81 $m.s^{-1}$)
GA	gibberellin
GC-MS	gas chromatography–mass spectrometry
GO	gene ontology
GOI	gene of interest
GPCR	G-protein-coupled receptor
h	hour
HK	histidine kinase
hpi	hours post infection
HR	hypersensitive response
HXK	hexokinase
IAA	indole-3-acetic acid
JA	jasmonic acid
KA	kinase-associated
kb	kilobase
KB	King‘s B
KIS	kinase interacting sequence
L	litre

LB	left border
LB	Luria Bertani
LD	long day
LRR-RLK	leucine-rich repeat receptor –like kinase
M	molar
MAPK	mitogen activated protein kinase
MAPKK	mitogen activated protein kinase kinase
MAPKKK	mitogen activated protein kinase kinase kinase
min	minute(s)
miRNA	microRNA
mL	millilitre
mM	millimolar
MOPS	4-morpholinepropanesulfonic acid
mRNA	messenger RNA
MS	Murashige and Skoog
MT	mutant
MW	molecular weight
NACR	NAC recognition
NaOAC	sodium acetate
NB-LRR	nucleotide-binding leucine-rich repeat
nL	nanolitre
nM	nanomolar
^{o}C	degrees Celsius
OD	optical density
p	probability
PAMP	pathogen associated molecular pattern
PCD	programmed cell death
PCR	polymerase chain reaction
P_i	inorganic phosphate
PP2C	protein phosphatase 2C
ppm	parts per million
PR	pathogenesis related
PRR	pattern recognition receptors
Pst	*P. syringae* pv *tomato* DC3000

PTI	PAMP triggered immunity
PYL	pyracbactin resistance-like
PYR	pyracbactin resistance
QTL	quantitative trait locus
R protein	resistance protein
RB	right border
RCAR	regulatory component of ABA receptors
RE	restriction endonuclease
RLCK	receptor-like cytoplasmic kinase
RLK	receptor-like kinase
RNA	ribonucleic acid
RNI	reactive nitrogen intermediate
ROS	reactive oxygen species
RT-qPCR	reverse transcriptase –quantitative PCR
S.D.	standard deviation
S.E.M	standard error of the mean
SA	salicylic acid
SAR	systemic acquired resistance
SD	short day
SDS	sodium dodecyl sulphate
sec(s)	second(s)
SIRT	sucrose induced translational repression
Snf	sucrose non-fermenting
SNP	single nucleotide polymorphism
SnRK	sucrose non-fermenting-1-related kinase
SRS	sugar response sequence
T-6-P	trehalose-6-phosphate
T-DNA	transfer DNA
TF	transcription factor
TFBS	transcription factor binding site
TMV	tobacco mosaic virus
T_n	n^{th} transformant generation
TOR	target of rapamycin
TPH	trehalose phosphate hydrolase

TPS	trehalose-P synthase
TTSS	type three secretion systems
UTR	untranslated region
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume
WRKY	WRKY DNA binding protein
WT	wild type
YFP	yellow fluorescent protein
ρ	Pearson‘s correlation coefficient

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CHAPTER 1

Introduction

CHAPTER 1: INTRODUCTION

1.1 BIOTIC AND ABIOTIC STRESS RESPONSES

The sessile nature of plant life cycles presents additional challenges during growth and development as unlike animals, plants cannot evade environmental stresses, and have instead evolved mechanisms to effectively deal with stressful conditions while maintaining growth. Environmental stress can be imposed via pathogen attack or herbivory (biotic stress) or can be a result of a change in the physical environment such as drought, oxidative stress, salinity, low and high temperatures, anaerobic conditions and high light intensity (abiotic stress) (Agarwal & Grover, 2006; Bailey-Serres & Voesenek, 2008; Chaves & Oliveira, 2004; Hirel *et al.*, 2007; Nakashima & Yamaguchi-Shinozaki, 2006; Wang *et al.*, 2003). Unlike in controlled experimental conditions, plants often experience multiple stresses simultaneously and unique combinatorial stress responses are often required to successfully survive (Atkinson & Urwin, 2012; Suzuki *et al.*, 2014). Ultimately, stress responses attempt to minimise stress-related damage while sustaining life through conservation of resources for vegetative and reproductive growth and development.

1.1.1 Plant Innate Immunity

1.1.1.1 Detection of non-self and response – PTI

It is general consensus that plant innate immunity occurs through two branches namely pathogen associated molecular pattern (PAMP) triggered immunity (PTI) and effector triggered immunity (ETI). Most pathogens have characteristic and evolutionary conserved molecules and structures associated with them that are essential for the organism‘s survival. The detection of these PAMPs and damage associated molecular patterns (DAMPs) by the host through transmembrane pattern recognition receptors (PRRs) leads to the induction of defence responses (Beck *et al.*, 2012). DAMPs are the by-products of pathogen directed damage to the host and include cell wall fragments (Darvill & Albersheim, 1984), cutin monomers (Kauss *et al.*, 1999) or peptides (Huffaker *et al.*, 2006) whose detection in the apoplast induce immune responses comparable to that activated by PAMPs (Henry *et al.*, 2012). Cognate P/DAMP:PRR interactions are very specific and dependent on recognition of particular epitopes within the P/DAMP, as is the case with recognition of the flagellin epitope, flg22, by FLS2 (Chinchilla *et al.*, 2006).

Although numerous PAMP/PRR interactions exist, the downstream effects are typically similar (Jones & Dangl, 2006). PAMP recognition triggers heteromerisation of PRRs which in turn leads to the activation of downstream MITOGEN-ACTIVATED PROTEIN KINASE (MAPK) signal transduction pathways that activate WRKY transcription factors which in turn regulate the expression of early defence response genes. PTI is characterised by a rapid alkalinisation of the pathogen growth environment (Garcia-Brugger *et al.*, 2006), the production of reactive oxygen and nitrogen intermediate species (ROS and RNI) (Muthamilarasan & Prasad, 2013), stomatal closure (Melotto *et al.*, 2006), callose deposition between the cell wall and plasma membrane (Brown *et al.*, 1998) and the biosynthesis of the phytohormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) (Tsuda *et al.*, 2008). SA signalling is typically associated with responses to biotrophic and hemi-biotrophic pathogen infection while detection of necrotrophs and herbivorous insects is more likely to activate JA/ET-dependent signalling (Kessler & Baldwin, 2002; Thomma *et al.*, 2001). Signalling crosstalk exists between the two networks and although this crosstalk is predominantly mutually antagonistic, multiple synergistic interactions between SA and JA/ET signalling have been revealed *in planta*, highlighting the complexity of hormone signalling networks (Koornneef & Pieterse, 2008; Mur *et al.*, 2006; Pieterse *et al.*, 2009). These early responses ultimately create a hostile environment for pathogen survival preventing entry into the cells, limiting carbon availability and actively targeting them with antimicrobial agents.

1.1.1.2 Successful infection via suppression of PTI – effector triggered susceptibility

Pathogenic bacteria can secrete effectors into the host cytoplasm via type III secretion systems (TTSS). Effector molecules (the majority of which are proteins) are useful during host colonisation and they function through the suppression of PTI and enable the acquisition of nutrients from the host. Effectors can function as host transcription factors directly regulating host gene function, target host transcription factors (TFs) or regulate DNA packaging and configuration all with the overall purpose of controlling nutrient availability (Abramovitch *et al.*, 2006; Feng *et al.*, 2012; Grant *et al.*, 2006; Mudgett, 2005). *P. syringae* mutants with defective TTSS are unable to utilise effectors in this regard and unlike the isogenic wild type strain, cannot successfully colonise the host (Jakobek *et al.*, 1993).

Many bacterial effectors have been characterised and their modes of function are just as diverse as their structures. Some effectors target vesicle transport (Nomura *et al.*, 2006), others inhibit signal transduction in the PTI, via interaction with the serine/threonine domain of PRRs upstream of MAPK kinase kinase (MAPKKK) (He *et al.*, 2006; Xiang *et al.*, 2008) while some regulate host protein degradation (Janjusevic *et al.*, 2006). Other effectors exploit the antagonism between the SA and JA/ET branches of the immune network, exemplified by the antagonistic suppression of SA-mediated signalling by coronatine, a mimic of JA that induces stomatal opening to facilitate pathogen entry (Brooks *et al.*, 2005; Melotto *et al.*, 2006; Navarro *et al.*, 2006; Zheng *et al.*, 2012). *B. cinerea* has been shown to secrete β-(1,3),(1,6)-D-glucan, an exopolysaccharide that abrogates plant immunity via manipulation of the antagonistic effect of SA on JA signalling (El Oirdi *et al.*, 2011). Eukaryotic pathogens also produce effector molecules that can either be secreted into host cells or like some *Cladosporium fulvum* effectors, function in the extracellular matrix (Rivas & Thomas, 2005).

1.1.1.3 Plant evolutionary response to pathogen evasion of PTI – ETI

In the arms race model of pathogen:host interactions, plants evolved RESISTANCE (R) proteins that can directly or indirectly detect effector molecules and mount an immune response similar to PTI but much more rapid and aggressive and this is effector triggered immunity (Tao *et al.*, 2003; Thilmony *et al.*, 2006; Truman *et al.*, 2006). Once detectable via R protein recognition, an effector is re-classified as an avirulence factor. A majority of R proteins belong to the family of intracellular nucleotide-binding leucine-rich repeat (NB-LRR) and the other major group classified as extracellular LRR (eLRR) proteins (Fritz-Laylin *et al.*, 2005; Jones & Dangl, 2006). NB-LRRs can recognise effectors directly or via effector modification of the host protein(s) that are guarded by the R protein. These host proteins are particularly interesting because they can be genuine targets of effectors or they can be decoys that the host has evolved to mimic the respective effector targets (Dangl & Jones, 2001; Dodds & Rathjen, 2010; van der Hoorn & Kamoun, 2008). Direct interaction with effectors causes two conformational changes in NB-LRRs that result in interaction and activation of downstream targets (Takken, 2009) while indirect interactions are slightly more complicated. For example, the host protein RIN4 can either be phosphorylated or degraded due to effector interaction and these modifications cause changes in the regulation of two NB-LRR proteins (Mackey *et al.*, 2003), RPM1 and RPS2 resulting in ETI induction (Kim *et al.*, 2005; Leister *et al.*, 1996; Mackey *et al.*, 2002).

ETI may be a faster response because its NB-LRR dependent signalling pathway is thought to have less components and functions directly in the nucleus (Altenbach & Robatzek, 2007; Cheng *et al.*, 2009). Similarly to PTI, ETI also activates downstream MAPK cascades and WRKY TFs resulting in the activation of *PATHOGENESIS RELATED* (*PR*) genes that induce phytohormone signalling, cell wall strengthening and lignification and production of antimicrobial agents eventually culminating in the hypersensitive response (HR) (Eichmann & Schäfer, 2012; Iwai *et al.*, 2006; Nomura *et al.*, 2012). HR results in localised programmed cell death (PCD) and is an attempt to quarantine and neutralise the pathogenic threat. Effector:R protein dynamics are constantly changing and through selective pressures, non-target alleles of effectors can become dominantly expressed resulting in R protein redundancy. Alternatively, novel effector molecules evolve that can directly suppress the ETI and since evolutionary pressure results in host counteractions the cycle perpetuates (Jones & Dangl, 2006).

1.1.1.4 Systemic acquired resistance

During infection, signalling molecules such as methyl-SA, glycerolipids and azeleic acid can be transported to sites distal to the infection site and cause the induction of a long lasting and broad spectrum disease response called systemic acquired resistance (SAR) (Liu *et al.*, 2011). SAR prepares the rest of the host organism for the pathogenic threat and requires the accumulation of endogenous SA leading to transcriptional reprogramming of *PR* genes (Park *et al.*, 2007b; van Loon *et al.*, 2006). The activated *PR* genes can be classified as either immediate-early genes that are activated early in responses to exogenous SA and pathogen attack or genes induced later exemplified by the hallmark gene of SA signalling, *PR1* (Horvath *et al.*, 1998; Lebel *et al.*, 1998).

1.1.2 Abiotic stress responses

Abiotic stress responses in plants are diverse and can vary across species and typically trigger changes in gene expression, metabolism, vegetative growth and plant development (Nuruzzaman *et al.*, 2013) in an attempt to maintain a balance between growth, development and survival (Mazzucotelli *et al.*, 2008). Abiotic stress can have implications on plant productivity and yield and so understanding mechanisms involved in abiotic stress response is critical for global food security, especially in the context of the ever-increasing human population and climate change. Due to the highly diverse and complicated response to abiotic stress only a general overview shall be described in this section with a

focus on key players of signalling responses such as ROS, receptor-like kinases (RLK) and abscisic acid (ABA).

1.1.2.1 Stress perception

Different stresses often have different effects on plant biology due to their different modes of action, ultimately resulting in cell damage. For instance, salt stress imposes specific Na^+ damage as well as osmotic stress, while UV-B treatment results in DNA damage through pyrimidine dimer formation (Hasegawa *et al.*, 2000; Kaiser *et al.*, 2009). Since there is a diverse array of abiotic stresses that plants can experience it makes sense that there would be an equally diverse number of stress-specific perception and response mechanisms. However, there is evidence that supports the simultaneous activation not only of specific but also general stress responses and reactive oxygen species have been identified as a universal component in the generalised perception of abiotic stress (Vaahtera & Brosché, 2011). Other general stress effects include osmotic stress caused by salt, drought and cold stress (Yamaguchi-Shinozaki & Shinozaki, 2006).

ROS were previously characterised as damaging agents in cells but more recent research has identified them as crucial signalling molecules in the regulation of abiotic stress responses. ROS are particularly useful in this regard since essentially all abiotic stresses result in the production of ROS even though the types of ROS produced are often varied and specific to the sub-cellular compartment (Jaspers & Kangasjärvi, 2010). ROS are unstable and have a short half-life which has made identifying ROS perception proteins *in planta* quite challenging and mechanisms of ROS perception are not well understood. Several putative ROS perception molecules have been investigated to date including the STIG1-like protein, GRIM reaper which is involved in the regulation of programmed cell death in response to ROS (Wrzaczek *et al.*, 2009). Other research has suggested that certain heat shock transcription factors could be functioning as direct sensors of ROS during oxidative stress in plants but the mechanisms of function are still unclear (Miller & Mittler, 2006).

An increase in cytosolic Ca^{2+} is a well characterised early response to both biotic and abiotic stress in plants and perception of these changes in Ca^{2+} concentrations results in signal transduction via calcium sensing and/or calcium binding proteins (Evans *et al.*, 2001; Knight & Knight, 2001; Sanders *et al.*, 2002). Of the several Ca^{2+} detecting family of

proteins, calcium-dependent protein kinases (CDPKs) and calcineurin B-like (CBL) interacting protein kinases (CIPKs) are able to translate this perception of calcium to signal transduction via protein phosphorylation and ultimately lead to transcriptome reprogramming (Franz *et al.*, 2011). When cytosolic Ca^{2+} concentrations are low, CDPKs exist in an inactive state due to an autoinhibitory domain. Rises in Ca^{2+} and a prerequisite direct binding of Ca^{2+} to the calmodulin-like domain of CDPKs, results in a conformational change that activates protein function (Christodoulou *et al.*, 2004; Harper *et al.*, 2004; Harper & Harmon, 2005).

Leucine-rich repeat receptor-like kinases (LRR-RLKs) and numerous other RLKs form a large family of proteins in plants and are involved in the perception of environmental cues and appropriate signal response transduction (through their protein kinase activity), in addition to regulation of growth and development (Gish & Clark, 2011; Shiu & Bleecker, 2001a; Shiu & Bleecker, 2001b; Shiu & Bleecker, 2003). Multiple transcriptome profiling experiments monitoring *RLK* gene expression under various environmental stresses have revealed that RLKs are differentially expressed in response to environmental stress, supporting their involvement in abiotic stress responses (Chae *et al.*, 2009; Hwang *et al.*, 2011; Lehti-Shiu *et al.*, 2009; Marshall *et al.*, 2012).

There are many RLKs involved in abiotic stress responses and they can both positively or negatively regulate responses to stress. An example of a positive regulator of water stress is RECEPTOR-LIKE PROTEIN KINASE1 (RPK1), whose expression is induced by dehydration, high salinity, cold stress and ABA (Osakabe *et al.*, 2013). RPK1 is thought to be a positive regulator of water stress responses via ABA signalling as *rpk1* null mutants are ABA insensitive and display lower levels of water stress gene induction under stress conditions. In agreement with this, overexpression of *RPK1* confers increased tolerance to drought correlated with constitutive expression of stress-responsive genes (Osakabe *et al.*, 2010).

CYSTEINE-RICH RECEPTOR LIKE PROTEIN KINASE36 (CRK36) is a member of the cysteine-rich RLKs (CRK) family and is a recently identified negative regulator of osmotic stress and ABA signalling in *Arabidopsis thaliana* (hereafter *Arabidopsis*) (Tanaka *et al.*, 2012). *CRK36* is up-regulated in response to abiotic stresses (Wrzaczek *et al.*, 2010) and *crk36* knockdown mutant seedlings have an increased sensitivity to ABA and osmotic

stress and induction of ABA-responsive genes (Tanaka *et al.*, 2012). There are several other subfamilies of RLKs thought to be involved in abiotic stress signalling such as the proline-rich extensin-like receptor kinase family, with *PERK4* identified as a positive regulator of ABA and Ca^{2+} dependent responses. Calcium/calmodulin modulated signalling pathways are crucial in response to cold and drought stress and an example of calcium/calmodulin regulated receptor-like cytoplasmic kinases (RLCKs), has been reported to regulate cold responses and induction of cold response genes (Yang *et al.*, 2010).

Histidine kinases (HKs) are another family of transmembrane proteins that are involved in abiotic stress signal transduction. Cytokinin receptor *Arabidopsis* histidine kinases (AHKs) have been shown to negatively regulate abiotic stress responses in a cytokinin-mediated manner (Kumar & Verslues, 2014; O'Brien & Benkova, 2013; Osakabe *et al.*, 2013) while the non-cytokinin receptor kinase AHK1 has been identified as an osmosensor with a positive regulatory role on abiotic stress signal transduction (Tran *et al.*, 2007b; Wohlbach *et al.*, 2008). Phenotypic analysis with *ahk1* and *35S::AHK1* revealed that AHK1 is required for tolerance of osmotic stress and that AHK1 regulates the downstream targets of osmotic stress signalling in both an ABA-dependent and an ABA-independent manner (Wohlbach *et al.*, 2008). AHK5 has also been implicated in the regulation of stomatal closure in response to ROS (H_2O_2) signalling in an ABA-independent manner. Interestingly AHK5 has recently been shown to positively regulate salt stress responses and is important for resistance to both virulent *P. syringae* and *B. cinerea (Pham et al., 2012)*.

1.1.2.2 Signal transduction

Following perception of an abiotic stress signalling pathways that integrate the stress perception into plant signalling networks are activated. Protein kinases such as MAPKs, phosphatases and Ca^{2+}-DEPENDENT PROTEIN KINASES (CDPK) (Ichimura *et al.*, 2000; Mehlmer *et al.*, 2010; Miller *et al.*, 2008; Mizoguchi *et al.*, 1997; Takahashi *et al.*, 2011) are a critical part of signal transduction and mediate responses to abiotic stress that result in transcriptional reprogramming (de la Fuente *et al.*, 2008; Nakagami *et al.*, 2010; Sugiyama *et al.*, 2008).

Up to 60 putative MAPKKK, 10 MAPKKs and 20 MAPKs have been identified in the *Arabidopsis* genome and it is thought that specific stresses can activate specific MAPKKK

which in turn converge on generalised MAPKKs which in turn can activate diverse MAPKs (through protein phosphorylation). For instance, the MAPK MPK6 is activated by both MKK2 and MKK3 but unlike MKK2, MKK3 is not required for salt tolerance responses during salt and cold stresses (Takahashi *et al.*, 2007; Teige *et al.*, 2004). MAPKs have additional regulatory roles outside of signal transduction cascades and can directly interact with TFs and their promoters, as is in the case of MEKK1 (MAPKKK) that phosphorylates the WRKY53 TF and also binds directly to its promoter to regulate gene function (Miao *et al.*, 2007).

SUCROSE NON-FERMENTING-1-RELATED KINASE3 (SnRK3) and CDPK families of proteins are thought to be the targets of Ca^{2+} based stress signalling. There are at least 25 SnRK3 protein complexes in *Arabidopsis* and SnRK3.11 is the most characterised of them all. SnRK3.11 functions in conjunction with SALT OVERLY SENSITIVE3 (SOS3) and activates SOS1; a membrane bound Na^{+}/H^{+} transporter that is essential for salt tolerance (Luan, 2008; Mahajan *et al.*, 2008). Of the more than 30 CDPK proteins identified in *Arabidopsis* several have been shown to be abiotic stress activated and can regulate ABA-dependent signalling, possibly through phosphorylation of ABA-responsive TFs such as AREB/ABFs.

1.1.2.3 ABA signalling during abiotic stress

The phytohormone ABA is a key signalling molecule in the plant responses to abiotic stress. SA, ET, JA and other plant hormones have also been shown to have links to abiotic stress responses through complex interactions with the ABA signalling network (Fedoroff, 2002; Fujita *et al.*, 2006; Grant & Jones, 2009; Pieterse *et al.*, 2009) and so although ABA is the most characterised hormone in abiotic stress responses, it is increasingly apparent that it does not function in isolation.

ABA levels are typically regulated by a balance between hormone synthesis and degradation (Nambara & Marion-Poll, 2005; Nilson & Assmann, 2007). Perception of abiotic stresses often leads to altered ABA levels resulting in the transcriptional reprogramming of ABA-dependent genes and physiological changes that are necessary for stress tolerance. For instance during drought stress, NINE-CIS-EPOXYCAROTENOID DIOXYGENASE3 (*NCED3*) expression is strongly up-regulated and in loss-of-function *nced3* mutants the typical stomatal closure require for water retention and associated with

NCED3 induction is abolished (Iuchi *et al.*, 2001). In addition to regulating stomatal closure, ABA signalling often results in the accumulation of osmoprotectants such as myoinositol, galactinol and fumarate that enhance the plants ability to retain water under water deprivation stress (Qin *et al.*, 2011)

Changes in ABA levels during stress responses typically occur in vascular tissue but many of the effects of ABA signalling are observed in distal cells and tissue and there is a prerequisite for ABA translocation if distal ABA responses are to occur (Kuromori *et al.*, 2010). Plasma membrane localised, ATP-binding cassette (ABC) and nitrate transporter 1/peptide transporter (NRT1/PTR) protein families have been recently implicated in ABA translocation in plants (Boursiac *et al.*, 2013; Kang *et al.*, 2010a; Kanno *et al.*, 2012; Kuromori *et al.*, 2010).

Signal transduction following detection of ABA by pyracbactin resistance/pyracbactin resistance-like/regulatory component of ABA receptors (PYR/PYL/RCARs) (Ma *et al.*, 2009; Melcher *et al.*, 2010; Nishimura *et al.*, 2009) is effected negatively by protein phosphatase 2Cs (PP2Cs) and positively by SnRK2 complexes (Mustilli *et al.*, 2002; Schweighofer *et al.*, 2004; Umezawa *et al.*, 2009). In the absence of ABA, PP2C proteins interact with SnRK2 and dephosphorylate a serine residue in the kinase activation loop whose phosphorylated state is required for kinase activity and signal transduction resulting in negative regulation of ABA responses (Ma *et al.*, 2009; Soon *et al.*, 2012; Umezawa *et al.*, 2009). Upon interacting with ABA, PYR/PYL/RCAR receptors interact with the catalytic site of PP2Cs and effectively block the interaction with SnRK2 (Melcher *et al.*, 2010; Miyazono *et al.*, 2009; Nishimura *et al.*, 2009; Santiago *et al.*, 2009). Without the interaction with PP2Cs SnRK2 proteins are activated via autophosphorylation (Boudsocq *et al.*, 2007) and/or phosphorylation by unidentified upstream kinases (Boudsocq *et al.*, 2007; Burza *et al.*, 2006), and downstream signalling can occur (Cutler *et al.*, 2010; Hubbard *et al.*, 2010; Umezawa *et al.*, 2010).

Several basic region/leucine zipper (bZIP) TFs have been identified as the downstream targets of SnRK2 activity, particularly in response to drought, salt and cold stresses. The promoters of ABA-induced genes typically have multiple copies of conserved motifs called ABA responsive elements (ABRE) that are the binding sites of a family of bZIP TFs called ABFs (ABRE binding factor) / AREBs (ABA responsive element binding protein) that are

required for gene activation (Gómez-Porras *et al.*, 2007; Umezawa *et al.*, 2010; Zhang *et al.*, 2005). MYB and MYC TFs are also involved in ABA-dependent gene regulation and they are capable of binding to a diverse range of *cis*-acting elements in order to effect a change in gene regulation (Abe *et al.*, 2003; Atkinson & Urwin, 2012).

It is worth noting that ABA-independent signalling pathways exist and the interaction of C-repeat binding factor/dehydration responsive element binding (CBF/DREB) TFs with the dehydration responsive element/C repeat (DRE/CRT) *cis*-acting elements in the promoters of cold, dehydration, drought and salinity stress responsive genes are a typical ABA-independent TF/*cis*-element interactions (Agarwal & Jha, 2010; Hirayama & Shinozaki, 2010; Qin *et al.*, 2011; Shinozaki & Yamaguchi-Shinozaki, 2007; Thomashow, 2001). Some of the members of the NAM ATAF1 CUC2 (NAC) protein family such as EARLY RESPONSIVE TO DEHYDRATION STRESS1 (ERD1) and ARABIDOPSIS NAC DOMAIN-CONTAINING PROTEIN2 (ANAC002, also known as ATAF1) are also part of a group of ABA-independent TFs that recognise and bind NAC recognition sites (NACRS) and induce changes in gene expression of drought and salinity stress response genes (Atkinson & Urwin, 2012; Nakashima & Yamaguchi-Shinozaki, 2006; Tran *et al.*, 2007a).

1.2 SUGAR SENSING AND SIGNALLING

Regulation of energy levels is critical in the homeostatic control of the plant internal environment and therefore survival and growth and is achieved through the coordination of environmental, metabolic and developmental cues (Eveland & Jackson, 2012; Ramon *et al.*, 2008). Energy-rich sugars generated from photosynthesis are involved in the regulation of photosynthesis itself and also in the regulation of other sugar content-altering metabolic processes such as respiration, sugar transport and immobilisation (e.g. in cell walls). Although the self-regulation of cellular carbon energy levels by sugars is well documented, it is becoming increasingly apparent that sugars are also crucial signalling molecules in a multitude of biological processes in autotrophic eukaryotes including but not limited to abiotic stress responses, plant innate immunity, seed germination and flowering of adult plants (Eveland & Jackson, 2012; Rolland *et al.*, 2006). It is likely that the high energy demands of these biological processes necessitate the integration with the energy status of the plant to allow for efficient growth and development.

1.2.1 Photoautotrophic generation, transportation and utilisation of sugars

An array of sugars are produced and utilised within the plant depending on temporal and spatial factors but the primary process responsible for sugar production is the photosynthetic conversion of CO_2 and water to carbohydrates and oxygen, utilising light as an energy source within the plant chloroplasts (Rolland *et al.*, 2002). Plastid sugars are typically converted to triose-phosphates before export to the cytosol where they are converted to hexose-phosphates, sucrose and starch. The hexose sugars and sucrose either remain in the cytosol for immediate use throughout the light period or excess sugar is stored as sucrose or converted to starch for subsequent use under energy limiting periods such as night time. Sugars are essential for metabolism across all plant tissue types and during the day sucrose is also transported to non-photosynthetic sink tissues and organs such as roots (Lemoine *et al.*, 2013). In sink tissues, the sucrose is converted to a range of hexose sugars by invertases or sucrose synthases for utilisation as carbon backbone in other metabolic pathways or stored as excess energy in the form or starch. At night, plastid stored starch is re-mobilized and converted into maltose and glucose which are then exported as a continual energy source in the absence of light-dependent carbon fixation (Lu *et al.*, 2005). Typically, photosynthetic tissue-based starch is a transitionary energy reserve while long-term starch storage occurs in non-photosynthetic cells such as roots, tubers and seeds.

1.2.2 Elucidating sugar signalling mechanisms

Different organisms have adapted various and complex regulatory mechanisms to consistently deal with the ever-changing environment. Effectively, these mechanisms involve control of genes and proteins and their by-products to tightly regulate overall plant responses to environmental changes. In plants, an increase in sugar availability is typically characterised by the induction of sink function genes involved in polysaccharide biosynthesis, generation of storage proteins as well as genes associated with respiration and defence response (Gupta & Kaur, 2005; Price *et al.*, 2004). On the other hand, sugar limitation results in the up-regulation of source function photosynthetic genes and resource remobilisation genes such as those regulating starch and lipid degradation (Ho *et al.*, 2001; Koch, 1996; Yu, 1999). While differential gene responses to nutrient availability have been documented in numerous experiments the fundamental mechanisms of sugar regulation are still poorly misunderstood (Cui, 2012; Dai & Chen, 2012; Eveland & Jackson, 2012; Price *et al.*, 2004; Price *et al.*, 2004; Tiessen & Padilla-Chacon, 2013).

1.2.2.1 Transcriptome profiling analysis utilising signalling mutants

Various microarray studies done on *Arabidopsis* ecotypes and sugar signalling defective mutants have revealed an overwhelming amount of novel information regarding sugar signalling. Global expression studies in response to glucose treatment show that a varying proportion of the transcriptome is glucose responsive. In one study, 534 and 444 genes were down-regulated and up-regulated in response to glucose, respectively and it was seen that transcriptional gene activation by glucose largely requires *de novo* protein synthesis and less so in gene repression (Price *et al.*, 2004). Glucose treatment largely represses the expression of monosaccharide transport, peptide transport and purine transport genes while additionally inducing expression of polysaccharide biosynthetic genes. Many of these polysaccharide biosynthesis genes were also shown to be repressed during extended dark periods in the absence of sugar implicating energy detection and signalling in the regulation of plant carbohydrate metabolism (Thimm *et al.*, 2004; Thum *et al.*, 2004). Trehalose-6-phosphate (T-6-P) has been described as being important in energy signalling under high energy conditions (Yadav *et al.*, 2014) and it was noted that glucose both represses and induces the expression of various T-6-P synthase genes and this is of potential significance as it shows cross talk between two sugar signalling pathways.

Carbon starvation in plants leads to transcriptional repression of biosynthetic pathways that utilise sugars such as starch synthesis, glycolysis, amino acid synthesis, nucleotide synthesis, and the induction of processes that salvage carbohydrates from other sources, using sugar invertases for instance (Usadel *et al.*, 2008). Signalling mutants have proved indispensible in the analysis of sugar detection and regulation. For example, characterisation of gene responses in the *Arabidopsis pho3* mutant helped identify sugar regulated genes and processes. The *pho3* mutant has a defective *SUCROSE-PROTON SYMPORTER2* (*SUC2*) gene and as such cannot load sucrose into the phloem from source tissue. This leads to an accumulation of the soluble sugars glucose, fructose and sucrose and the storage sugar starch in shoot tissue. Genes involved in anthocyanin biosynthesis are induced in this mutant much like in sugar treated wild type plants suggesting a sugar-dependent regulation of anthocyanin in plants. The starchless mutant *phosphoglucomutase* (*pgm*) also displays similar anthocyanin specific responses as *pho3* and it also accumulates soluble sugars but cannot convert them to starch (Solfanelli *et al.*, 2006).

1.2.2.2 Transcriptional control through transcription factors and promoter elements

Sugar treatment or limiting conditions are known to regulate an array of genes in plants. In *Arabidopsis*, there is a relative overrepresentation of glucose responsive TFs (8.3%) compared to the estimated 5 to 7% total TF count in the entire genome. There is an additional enrichment of stress related transcription factors in this subset when compared to the entire genome (Price *et al.*, 2004). Group-S bZIPs are generally considered to energy and stress signalling TFs (Jakoby *et al.*, 2002; Llorca *et al.*, 2014; Matiolli *et al.*, 2011). Different members of the S1 sub-group have been shown to be either sugar-inducible or sugar repressible (Price *et al.*, 2004) and specifically *bZIP1* in *Arabidopsis* is sugar repressed in a reversible manner. bZIP1 has been shown to interact with the ACGT cores of C-box, G-box and C/G hybrid Hex-box promoter motifs implicated in sugar signalling (Kang *et al.*, 2010b).

The WRKY transcription factor SUGAR SIGNALING IN BARLEY2 (SUSIBA2) has been shown to be involved in sugar signalling in barley. Not only is the *SUSIBA2* gene sugar inducible but the protein only actively interacts with the SURE (sugar responsive element) and W-box of the *ISO1* promoter upon detection of sugars and activates gene expression

(Sun *et al.*, 2003). Of further significance is the observation that SUSIBA2 directly regulates starch biosynthesis which is a typical, glucose-induced sink response.

In sweet potato the transcription factor SPF1 has been identified as being sucrose repressed in leaf tissue and it binds to the SP8a and SP8b 5‘ UTR sequences of three different sporamin and β-amylase genes which regulate carbohydrate levels in potatoes (Ishiguro & Nakamura, 1994). SPF1 has putative orthologues in *Arabidopsis* and other plant species implying its significance in plant sugar signalling regulation. Another transcription regulator identified in potato research is STOREKEEPER (STK), a TF that specifically targets the B-box motif and regulates the sucrose induced accumulation of patatin, a storage molecule in potatoes (Zourelidou *et al.*, 2002).

During sugar starvation α-amylase genes are typically induced and all cereal-associated α-amylase genes contain a TATCCA domain or its variants in the 5‘ UTR. Three rice MYB transcription factors have been shown to interact with this domain in a sugar availability-responsive manner, directing the accumulation or depletion of α-amylase gene transcripts (Lu *et al.*, 2002). This α-amylase gene promoter study revealed a novel sugar response sequence (SRS) that is required for regulation of α-amylase genes in rice. The SRS is characterised by the already mentioned TATCCA domain, the GC-box and the G-box which are all crucial for successful sugar starvation/abundance based gene regulation (Lu *et al.*, 2002).

More recently, ARABIDOPSIS NAC DOMAIN CONTAINING PROTEIN60 (ANAC060) was identified as glucose responsive transcription factor. Interestingly, the protein was identified through quantitative trait locus (QTL) analysis of a Col-0/C24 F_2 population and a single nucleotide polymorphism (SNP) in Col-0 results in a truncated splice variant that leads to a sugar insensitivity phenotype while the C24 full variant produces a hypersensitivity phenotype (Li *et al.*, 2014). Glucose induces *ANAC060* in an ABA signalling-dependent manner as the ABA INSENSITIVE4 (ABI4) TF actively binds to the *ANAC060* promoter and drives expression. Glucose signal transduction can occur in either an ABA-dependent or independent manner. Curiously, Col-0 ANAC060 reduces glucose induced ABA accumulation and *ABI4* accumulates to a lesser extent in this ecotype. Although sugar signalling induces *ANAC060* expression, it is thought that the truncated

variant additionally attenuates ABA induction and signalling and this results in the glucose insensitivity.

1.2.2.3 Transcript stability and processing

mRNA levels are not only determined by the regulation and rate of transcription but also by post-transcriptional modifications and transcript stability. Several post-transcriptional regulatory mechanisms have been identified in sugar signalling. Studies utilising actinomycin D (Act D) transcriptional inhibition have shown that sugar responsive regulation can occur post-transcriptionally. Specifically, sucrose has been shown to decrease the stability of sucrose down-regulated mRNA while increasing the stability of most sucrose induced mRNA in *Oryza sativa* cell culture lines (Ho *et al.*, 2001). The entire *α-AMYLASE3* (*αAmy3*) 3′ UTR has been shown to independently mediate sugar-dependent repression of a heterologous reporter gene transcript (Chan & Yu, 1998). Glucose regulates the expression of miR156 through the controlled regulation of degradation of *pri-MIR156* transcript (Yu *et al.*, 2013). Several other instances of mRNA stability have been cited in literature and it is clear that post-transcriptional regulation may be an important factor in sugar regulation of gene expression.

1.2.2.4 Translational regulation

Another key regulatory site during sugar responses is the control of translation of mRNA into peptides and proteins. A strong example of the significance of this is the regulation of S-class bZIP transcription factors. Sucrose induced translational repression (SIRT) has been shown for several of the TFs in this family. *bZIP11* transcript levels are known to be sugar induced but interestingly the subsequent translation into protein can be repressed by excess levels of sucrose. *bZIP11* has a very long transcribed 5‘ UTR and it is thought that the sucrose controlled uORF2 (SC-uORF2) in this UTR is essential for the observed sugar repression of translation. SC-uORF-directed SIRT has also been shown for the bZIP2 protein whose mRNA levels are not sucrose responsive but TF translation is repressed (Wiese *et al.*, 2005). The chloroplast protein disulphide isomerase RB60 may be involved in translational regulation of sugar responsive genes. RB60 controls the binding of RB47 (chloroplast poly(A)-binding protein) to the 5‘ UTR of *PSBA* mRNA in the green algae *Chlamydomonas reinhardtii* allowing for the reversible regulation of *PSBA* in a light dependent manner (Kim & Mayfield, 2002). Although there is no direct evidence linking

RB60 to sugar regulation of *PSBA* translation, *PSBA* and its protein D1 are known to be sugar repressed in *Arabidopsis* (Sulmon *et al.*, 2004).

1.2.2.5 Post-translational modification and protein stability

Sugar signalling can be regulated at the protein function level and one mechanism for such regulation is the control of protein availability. Endocytosis of the seven-transmembrane G-protein-coupled receptor (7-TM GPCRs) is a classic example of this type of regulation. Across many plant species the detection of glucose leads to the internalisation of 7-TM GPCRs through endosome formation and it is this process that causes the uncoupling of the α, β and γ sub-units of G proteins from the GPCRs resulting in sugar signal transduction (Phan *et al.*, 2013; Urano *et al.*, 2012). Proteins can be post-translationally regulated via redox activation. During trehalose and sucrose feeding the starch biosynthesis protein ADP-glucose pyrophosphorylase (AGPase) is activated via redox activation. Interaction with the sugar substrates results in a conformational change in the protein through a reduction of the intermolecular disulphide bridges between the protein sub-units and effectively results in the activation of the protein (Fu *et al.*, 1998; Kolbe *et al.*, 2005).

1.2.3 Interaction of sugar signalling with other signalling networks

1.2.3.1 Plant immunity

The metabolic profiles of plants are known to vary quite considerably during biotic stress and this can be as a result of changes in gene expression induced by the host or the pathogen. Pathogen interactions can cause changes in numerous metabolites including ascorbate, glucose, sucrose, raffinose and trehalose and combined inoculation with both pathogen and these metabolites has been shown to alter disease severity when compared to pathogen inoculation alone (Bolouri *et al.*, 2012; Botanga *et al.*, 2012). These changes in metabolites can be a result of both manipulation of the host by the pathogen (e.g. infection of plants by *Agrobacterium tumefaciens*) or be part of the host‘s defence response (Berger *et al.*, 2007).

Sugars and sugar-related genes are differentially regulated during defence responses and are crucial to successful disease resistance. Hexose sugars levels can vary depending on the effect of infection on photosynthesis as some infections result in suppression of photosynthesis while others promote it (Berger *et al.*, 2007). Sugar levels can also vary

depending on the type of infection and proximity to the site of infection and cause varying signal transduction pathways (Berger *et al.*, 2007). During nematode infection, raffinose, trehalose and galactinol are known to accumulate to high levels not only in infected tissue but are also utilised as mobile stress signals through phloem loading, to prime defence responses in other tissues (Hofmann *et al.*, 2010). *TPS11*, a putative T-6-P synthase/phosphatase is up-regulated in response to tobacco mosaic virus (TMV) infections in *Arabidopsis* and *tps11* knockout mutants are increasingly susceptible to green peach aphids, with this phenotype being rescued via trehalose supplementation and implicating trehalose as a signalling molecule during defence (Golem & Culver, 2003; Singh *et al.*, 2011).

The transport sugar, sucrose has a specific role in the induction of the defence-related, additional function of anthocyanin. Glucose and fructose sub-units do not induce this anthocyanin production and the pathogen-associated elicitor flg22 can regulate this sucrose-dependent induction of anthocyanin. Conversely, several saccharides have been implicated in regulatory roles during plant immune responses to pathogen attack. Defence gene expression is induced in response to exogenous sugars in several species. Galactinol, a precursor for raffinose induces *PR1a*, *PR1b* and *NtACS1* in tobacco (Kim *et al.*, 2008), *PR1*, *PR2*, *PR5*, *OsPR1a*, *OsPR1b*, *PBZ1* are induced in response to sucrose in rice (Gómez-Ariza *et al.*, 2007; Thibaud *et al.*, 2004), *PR-Q* and *PR1* are induced in response to sucrose, glucose and fructose in tobacco (Herbers *et al.*, 1996) and in *Arabidopsis PR1* and *PR5* are glucose responsive (Jossier *et al.*, 2009; Xiao *et al.*, 2000). Interestingly, some cell wall invertases responsible for breakdown of sucrose to fructose and glucose are also considered to be PR proteins (Roitsch *et al.*, 2003).

1.2.3.2 Abiotic stress

Plants experience various stresses imposed by water availability, temperature variances, nutrient deficiencies, anoxic conditions and osmotic pressures amongst others that often result in energy stress and ultimately inefficient growth, development and maturation. It is therefore critical to have mechanisms in place that can simultaneously regulate abiotic stress responses and maintain energy status for the maintenance of life.

Sugars have been shown to provide both a protective and signalling function during abiotic stress responses. It is well established that abiotic stress-associated genes are

differentially expressed in response to exogenous sugar application and in plant mutants with altered carbohydrate regulation and metabolite profiles (Li *et al.*, 2006; Price *et al.*, 2004). One microarray expression study revealed that more than 12% of glucose and ABA responsive genes are associated with stress responses including key regulators of abiotic stress responses, *CBF3, COR15A* and *RD29A (Li et al., 2006)*. Over-expression of *CBF3* in *Arabidopsis* induces expression of *COR* (*COLD REGULATED*) genes including *COR15A* and *RD29A* causing freezing tolerance in non-acclimated plants (Gilmour *et al.*, 2000). This is evidence for the possible involvement of both glucose and ABA in cold stress signalling. Fluctuations in internal carbohydrate concentrations are observed during various abiotic stress responses and this all indicative of the overlaps between carbon signalling and abiotic stress responses. Plants with altered activities of starch and soluble sugar metabolism enzymes often have altered tolerances to abiotic stress (Krasensky & Jonak, 2012). One of the most abiotic stress vulnerable points during the plant growth cycle is the initial onset of fruit and seed development (Barnabás *et al.*, 2008; Hedhly *et al.*, 2009; Thakur *et al.*, 2010). Challenge with cold, heat and drought stress at this stage typically ends in failure of fertilisation or seed and fruit abortion (Thakur *et al.*, 2010). It has been suggested that during stress low levels of glucose caused by a decrease in sugar export to reproductive organs may lead to the induction of senescence hormone signalling mediated by GA and ABA and causing cell division arrest and ultimately abortion of seeds and fruit (Liu *et al.*, 2013). Sugars accumulate in cold treated plants (Cook *et al.*, 2004; Kaplan *et al.*, 2007) and during cold acclimation both ABA-dependent and independent responses are observed (Yang *et al.*, 2005). Some cold response genes are both ABA and sugar inducible suggesting overlaps among ABA, sugar and cold acclimation responses and that sugar accumulation reinforces cold acclimation (Masclaux-Daubresse *et al.*, 2007).

Two notable studies have been conducted with transgenic plants that have enhanced accumulation of trehalose. *Indica* rice plants expressing the trehalose biosynthetic fusion gene generated from two bacterial trehalose biosynthetic genes and under stress-inducible promoter regulation were evaluated for their stress responses. The transgenic plants showed normal growth and fertility phenotypes and only showed elevated trehalose levels under stress conditions. The plants were shown to be salt and drought tolerant and this increased tolerance was attributed to increased soluble sugars concentrations driven by higher photosynthetic capacity during both non-/stress conditions implicating trehalose in

sugar signalling during salt and drought stress (Garg *et al.*, 2002). In *Arabidopsis* over-expression of the native *TPS1* gene results in minor changes in trehalose and T-6-P concentrations that have profound effects on stress tolerance. The transgenics showed an increased tolerance to dehydration, developed insensitivity to exogenous glucose and ABA treatment and differential expression of two typical glucose and ABA insensitivity genes, *ABI4* and *CHLOROPHYLL A/B BINDING PROTEIN1* (*CAB1*). *TPS1* expression seems to occur in a HXK1-depdendent manner and all this evidence strongly implicates trehalose in glucose and ABA regulation during dehydration stress (Avonce *et al.*, 2004).

1.2.3.3 Hormone signalling

Overlaps between sugar signalling and hormonal transduction pathways have been shown to be critical for plant growth and development. As a general rule, plants defective in ABA, ethylene and auxin hormonal signalling tend to have altered sugar responses as well providing evidence for an overlap of regulatory function.

Abscisic acid

Interactions between sugar and ABA signalling are most obvious during early seedling development (Arenas-Huertero *et al.*, 2000; Gibson, 2005; León & Sheen, 2003) and interactions between the two pathways were initially identified through the observation that several sugar response mutants also displayed altered ABA signalling and metabolic responses.

Sugar response mutants are often allelic to ABA synthesis and ABI insensitive mutants and their characterisation has led to a greater understanding of sugar/hormone signalling overlap (Arenas-Huertero *et al.*, 2000; León & Sheen, 2003; Nishimura *et al.*, 2007). For instance, *glucose insensitive5* (*gin5*), *gin6/sucrose insensitive5* (*sis5*) and *gin1/sis4* mutants are allelic to *aba3* (*ABA deficient3* – ABA biosynthesis defective), *abi4* (*ABA insensitive4*) and *aba2* suggesting and overlap between sugar and ABA signalling (Arenas-Huertero *et al.*, 2000; Gibson, 2005; León & Sheen, 2003). *CAB1* and *PLASTOCYANIN* (*PC*) are known glucose responsive genes whose expression is not repressed by glucose treatment in the *gin1/aba2* and *gin5/aba3* mutants implying an ABA-dependent glucose response. Exogenous glucose treatment of seedlings is known to induce the expression of ABA biosynthetic and signalling genes, suggesting a glucose-specific accumulation of ABA during sugar signalling (Cheng *et al.*, 2002). Sugars and

ABA can act synergistically - as is seen by the ABA based enhancement of starch biosynthesis gene expression in response to sucrose – or antagonistically as shown by the alleviation of wild type seedling sensitivity to ABA by exogenous glucose (León & Sheen, 2003; Rook *et al.*, 2001). It is important to note not all *abi* mutants have altered sugar response phenotypes suggesting that even with all the overlap in signalling, independent sugar and ABA pathways exist (Arenas-Huertero *et al.*, 2000).

Ethylene

Seedling development is also known to be regulated through the interaction of sugar and ethylene signalling pathways. The ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) prevents sugar dependent inhibition of cotyledon greening and expansion, even at high glucose concentrations, and ethylene insensitive mutants including *ethylene receptor1* (*etr1*), *ethylene insensitive2* (*ein2*) and *ein3* are hypersensitive to glucose, Alternatively, *ethylene overproducer1* (*eto1*) and the constitutive ethylene triple response mutant *ctr1/gin4/sis1* are glucose insensitive (Cheng *et al.*, 2002; Gibson *et al.*, 2001; Zhou *et al.*, 1998). A molecular link between glucose and ethylene signalling was discovered during characterisation of the transcription factor EIN3. The TF is a crucial regulator of ethylene signalling that is downstream of the ETR1 receptor and is antagonistically regulated by glucose and ethylene treatment (Yanagisawa *et al.*, 2003). In maize and *Arabidopsis* protoplasts, glucose activates the degradation of EIN3 in the nucleus in a HXK1-dependent manner while treatment with ACC inhibits degradation. Constitutive expression of *EIN3* affords insensitivity to glucose and as already mentioned, *ein3* mutants are hypersensitive to glucose (Yanagisawa *et al.*, 2003).

Auxin

Transcriptome profiling analysis has revealed overlap of glucose and auxin response pathways regulating *Arabidopsis* root growth and development (Mishra *et al.*, 2009). Sixty-two percent of auxin responsive genes are also glucose responsive and glucose and auxin have both synergistic and antagonistic regulatory roles. Auxin and glucose can have additive effects as some auxin responsive genes that did not respond to glucose were regulated by the combination of both glucose and auxin. *gin2* mutants are known to be auxin insensitive and other auxin resistant mutants (*axr1*, *axr2* and *tir1*) are also insensitive to high glucose levels (Moore *et al.*, 2003). A study of *hookless1* (*hls1*) revealed additional sugar and auxin overlap (Ohto *et al.*, 2006). The *hls1* mutant is

insensitive to both sugar and auxin and HLS1 is thought to negatively regulate sugar and auxin signalling as exogenous indole-3-acetic acid (IAA) partially repressed sugar-induced gene expression while activating auxin response genes.

Cytokinin

The *gin2* mutant once again helped reveal a link between sugar signalling and another hormone, cytokinin which is associated with plant senescence. *gin2* mutants experience a delay in senescence and cytokinin and sugar appear to have antagonistic functions as exogenous cytokinin alleviates the glucose sensitivity of wild type plants (Moore *et al.*, 2003). Furthermore, constitutive cytokinin signalling in transgenic plants leads to a subversion of the glucose repression response. Cytokinins promote greening in the ethylene insensitive mutants, *etr1* and *ein2*, suggesting ethylene and cytokinin responses to glucose are independent of each other.

Gibberellin

Gibberellin (GA) and glucose target the expression of genes in rice via the same promoter elements (Chen *et al.*, 2002; Chen *et al.*, 2006; Morita *et al.*, 1998). The glucose and GA responsive element TATCCA is a target of three MYB transcription factors in rice and is found in the promoters of α-amylase genes. All three TFs have glucose and GA specific regulatory roles and could be an interaction point for the two pathways (Lu *et al.*, 2002). It has also been observed that two negative regulators of GA signalling, RGL2 (RGA-like) and SPY (SPINDLY) may be important in sugar signalling as *rgl2* and *spy* mutants germinate normally in high levels of glucose that limit germination in the wild type (Yuan & Wysocka-Diller, 2006). In a recent study it was observed that exogenous GA treatment can rescue the dwarf phenotype of starch synthesis and starch degradation mutants, *pgm and sex1* respectively (Paparelli *et al.*, 2013). GA treatment only affects fresh weight (FW) tissue gains and not dry weight (DW) indicating regulation of cell elongation and not changes in photosynthetic rates. In *pgm* GA significantly reduced sucrose and glucose levels suggesting a diversion of energy resources to GA-mediated cell expansion. Finally, GA biosynthesis was found to be diminished in both *pgm* and *sex1* confirming the GA/sugar overlap (Paparelli *et al.*, 2013). Another study has also identified the glucose-specific induction of CALCINEURIN B-LIKE1 (CBL1) and showed that the *cbl1* mutant is hypersensitive to glucose and paclobutrazol, a GA biosynthetic inhibitor during seed germination and development. A number of sugar-responsive and GA biosynthetic genes

were differentially expressed in the *cbl1* mutant and the direct interaction of CBL1 protein with the AKINβ1 subunit of SnRK1 implicate it in sugar signalling (Li *et al.*, 2013).

Salicylic acid

There is a limited amount of research linking salicylic acid signalling and sugar regulatory pathways. The sugar-responsive induction of *PR* genes is abolished in *Arabidopsis* plants expressing a bacterial SA hydroxylase (NahG) suggesting a role of SA in sugar up-regulation of *PR* genes (Thibaud *et al.*, 2004; Xiao *et al.*, 2000). SA treatment has also been shown to cause an increase in total soluble sugars in several genotypes of cowpea at the flowering stage (Chandra *et al.*, 2007).

1.2.3.4 Diurnal and clock regulation of sugar responsive genes

Diurnal control of genes and metabolites is crucial in the regulation of many metabolic and physiological processes. Plant sugars are known to display fluctuations that correlate with the time of day and optimisation of carbon availability is essential for maximal vegetative growth and development. Sucrose, reducing sugars and starch all rise during the day and fall at night. Starch is remobilised in a near-linear manner at night through enzymatic degradation such that starch levels are almost completely depleted before dawn (Graf *et al.*, 2010). This tight regulation of carbohydrate levels means that most of the available sugars are invested into active plant growth while minimising the risk of starvation under energy limiting conditions. The elegance of this system is seen in the immediate adjustments of starch degradation in response to energy regulating environmental cues such as temperature and light, and the fact that starch biosynthesis and degradation rates adjust to changes in photoperiod and light intensity (Graf *et al.*, 2010; Pyl *et al.*, 2012; Stitt *et al.*, 2007) in a circadian clock-dependent manner (Graf *et al.*, 2010).

A global gene regulation study in *Arabidopsis* identified numerous sugar-responsive genes that displayed diurnal expression patterns (Bläsing *et al.*, 2005). These diurnal patterns mirrored those of the internal carbohydrate profiles and it is thought that sugar levels are the ones responsible for gene regulation and not vice-versa. In wild type plants it was observed that both circadian and sugar responses are the major inputs into diurnal regulation but in the starchless mutant *pgm* the misregulation of sugar levels overrides existing circadian regulatory mechanisms signifying the importance of sugars in regulation. It has been recently revealed that endogenous sugar oscillations can entrain the circadian

clock in *Arabidopsis* through the morning-expressed gene, *PSEUDO-RESPONSE REGULATOR7* (*PRR7*) and the effects of sucrose on the circadian period are abolished in *prr7* mutants (Haydon *et al.*, 2013).

Periods of nutrient abundance are probably less stressful to a plant than starvation or the threat of it and this hypothesis is reflected on a transcriptional level. The transcriptome is rapidly and hugely modified in response to sugar starvation while it is not as widely affected during nutrient abundance, exemplified by the intense responses of *pgm* mutants at night and the repression of these night responses through sugar supplementation (Bläsing *et al.*, 2005). Short periods of starvation trigger rapid repression of plant growth (Gibon *et al.*, 2004) and as such many sugar-related genes start to respond to declining sugar levels towards the end of the night (Bläsing *et al.*, 2005).

1.2.3.5 Interaction with other nutrients

Sugar and nitrogen networks needs to be tightly regulated to allow for optimal growth and development in plants and indeed other cellular organisms. Nitrogen is a crucial component of the photosynthetic machinery that drives carbon fixation, and is required for the biosynthesis of proteins and nucleic acids. On the other hand, carbohydrates are crucial as energy molecules for the active uptake of nitrogen from the external environment and also form the carbon backbone of all nitrogen based molecules. Given this reciprocal dependency of one nutrient on the other it is pertinent that signalling crosstalk occur for successful maintenance of life in the ever-changing environment.

Several microarray studies to date have shown genes that are thought to be involved in both carbon and nitrogen signalling (Gutiérrez *et al.*, 2007; Palenchar *et al.*, 2004; Price *et al.*, 2004). The general observation was a relatively higher response to carbon and carbon/nitrogen treatment compared to nitrogen alone. Most of the nitrogen responsive genes were also carbon responsive and a smaller number of carbon responsive genes are also differentially expressed after nitrogen treatment.

Nitrogen regulation plays a much more important role in the root tissue, the site of nitrogen uptake into plants (Gutiérrez *et al.*, 2007). NITRATE TRANSPORTER 2.1 (NRT2.1) was first identified as a player in carbon/nitrogen sensing when the *lin1* (*lateral root initiation 1*) mutant lacking functional NRT2.1 showed an insensitivity to high carbon/low nitrogen

conditions implicating it as a regulatory point utilised during carbon/nitrogen signalling (Malamy & Ryan, 2001). A broad range of carbon/nitrogen ratios were exogenously supplied to *osu1* (*oversensitive to sugar1*) mutants and they were seen to be sensitive to both high C/low N and low C/high N imbalances but not to balanced C/N ratios. Curiously, *osu1* mutants are also sensitive to high C/low P and high C/low S implicating them not only in C/N regulation but also phosphate and sulphur overlaps with carbon signalling and regulation (Gao *et al.*, 2008b).

Inorganic phosphate (Pi) is an essential nutrient in plants whose signalling often overlaps with carbon assimilation and energy regulation and it is a crucial component of numerous metabolites. Pi is often a scarce nutrient in plant environments and plants have evolved mechanisms to perceive and respond to Pi starvation. Phosphate starvation has been shown to induce expression of sugar responsive genes (Ciereszko & Kleczkowski, 2002) while phosphate starvation responsive genes appear to be sucrose responsive (Lejay *et al.*, 2003; Lejay *et al.*, 2008). Altered sucrose and Pi availability result in dramatic changes in the metabolite profiles of plants. For instance, Pi starvation results in down-regulation of photosynthesis, increased soluble sugar and starch levels (Ciereszko *et al.*, 2005; Müller *et al.*, 2007), an increase in the flux of sucrose from source tissue to sink tissue and an overall increase of the root/shoot biomass ratio (Al-Ghazi *et al.*, 2003; Ciereszko *et al.*, 2005; Hammond & White, 2008; Hermans *et al.*, 2006). Additionally, there is an obvious increase in root sucrose concentrations preceding typical phosphate starvation genetic responses under Pi limiting conditions (Hammond & White, 2008). On the other hand, inhibition of sucrose biosynthesis and/or phloem translocation by reduced photosynthesis, dark treatment or stem girdling supress typical plant responses to Pi unavailability (Liu *et al.*, 2005).

1.2.4 Energy perception and central integrators of sugar signalling

1.2.4.1 Sucrose and trehalose signalling

Although the major function of sucrose in plants has been attributed to sugar transport, several studies have now implicated the disaccharide in specific sugar signalling roles. The role of sucrose in sugar sensing is not as well understood as that of glucose but there have been some breakthroughs in recent times that have shed light on the subject. The sucrose transporter SUT4 and its interacting partner Cyb5-2 have been shown to to regulate seed germination in response to both sucrose and glucose (Li *et al.*, 2012).

Sucrose is known to inhibit the interaction between SUT4 and Cyb5-2 and the *sut4* and *cyb5-2* knockdown mutants are insensitive to both glucose and sucrose during germination and display wild type germination efficiency on the mannitol control media. However, the germination response is more severe in response to sucrose than glucose and can be rescued by over-expressing either protein in the respective knockdown mutants implicating the two genes in sugar sensing during germination.

In a sugar beet study it has been shown that exogenous treatment with sucrose and not glucose, mannitol or KCl has a direct effect on the repression of proton motive-force driven sucrose transport out of source tissue (Chiou & Bush, 1998). This repression of sucrose transport has been shown to be concentration dependent implying a dosage appropriate response to sucrose levels in sugar transport. The *Arabidopsis* transcription factor ATB2 has also been shown to be sucrose responsive. Promoter activity of *ATB2* is seen to be repressed specifically by sucrose treatment and not by other sugars include the subunits of sucrose, glucose and fructose implicating the TF in sucrose sensing (Rook *et al.*, 1998). Further evidence for sucrose mediated regulation is seen through the sucrose-specific induction of several genes including a UDP-glucose phosphorylase and anthocyanin biosynthesis enzymes through regulation of the transcription factor MYB75/PAPI (Hummel *et al.*, 2009).

Trehalose and T-6-P are emerging as major regulators of carbohydrate metabolism and development in plants. Trehalose has been implicated in stress tolerance in desiccation tolerant plants (Márquez-Escalante *et al.*, 2006) and as a storage sugar but its presence in minute concentrations in *Arabidopsis* and other desiccation sensitive plants suggests more of a regulatory role in metabolism (Avonce *et al.*, 2006; Fernandez *et al.*, 2010). T-6-P has been shown to be essential for embryo development through the *tps1* (*trehalose phosphate synthase*) mutation as homozygosity results in lethality (Gómez *et al.*, 2006). Transgenic *Arabidopsis* lines with varying levels specifically of T-6-P and not trehalose have been shown to have altered sugar responses. Mutants over-expressing the *E.coli* trehalose-P synthase (TPS) accumulate T-6-P while mutants over-expressing trehalose phosphate hydrolase (TPH) have decreased levels of T-6-P (Schluepmann *et al.*, 2003; Schluepmann *et al.*, 2004). The implications of this are seen as polarised growth phenotypes between T-6-P accumulators and plants with depleted levels, under normal conditions (Schluepmann *et al.*, 2003). Furthermore, the mutants with elevated T-6-P are

hyposensitive to exogenous sugar application while the *tph* mutants are hypersensitive to sugars. This response is seen regardless of treatment with glucose, sucrose or fructose, with sucrose producing the most potent responses but not evident in response to sorbitol. *TREHALOSE PHOSPHATE SYNTHASE 1* (*TPS1*) is induced in response to glucose and *Arabidopsis TPS1* over-expression confers glucose insensitivity implicating T-6-P in glucose signalling (Avonce *et al.*, 2004). Interestingly, low amounts of exogenous trehalose lead to the induction of *APL3,* the first enzyme in starch biosynthesis and the repression of starch breakdown genes *SEX1* and *BAM3* leading to high levels of starch at the end of the night period, suggesting detection of trehalose leads to altered carbohydrate metabolism. This starch accumulation is coupled with complete halting of seedling development in response to trehalose (Wingler *et al.*, 2000).

T-6-P has been recently described as being crucial in sucrose-dependent energy signalling and its role in energy signalling is more significant under high energy conditions (Yadav *et al.*, 2014). Energy starved plants contain low levels of T-6-P which rapidly rise (in parallel with internal sucrose) within 15 min of exogenous sucrose application (Lunn *et al.*, 2006). Additionally, diurnal fluctuations in T-6-P closely mirror the changes in sucrose levels throughout the day (Lunn *et al.*, 2006; Sulpice *et al.*, 2014). This sucrose specificity is well described by Yadav *et al.* (2014) who showed that not only is T-6-P signalling sucrose specific but also that other sugar effects in T-6-P signalling are indirect and are a consequence of changes in exogenous sugar-driven, sucrose level changes. Finally, Yadav *et al.* (2014) also suggested a synergistic relationship between sucrose and T-6-P levels whereby sucrose controls T-6-P and vice versa emphasising the importance of the internal T-6-P:sucrose ratio for the regulation of high energy status signalling.

1.2.4.2 HEXOKINASE1 (HXK1)-dependent glucose signalling

Regarding plant carbohydrates, cellulose is important for structural function, starch important for energy storage, sucrose thought to be the most important transport sugar while glucose has the most significant regulatory role in sugar signalling. Three master regulators of glucose signalling have been identified in plants, namely HXK1, TARGET OF RAPAMYCIN (TOR) and SUCROSE NON-FERMENTING-1-RELATED KINASE1 (SnRK1) and are thought to be crucial in the governance of energy responses in plant species (Sheen, 2014). Hexokinases are glucose sensors evolutionarily conserved across eukaryotes with four known hexokinases and two hexokinase-like kinases previously

identified in *Arabidopsis* (Karve *et al.*, 2008). Of particular interest is HXK1 that has been shown to be central in metabolism via the phosphorylation of hexose sugars in the plant cell mitochondria (Karve *et al.*, 2008) and additionally crucial in sugar signalling in the nucleus (Cho *et al.*, 2006). The HXK1-dependent glucose sensing was discovered in the HXK1 null mutants, *glucose insensitive2* (*gin2*) and *anti-HXK1* plants were incapable of detecting glucose during seed germination and germinated more successfully than wild type plants. Conversely, plants with higher than normal levels of *HXK1* are hypersensitive to exogenous glucose (Jang *et al.*, 1997; Moore *et al.*, 2003). Interestingly, when grown under low light conditions in the absence of glucose, *gin2* mutants phenocopy the wild type while treatment with higher light doses (higher energy conditions due to photosynthesis dependent sugar accumulation) results in stunted growth rates in the mutants. This suggests a role for HXK1 in plant growth stimulation during vegetative development that contrasts with the role in repression of seed germination under high energy conditions. It was experimentally determined that *gin2* mutants retain 50% of their catalytic activity and have normal hexose-phosphate levels which could possibly be attributed to the other HXKs in the genome and suggests the mutant phenotypes described were caused by the glucose detection properties of *HXK1* and not by metabolic changes caused by loss of HXK catalytic activity. Additionally, *gin2* mutants complemented with catalytically inactive (ATP binding and phosphoryl transfer activity) *HXK1* alleles (that can still bind glucose) display the hypersensitivity to glucose seen in the *sense-HXK1* transgenic lines and the ability to regulate the glucose-dependent repression of chlorophyll accumulation and photosynthetic gene expression (Moore *et al.*, 2003).

1.2.4.3 Signalling dependent on HEXOKINASE catalytic activity

PATHOGENESIS RELATED genes (*PR*) have been shown to be induced in response to glucose treatment in a HXK-dependent manner. *35S::HXK1* transgenics have higher levels of *PR1* and *PR2* gene expression and there is a corresponding loss of *PR* gene induction in the *35S::antiHXK1* indicating a hexokinase dependent regulation of *PR* genes in response to glucose. Transgenic *35S::YHXK2* lines over-expressing a yeast-based hexokinase showed a similar *PR* response to glucose and this is of great interest because yeast *HXK2* only contains the catalytic HXK domain and not the glucose binding domain. This result then suggests that the induction of the *PR* genes is occurring in a manner dependent on the catalytic domain of HXK and not on the established glucose signalling function of the gene. Since glucose has many downstream metabolic products it is

possible that these subsequent metabolites may be more directly responsible for the sugar induction of *PR* genes (Xiao *et al.*, 2000).

1.2.4.4 TOR-*mediated glucose signalling*

TOR is conserved in eukaryotes and much like the other members of the phosphoinositol 3-kinase-related kinases class, the protein is structurally similar to lipid kinases but functions as a serine/threonine protein kinase (Caldana *et al.*, 2013; Wullschleger *et al.*, 2006). The molecular function and regulatory mechanisms of TOR in plants are largely unclear mostly due to the fact that homozygosity for null *tor* alleles results in embryonic lethality, which limits knockout mutation analysis (Ren *et al.*, 2011). It is only in recent times that research with ethanol/estradiol-induced *TOR* silencing mutants has revealed new insights into TOR function in glucose signalling (Caldana *et al.*, 2013; Deprost *et al.*, 2007; Liu *et al.*, 2012; Ren *et al.*, 2012; Xiong & Sheen, 2012; Xiong & Sheen, 2012; Xiong *et al.*, 2013).

TOR-dependent signalling is activated during high energy conditions and transcriptome comparisons between wild type plants and the estradiol-inducible null *tor-es* mutants treated with glucose at dawn, revealed 1318 and 1050 genes induced and repressed, respectively in response to an increase in glucose (Xiong *et al.*, 2013). Importantly, TOR-dependent signalling is specifically responsive to glucose and substitution with other sugars, amino acids or plant hormones is not sufficient to drive TOR signalling (Xiong *et al.*, 2013). Suppression of *TOR* expression or protein activity produces responses similar to those experienced in plants undergoing nutrient starvation. TOR RNAi lines display elevated levels of soluble sugars and amino acids, and reduced growth, cell size and fresh weight, as well as decreased polysome/ribosome content (decrease in translation) (Deprost *et al.*, 2007).

TOR null mutants also display an increase in autophagy which is interpreted as a nutrient scavenging response during the illusion of nutrient deprivation in the absence of *TOR* mRNA, implicating TOR in nutrient perception (Robaglia *et al.*, 2012). Suppression of TOR protein kinase activity also causes similar responses and an inability to respond to increasing nutrient and light energy implicating TOR in energy perception and response (Ren *et al.*, 2012). Interestingly, there are strong correlations between the transcriptome profiles mediated by TOR signalling and expression responses regulated by soluble

sugars and photoautotrophism (Bläsing *et al.*, 2005; Gonzali *et al.*, 2006; Li *et al.*, 2006) and partial overlaps with SNF1 KINASE HOMOLOG10 (KIN10) (Baena-González *et al.*, 2007; Xiong *et al.*, 2013) responses reinforcing TOR functionality in energy responses and suggesting interaction with other energy responses and SnRK1-regulated signalling pathways.

1.2.4.5 SnRK1-*dependent signalling*

The SnRK1 complex is a member of the sub-family of serine/threonine protein kinases conserved in plants together with SnRK2 and SnRK3. The SnRK1 complex sub-unit proteins KIN10/KIN11/KIN12 (also known as SnRK1.1/SnRK1.2/SnRK1.3, AKINα1/AKINα2/AKINα3, or AKIN10/AKIN11/AKIN12) are orthologous to yeast Snf1(Sucrose non-fermenting1) and mammalian AMPK (AMP activated protein kinase) protein kinases that are well established sugar starvation response kinases (Hardie, 2007; Hardie, 2004; Young *et al.*, 2003). Nutrient deprivation and most a/biotic stresses have detrimental effects on overall energy status in plants that results in sugar sensing and signal transduction pathways that can utilise SnRK1 proteins to effect survival strategies during stress (Baena-González & Sheen, 2008).

SnRK1 *structure*

A working model of the plant SnRK1 complex has been outlined based on studies in *Arabidopsis* and other plants and homology to yeast SNF1 and mammalian AMPK. SnRK1 proteins are heterotrimeric complexes comprised of catalytic α, scaffolding/regulatory β and regulatory γ sub-units (Polge & Thomas, 2007), (Figure 1.1). Although KIN10/KIN11/KIN12 sub-units have been genetically identified in *Arabidopsis,* only KIN10 and KIN11 are known to be expressed in plants as the two catalytic α-related sub-units (Baena-González *et al.*, 2007) and require phosphorylation at a conserved activation threonine loop (T-loop) for kinase activity (Hardie, 2011; Hedbacker & Carlson, 2008; Polge & Thomas, 2007). The T-loop is within the kinase domain that displays a canonical fold with 11 sub-domains (Hanks & Hunter, 1995). A separate kinase-associated1 (KA1) domain is also found within the α sub-unit and it is thought to be responsible for interaction with the regulatory sub-unit and upstream phosphatases (Crozet *et al.*, 2014; Rodrigues *et al.*, 2013; Xiao *et al.*, 2013).

Three plant specific β sub-units have been described with AKINβ1 and AKINβ2 belonging to one class and AKINβ3 to another (Bouly *et al.*, 1999; Gissot *et al.*, 2004). AKINβ1 and AKINβ2 are responsible for the interaction with γ subunits through an internal domain of the β sub-unit known as the KIS (kinase interacting sequence) domain (Jiang & Carlson, 1997). AKINβ3 protein is a truncated version of the β sub-units lacking an entire CBM (carbohydrate binding motif, also known as a glycogen-binding domain) and a variable N-terminal region, both of which are conserved in all other β sub-units. These two features are poorly characterised but have been linked to binding of glycogen (CBM in mammals and yeast) and facilitate association with downstream targets and sub-cellular localisation (N-terminal region in yeast) (Hedbacker & Carlson, 2008; Hudson *et al.*, 2003; Polekhina *et al.*, 2003; Polge & Thomas, 2007; Vincent *et al.*, 2001).

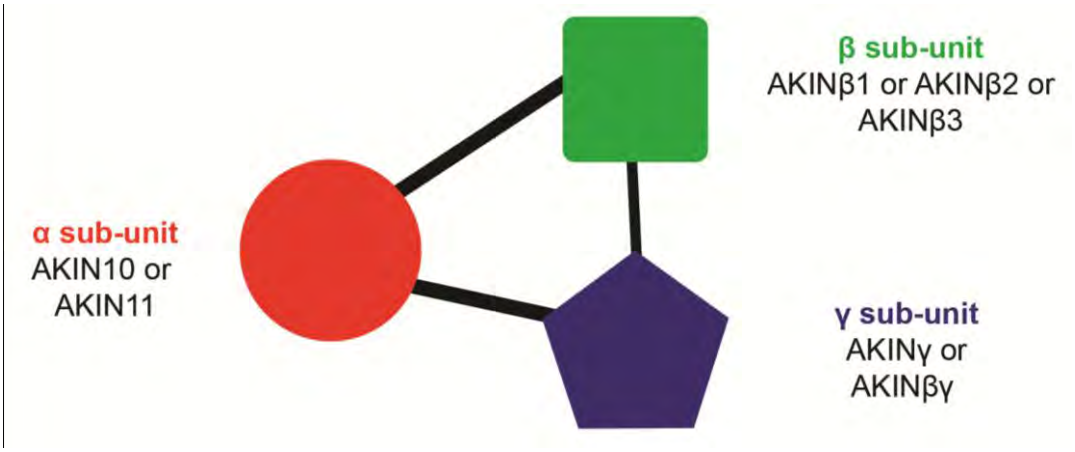


Figure 1.1: Overall structure of the SnRK1 complex in *Arabidopsis*.
Multiple heterotrimeric complexes are possible due to the available alternative sub-units of each type.

There is a large family of γ-like proteins in plants and two are of particular interest. AKINγ and AKINβγ both contain the γ sub-unit conserved, four in-tandem CBS (cystathionine β-synthase) motifs which are the site of AMP/ATP regulatory action on AMPK in mammals (Kemp, 2004; Scott *et al.*, 2004). AKINγ appears to have a classical γ sub-unit structure but fails to complement the yeast *snf4* mutant (SNF4 is a yeast γ sub-unit equivalent) while on the other hand, AKINβγ can complement this mutation. AKINβγ is unique to plants and it has a modified structure that appears to be an amalgamation of a γ type protein and a KIS domain-like sequence from β sub units (Lumbreras *et al.*, 2001). Curiously, this KIS-like domain matches perfectly with a CBM and has been shown to interact with the ORTHOLOG OF SUGAR BEET HS1 PRO-1 1 (HSPRO1) and ARABIDOPSIS ORTHOLOG OF SUGAR BEET HS1 PRO-1 2 (HSPRO2) proteins (hereafter referred to

as HSPRO collectively) which are the focus of this study (Gissot *et al.*, 2006). It is clear that several α, β and γ sub-units are available, resulting in the potential for the existence of multiple heterotrimeric SnRK1 configurations in eukaryotes and up to 12 in *Arabidopsis*. Multiple configurations are perhaps the first layer of regulation of the complexes as evidenced through differential localisation caused by different β sub-units of SNF1 in yeast (Crozet *et al.*, 2014; Hedbacker & Carlson, 2008).

Regulation by sugars

As previously mentioned the SnRK1 complex has two α sub-unit variants (KIN10 and KIN11) and these proteins are 512 amino acids long, with 89% sequence similarity in the N-terminal kinase domain and 64% similarity in the C-terminus (Mohannath *et al.*, 2014). SnRK1 studies implicating both KIN10 and KIN11 in sugar regulation have been conducted and according to one publication 90% of SnRK1 kinase activity can be attributed to KIN10 (Jossier *et al.*, 2009). However, for the context of this thesis, SnRK1.1 and SnRK1.2 will be used to distinguish between KIN10 and KIN11-based variants, respectively. SnRK1 function is regulated through several mechanisms including but not limited to post-translational modifications such as phosphorylation through interaction with upstream kinases and phosphatases, acetylation, ubiquitination, oxidation and myristoylation. Of contextual interest is the energy based regulation, which is a topic of serious contention. Evidence has been published supporting two opposing theories, one that proposes SnRK1 activity is induced under energy limiting conditions (as in yeast) while the other concludes that the complex is in fact activated by sugars. According to Jossier *et al.* (2009), exogenous treatment with glucose led to an increase in SnRK1.1 specific kinase activity in both wild type and *35S::SnRK1.1* plants with the over-expressor showing higher kinase activity. Additionally, increases in phosphorylated SnRK1.1 were only detected (via western blot analysis) in glucose treated plants and more so in the over-expressors and so protein kinase activity appears to be sugar regulated. Several other studies also lend support to the hypothesis that SnRK1 activity is induced by sugars which is a contrasting story with that of the orthologous systems in yeast and mammalian eukaryotes (Bhalerao *et al.*, 1999; Halford *et al.*, 2003; Purcell *et al.*, 1998; Tiessen *et al.*, 2003).

However, there is also substantial evidence from more recent work indicating conservation of Snf1/AMPK-like signal induction in plants with SnRK1 shown to be activated by energy

deprivation (Baena-González & Sheen, 2008; Ghillebert *et al.*, 2011; Halford & Hey, 2009; O'Hara *et al.*, 2013). For instance, SnRK1 kinase activity has been shown to be inhibited by glucose, sucrose, glucose-1-phosphate, glucose-6-phosphate and T-6-P and this sugar repression is seen across several species including *Arabidopsis,* spinach and sugar cane (Crozet *et al.*, 2014). Recent research also highlights the cooperative nature of the inhibition of SnRK1 activity by T-6-P and glucose-1-phosphate (Nunes *et al.*, 2013). T-6-P is activated in response to high energy conditions and so in this context it would make sense that SnRK1 is deactivated by T-6-P (Sonia & Allen, 2014). There is potential for feedback regulation between SnRK1 and T-6-P as a subset of *TPS* genes are regulated through SnRk1 (Baena-González *et al.*, 2007; Usadel *et al.*, 2008). Gene expression analysis also reveals *SnRK1* repression at the genomic level in response to glucose and sucrose (Baena-González *et al.*, 2007). It is thus generally accepted that SnRK1 in plants plays an analogous role to the SNF1 and AMPK systems in yeast and animals respectively.

SnRK1-*dependent regulation*

Several transcription factors have been identified as direct or indirect interacting partners of the SnRK1 complex in energy responsive signalling. bZIP S-group members have been shown to interact with the complex and mediate the regulation of target genes through G-box motif binding (Baena-González *et al.*, 2007). SnRK1 also interacts with the plant specific ATAF1 TF. *ATAF1* is known to be induced under a variety of carbon altering stresses and plants over-expressing ATAF1 are drought tolerant, possibly through regulation via SnRK1 (Wu *et al.*, 2009). SnRK1 acts upstream of the MYBS1 TF which induces the expression of *αAmy3*, an α-amylase that generates reducing sugars from starch during early embryo germination in rice and also allows the crop to survive during energy starvation conditions (Lee *et al.*, 2009; Lu *et al.*, 2007). Recently, miRNA dependent regulation of TCP (named after the TEOSINTE BRANCHED1, CYCLOIDEA and THE PROLIFERATING CELL NUCLEAR ANTIGEN FACTOR (PCF) TFs in *Zea mays*, *Anthirrinum majus* and *Oryza sativa* respectively) TFs by SnRK1 has been postulated (Confraria *et al.*, 2013). TCPs direct the diurnal regulation of mitochondrial proteins connected to energy metabolism (Confraria *et al.*, 2013; Giraud *et al.*, 2010). SnRK1 regulates other proteins directly through phosphorylation and has been shown to inactivate the enzymatic activity of SUCROSE PHOSPHATE SYNTHASE (sucrose biosynthesis) and TREHALOSE PHOSPHATE SYNTHASE5 (T-6-P biosynthesis) through

phosphorylation (Harthill *et al.*, 2006; Sugden *et al.*, 1999). SnRK1 stimulates the redox activation of ADP-glucose pyrophosphorylase (AGPase), a key enzyme in starch biosynthesis in potatoes (Geigenberger, 2003). Additional regulation of carbohydrate metabolism by SnRK1 is through mediation of gene expression of carbohydrate metabolism proteins such as sucrose synthase (sucrose degradation) (Purcell *et al.*, 1998). Amazingly, SnRK1 does not only regulate a small subset of genes and pathways as transient induction of SnRK1.1 in *Arabidopsis* protoplasts results in global differential gene expression affecting thousands of genes (Baena-González *et al.*, 2007). It is of added significance that there is a strong positive correlation between SnRK1.1 inducible genes and those induced under sugar limiting conditions and a negative correlation of SnRK1.1 targets with sugar responsive and energy rich response expression profiles is also seen (Baena-González *et al.*, 2007; Bläsing *et al.*, 2005; Palenchar *et al.*, 2004; Thimm *et al.*, 2004).

1.3 IDENTIFICATION OF HSPRO1 AND HSPRO2

The characterisation of the group of HSPRO and HSPRO-related proteins began with the identification of $Hs1^{pro-1}$ (from the wild species of sugar beet, *Beta procumbens*) as a nematode (*Heterodera schachtii*) resistance-conferring gene when heterologously expressed in the sugar beet, *Beta vulgaris* (Cai *et al.*, 1997). To date, *HSPRO* genes have been implicated as abiotic and biotic stress response genes in several plant species including tobacco and *Arabidopsis*.

In tobacco (*Nicotinia attenuata*) *NaHSPRO* has been identified as the only homologue of $Hs1^{pro-1}$ (Gilardoni *et al.*, 2010; Schuck *et al.*, 2012) while two homologues, namely *HSPRO1* (At3g55840) and *HSPRO2* (At2g40000) have been identified in *Arabidopsis* (Gissot *et al.*, 2006). The molecular roles of the HSPRO proteins are presently not known but in tobacco *NaHSPRO* is induced in response to several biotic stress treatments including simulated herbivory, and infection with the bacteria *P. syringae* pv *tomato* DC3000 (*Pst*) and the fungus *Piriformospora indica* (Schuck *et al.*, 2012). Suppression of *NaHSPRO* expression does not lead to altered regulation of defence responses against the herbivore *Manduca sexta* or altered disease resistance to *P. syringae*. However, *NaHSPRO* repression leads to increased seedling growth during the interaction with *P. indica*, which is a consequence of successful pathogenesis. This suggests that NaHSPRO negatively regulates seedling growth in wild type (WT) *N. attenuata* during interaction with *P. indica* and there is evidence that this may be mediated via SnRK1 signalling (Schuck *et al.*, 2013). Perhaps the interaction between HSPRO and SnRK1 is important for regulation and allocation of energy for vegetative growth versus defence response.

Although *HSPRO* genes are not differentially expressed in response to nematode attack in *Arabidopsis*, they are up-regulated in response to salicylic acid, virulent *P. syringae*, the bacterial elicitor flg22, phosphate starvation, salt stress, wounding, drought and UV-B (Cominelli *et al.*, 2005; Gissot *et al.*, 2006; Hammond *et al.*, 2003; Luhua *et al.*, 2008; Murray *et al.*, 2007; Walley *et al.*, 2007). *HSPRO2* overexpression confers increased resistance to oxidative stress and sensitivity to salt and osmotic stress (Luhua *et al.*, 2008). Transcriptome profiling of the *constitutively induced resistance1* (*cir1*) *Arabidopsis* mutant in the absence of disease revealed that *HSPRO2* mRNA levels were higher in *cir1* compared to the wild type and so might contribute to the increased resistance to pathogen attack observed in *cir1* (Murray *et al.*, 2007). Furthermore, *hspro2* mutants display

increased susceptibility to infection with virulent *P. syringae* and PR1 protein expression is misregulated in *hspro2* following pathogen infection (Murray *et al.*, 2007). Presently, *HSPRO1* has not been as characterised as *HSPRO2* but much like in tobacco, HSPRO proteins in *Arabidopsis* have been shown to interact with the central regulator of metabolism, SnRK1 (Gissot *et al.*, 2006). Yeast-two-hybrid analysis with AKINβγ as the bait protein and *in vitro* binding assays revealed a KIS domain specific interaction between AKINβγ and the HSPRO proteins. This interaction was confirmed *in planta* via bimolecular fluorescence complementation (BiFC) and through the co-transformation of *Nicotiana benthamiana* plants with *YN-AKINβγ/YC-HSPRO1* or *YN-AKINβγ/YC-HSPRO2* DNA.

The work described in this thesis represents the first characterisation of HSPRO1 in *Arabidopsis* and reveals further insights into the biological roles of HSPRO2. The reverse genetics approach taken here utilised several transgenic *Arabidopsis* lines, and the generation and isolation of these lines is described in chapter 3. This included isolation of homozygous *hspro* null mutants and generation of a *hspro1/hspro2* double knockout line. This chapter attempted to identify the biological functions of HSPRO proteins in biotic and abiotic stress responses. Gene co-expression analysis of publicly available microarray data was utilised to identify genes co-expressed with either *HSPRO1* or *HSPRO2* with the overall intention of revealing biological processes and signal transduction networks the HSPRO proteins may regulate. Promoter content analysis of genes co-expressed with *HSPRO* revealed potential *cis* regulatory elements driving *HSPRO* gene expression together with the co-regulated genes. Mutational phenotypic and gene expression analysis provided supporting evidence for biological responses during stress that require *HSPRO* expression. This study is also the first to describe the subcellular localisation of *Arabidopsis* HSPRO proteins in *Arabidopsis* leaf mesophyll cells and utilising confocal microscopy.

The work in chapter 4 is largely based on the expression profiling of *hspro* mutants under standard growth conditions using CATMA microarrays, the fact that the two proteins are known to interact with the SnRK1 complex (Gissot *et al.*, 2006) and that *HSPRO* genes are KIN10 targets (Baena-González *et al.*, 2007). As SnRK1 is integral to energy and stress signalling microarray analysis was utilised to identify genes and promoter regulatory elements utilised in energy signalling (and other signalling networks) that may be HSPRO targets. Gene expression analysis in *hspro* mutants was utilised to investigate whether

HSPRO1 and HSPRO2 are required for HXK1-dependent and independent glucose-mediated gene expression. This combined with the observed effects of a high energy environment on seed germination revealed previously undescribed insights into the potential role of HSPRO2 in energy signalling. Additionally, microarray analysis also revealed a circadian component to *HSPRO2* regulation which was confirmed via *in planta* luciferase imaging.

CHAPTER 2

Materials and Methods

CHAPTER 2: MATERIALS AND METHODS

2.1 PLASMIDS

2.1.1 pGem®-T Easy

pGemT-Easy (Promega Corporation, Madison, USA) is a linearised cloning vector and selection of positive transformants was carried out in *E. coli* cultures grown on Luria Bertani (*LB*) media (1% w/v tryptone; 0.5% w/v yeast extract and 1% w/v NaCl, pH 7) (Sambrook *et al.*, 1989) containing 100 µg/mL ampicillin. Solid media additionally contained 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 80 µg/mL 5-bromo-4-chloro-2-indolyl-β-D-galactoside (X-gal) for blue/white colony screening of transformants.

2.1.2 pENTR™4 Dual selection vector

pENTR™4 Dual selection vector (pENTR4-D), (Life Technologies, California, USA) is a suicide entry vector containing a *ccdB/chloramphenicol* fusion gene for negative selection in *E. coli* strains sensitive to the ccdB protein, e.g. DH5α. Propagation of the empty vector was carried out in One Shot ® *ccdB* Survival™2 $T1^R$ cells (Life Technologies, California, USA) grown on *LB* agar containing 25 µg/mL chloramphenicol and selection of positive transformants was carried out in *LB* media with 50 µg/mL kanamycin.

2.1.3 pFAST-G02

The empty destination vector was donated by the Shimada group (Shimada *et al.*, 2010) and maintained in One Shot ® *ccdB* Survival™2 $T1^R$ cells and confers resistance to spectinomycin (100 µg/mL) and streptomycin (50 µg/mL). Positive transformants were transformed into *ccdB* sensitive DH5α *E. coli* and selected for with either spectinomycin or streptomycin. In plants, positive transformants were identified via spraying seedlings germinated on soil with DL- phosphinothricin commercially sold as Basta™ herbicide (Bayer CropScience Group, Hertfordshire, UK) (30 mg/L) or identifying fluorescent, green fluorescent protein (GFP)-fluorescent seeds under a fluorescent microscope (Shimada *et al.*, 2010).

2.1.4 pFGC5941

Laboratory stocks of pFGC5941 were maintained in *E. coli* DH5α and selected in *LB* media with 50 µg/mL kanamycin. *A. tumefaciens* GV3101 transformants were similarly

screened for with kanamycin and the respective antibiotics of the strain transformed. In *Arabidopsis*, positive transformants were identified via spraying seedlings germinated on soil with Basta™ (30 mg/L).

2.1.5 pART27

Laboratory stocks of pART27 ((Murray *et al.*, 2002)) were maintained in *E. coli* DH5α and selected in *LB* media with spectinomycin (100 µg/mL). *A. tumefaciens* transformants were similarly screened for with spectinomycin and the respective antibiotics of the transformed strain. In *Arabidopsis*, positive transformants were identified via germination on 0.5 x Murashige and Skoog (MS) (Highveld Bioligical, Johannesburg, SA) agar containing kanamycin (25 µg/mL).

2.1.6 35S-YFP-NosT

The plasmid was a gift from the Seidel group (Seidel *et al.*, 2005) and grown and screened in *E. coli* on *LB* media containing 100 µg/mL ampicillin.

2.2 GROWTH OF MICROORGANISMS

2.2.1 Escherichia coli growth and competent cell preparation

E. coli DH5α was grown on *LB* agar (1.5% (w/v)) at 37°C overnight. To make competent cells a single colony was inoculated into 5 mL *LB* media and grown shaking at 37°C overnight. The following day 2 mL of the overnight culture were subcultured into 250 mL *LB* media supplemented with 20 mM $MgSO_4$ and grown at 37°C, shaking until OD_{600} was 0.4-0.6, measured using the Beckman DU 650 Spectrophotometer (Beckman Coulter, Inc., CA, USA). The cells were recovered by centrifugation at 5000 x *g* for 5 min at 4°C in a JA-21 Beckman centrifuge and the supernatant discarded. The cells were gently resuspended in 100 mL ice cold TFB1 buffer (30 mM potassium acetate, 100 mM RbCl, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15% (v/v) glycerol, which had been adjusted to pH 5.8 with glacial acetic acid and then filter sterilized) and incubated on ice for 5 min. The cells were collected again by centrifugation at 5000 x *g* for 5 min at 4°C and the supernatant discarded. Finally the pellet was gently resuspended in 10 mL ice cold TFB2 buffer (10 mM MOPS, 75 mM $CaCl_2$, 10 mM RbCl and 15% (v/v) glycerol, filter sterilized) and incubated on ice for 15-60 min. Competent cells were dispensed in 100 µL aliquots into pre-cooled 1.5 mL microfuge tubes, frozen immediately in liquid nitrogen and stored at -70°C. One Shot® ccdB Survival™ 2 T1R chemically competent cells were purchased from Life Technologies (California, USA) and propagated as described above.

2.2.2 Agrobacterium tumefaciens growth and competent cell preparation

A. tumefaciens GV3101 (Holsters *et al.*, 1980) was cultured on *LB* agar (1.5% (w/v)) containing 150 µg/mL rifampicin and 15 µg/mL gentamicin and grown at 30°C for two days. To make competent cells a single colony was inoculated into 10 mL YEP media (1% (w/v) peptone; 1% (w/v) yeast extract and 0.5% (w/v) NaCl) supplemented with 100 µg/mL rifampicin and 15 µg/mL gentamicin and incubated with shaking at 28°C overnight. The following day 2 mL of the overnight culture was transferred to 50 mL fresh YEP media with antibiotic selection and incubated with shaking at 28°C until the OD_{600} reached 0.5–1.0, measured using the Beckman DU 650 Spectrophotometer. The culture was chilled on ice before the cells were harvested by centrifugation at 3000 x *g* for 5 min at 4°C in a JA-21 Beckman centrifuge. The supernatant was discarded and the pellet resuspended in 1 mL ice cold 20 mM $CaCl_2$. Aliquots of 100 µL were then dispensed into pre-cooled 1.5 mL microfuge tubes, immediately frozen in liquid nitrogen and stored at -70°C.

2.2.3 Pseudomonas syringae growth

Virulent *P. syringae* DC3000 and avirulent *AvrB* strains were both gifts from Barbara Kunkel (Washington University, Missouri, USA) grown on King‘s B (KB) (King *et al.*, 1954) agar (1.5% (w/v)) supplemented with 50 µg/mL rifampicin for *Pst* and 50 µg/mL rifampicin plus 20 µg/mL tetracycline for *AvrB* selection; for two days at 28°C.

2.2.4 Botrytis cinerea

The *Botrytis cinerea* GLUK-1 (pepper) (Kliebenstein *et al.*, 2005) isolate was maintained on sugar free apricot halves (Weigh-Less, SA) at 25°C in the dark. Every four weeks *B. cinerea* was subcultured by inoculating a small piece of infected apricot onto a fresh apricot using a sterile toothpick.

2.3 PLANT GROWTH

2.3.1 Seed storage

Dry seeds harvested from mature siliques were threshed to separate seeds from debris, transferred to microfuge tubes that were then sealed with micropore tape (3M, SA) instead of the tube lid to allow the seeds to air dry for an additional 1-3 weeks. Once seeds were dried down the microfuge tubes were capped with lids and the seeds stored at 4^{o}C.

2.3.2 Seed sterilization

For single seed sowing, seed was sterilized by shaking for 5 min in 70% (v/v) ethanol (EtOH) after which time the EtOH was aspirated and replaced with 100% (v/v) EtOH. The EtOH was immediately aspirated and the seeds air dried in a laminar flow cabinet before resuspending in sterile 0.1% (w/v) agar. For stratification, seeds were incubated at 4°C, in the dark for 2-3 days prior to sowing. Alternatively for scattered sowing, seed was sterilized by shaking in 70% (v/v) EtOH and then air dried on sterile filter paper in a laminar flow cabinet. Seeds were stratified for 2-3 days at 4°C, in the dark after plating onto sterile media.

2.3.3 Arabidopsis seedling growth conditions

Arabidopsis Columbia-0 (Col-0) ecotype and transgenic seedlings were grown in petri dishes (vertically or horizontally) on half strength MS agar (8% (w/v)), pH adjusted to 5.7 with 0.1 M KOH, under a 16 h light (100 µM photons m^{-2} s^{-1}) and 8 h dark cycle at 22 °C for a maximum of two weeks.

2.3.4 Arabidopsis growth on soil

Plants were grown on soil that is a 1:1 mixture of peat (Jiffy Products, International AS, Norway) and vermiculite (Stark Ayres, Cape Town, SA). Seeds were sown onto soil or in some cases seedlings were transplanted from petri dishes onto soil and thereafter covered with Clingfilm for one week to ensure high humidity, optimal for cotyledon expansion. Plants were grown under a 16 h light (100 µM photons m^{-2} s^{-1}) and 8 h dark cycle at 22 °C. After a week the Clingfilm was removed and the plants were fertilized with Phostrogen (Bayer CropScience Group, Hertfordshire, UK). Thereafter plants were watered every few days, as required, until they reached four weeks of age, at which time experiments were conducted.

2.4 NUCLEIC ACID MANIPULATIONS

2.4.1 Plant genomic DNA extraction and purification

Genomic DNA was extracted from *Arabidopsis* seedlings or leaf tissue using a rapid DNA extraction procedure adapted from the method of Edwards (Edwards *et al.*, 1991). Briefly, a single seedling or leaf was homogenized in 250 µL extraction buffer (200 mM Tris pH 7.5, 250 mM NaCl, 25 mM EDTA pH 8.0 and 0.5% (w/v) SDS) and then incubated at 60°C for 10 min. An equal volume of chloroform:isoamylalcohol (24:1 (v/v)) was added to each sample, mixed and then centrifuged at 10 000 x *g* for 10 min in a bench top centrifuge. The aqueous phase was collected and precipitated with 0.1 x vol sodium acetate (pH 5.2) and 2.5 x vol ice cold 100% EtOH at 4°C for 15 min. The following day the DNA was collected by centrifugation at 10 000 x *g* for 10 min. The pellet was washed with 70% (v/v) EtOH and the centrifugation repeated. The EtOH was aspirated and the DNA pellet allowed to air dry. Finally the DNA was resuspended in 50 µL TE buffer (10 mM Tris and 1 mM EDTA pH 8.0) and stored at -20°C.

2.4.2 Standard total RNA extraction and purification

Unless stated all plant tissue for gene expression was harvested 2 hr after dawn. Total RNA was extracted using a modified TRI reagent procedure for RNA isolation (Chomczynski & Mackey, 1995). Plant tissue was added to 1 mL ice cold TRIzol reagent (100 mM sodium acetate pH 5.2, 800 mM guanidium thiocyanate, 400 mM ammonium thiocyanate, 5% (v/v) glycerol and 38% phenol (v/v) pH 4, made up in DEPC-treated dH_2O). The tissue was disrupted and homogenized in the buffer by adding 3 ball bearings to the microfuge tubes and mechanically shaking in a tissue homogenizer for 3 min. The homogenate was incubated at room temperature for 5 min, followed by the addition of 200 µL of chloroform and a 30 sec vortex cycle. The homogenate was then incubated at room temperature for another 5 min and then centrifuged for 15 min at 13 600 x *g* and 4°C in a bench top centrifuge. The aqueous phase was transferred to a microfuge tube containing 500 µL of isopropanol and incubated at room temperature for 10 min. The sample was pelleted by centrifugation for 15 min at 13 600 x *g* and 4°C. The supernatant was carefully aspirated and discarded and the pellet washed in 75% (v/v) EtOH. The pellet was re-centrifuged for 5 min at 3420 x *g* and 4°C and the supernatant aspirated. The RNA pellet was air dried for 5 min, resuspended in 20 µL of DEPC-treated dH_2O and heated at 55°C for 10 min to facilitate resuspension. Finally, the RNA was centrifuged for 5 min at 9 500 x

g and room temperature to pellet any insoluble debris and the RNA-containing supernatant transferred to a fresh microfuge tube. The RNA was stored at -70°C.

2.4.3 Determination of RNA quality

RNA purity was analysed using the NanoDrop (NanoDrop Technologies, Wilmington, USA). Additionally, RNA integrity was determined using gel electrophoresis. Briefly, 2.5 µg RNA was mixed with 10 µL loading dye (1 x MOPS buffer (40 mM MOPS, 10 mM NaOAC, 1 mM EDTA, pH 8), 60% (v/v) formamide, 67 µg/mL ethidium bromide (EtBr) and 9% (v/v) formaldehyde) and run on a 1.2% (w/v) agarose gel made up in 1 x MOPS with 6.2% (v/v) formaldehyde in a 1 x MOPS running buffer.

2.4.4 DNase treatment of RNA for removal of contaminating DNA

To remove any contaminating DNA, all RNA samples were treated with DNase from the Turbo DNA-free™ kit (Life Technologies, California, USA). Two micrograms total RNA were mixed with 0.1 volume 10 x TURBO DNase buffer and 1 µL of enzyme and incubated at 37^{o}C for 30 min. 0.1 volume of DNase inactivation reagent was added after incubation to stop the reaction and a further incubation step performed at room temperature for 5 min, with occasional mixing. The final reaction was centrifuged at 10 000 x *g* for 1.5 min and the supernatant transferred to fresh microfuge tubes.

2.4.5 cDNA synthesis

For RT-PCR, cDNA was synthesized using Superscript III Reverse Transcriptase (Life Technologies, California, USA). One microgram RNA, together with 1 µL of 500 ng/µL oligo dT_{18} primer and 1 µL of 10 mM dNTPs in a total volume of 14 µL (DEPC-treated water) was denatured by heating at 65°C for 5 min and then snap cooling on ice for 2 min. To each reaction 4 µL 5 x First Strand buffer, 1 µL 0.1 M DTT and 1 µL Superscript III enzyme was then added and the reaction was incubated at 50°C for 1 h and inactivated by heating at 70°C for 15 min.The cDNA was diluted 1:10 before use in downstream PCR reactions and stored at -20^{o}C or -70^{o}C for short or long term periods respectively.

2.4.6 Reverse Transcriptase-quantitative Polymerase Chain Reaction (RT-qPCR)

RT-qPCR was conducted in accordance with the KAPA SYBR® FAST qPCR Kit protocol (Kapa Biosystems, Cape Town, SA). All reactions were conducted in 10 µL reactions

containing 1 x master mix, the forward and reverse primers (see Table 2.1 for concentrations), template cDNA and PCR-grade water. The amplification reactions were conducted in the Rotor-Gene® 6000 Real-Time PCR machine (QIAGEN, Limburg, Netherlands) with the following cycling conditions: enzyme activation at 95°C for 3 min, followed by 40 cycles of 95°C denaturation for 3 sec, 60°C annealing for 20 sec, 72°C extension/data acquisition for 1 sec and concluded with a final melt step at 72-95°C.

Table 2.1: RT-qPCR primers for gene expression studies

Gene name	Gene ID	Primer pair sequences	Final concentration (nm)	Product size (bp)
ACT2	At3g18780	5'AGTGGTCGTACAACCGGTATTGT3' 5'CATGAGGTAATCAGTAAGGTCACGT3'	900 300	138
DIN6	At3g47340	5'TCACGCTGCTCAAAATGTCA3' 5'TCTGCGGGAAGAACCTTTCA3'	900 300	125
HSPRO1	At3g55840	5'TTGGGAATGCAGAGGCGAAT3' 5'CTCGGGTAATACGGTGGCTC3'	100 100	97
HSPRO2	At2g40000	5'GCGATGAAGCTTTACGCGAG3' 5'GTTCATCTCCGCACTTCCCA3'	200 200	85
KIN2	At5g15970	5'GCTGGCAAAGCTGAGGAGAAG3' 5'CCGCCTGTTGCGCGG3'	200 200	88
PR1	At2g14610	5'CATGGGACCTACGCCTACC3' 5'TTCTTCCCTCGAAAGCTCAA3'	200 200	92
SAUR78	At1g72430	5'TCATTCGATGCGGTGTGTGA3' 5'TGCTCAGAAAAACGGAAGCA3'	200 200	70
WRKY70	At3g56400	5'TGCCAAATTCCCAAGAAGTTAC3' 5'TCGAGCTCAACCTTCTGGAC3'	200 200	90
XBAT34	At4g14365	5'TGGTTTTCTGAGGCATGTAAA3' 5'AGTGCAGGAACATTGGTTGT3'	200 200	101

2.4.7 Microarray analysis

2.4.7.1 RNA amplification and CATMA array experiments

Arabidopsis plants were grown on soil for four weeks under long day (16 hr light/8 hr dark) conditions and rosette leaf tissue was harvested two hours after dawn. RNA was extracted from 4 pools of leaves (with 4 plants per pool) for wild type and *hspro* mutants. For microarray analysis, the 4 RNA samples per plant line were pooled and 1 µg of total RNA was amplified using the Ambion MessageAmp™ II aRNA Amplification kit (Life Technologies, California, USA) in accordance with the kit protocol with a single round of amplification. Cy3- and Cy5-labeled cDNA probes were prepared by reverse transcribing 5 µg of aRNA with Cy3- or Cy5-dCTP (GE Healthcare, London, UK) and a modified dNTP mix (10 mM each dATP, dGTP, and dTTP; 2 mM dCTP) using random primers (Life Technologies) and SuperScript III reverse transcriptase (Life Technologies, California,

USA), with the inclusion of RNase inhibitor (RNaseOUT; Life Technologies) and DTT. Labelled probes were purified using QiaQuick PCR Purification columns (QIAGEN, Limburg, Netherlands), freeze-dried, and resuspended in 50 µL of hybridisation buffer (25% formamide, 5 × SSC, 0.1% (w/v) SDS, and 0.5 µg/µL yeast tRNA; Life Technologies). Pairs of labelled samples (Col-0 and either *hspro1-2*, *hspro2* or *hspro1-2/hspro2*) were hybridized to slides overnight at 42°C. A dye-swap design was employed such that each comparison consisted of 4 slides (two with Col-0 labelled with Cy3, and hspro with Cy5, and two with Col-0 labelled with Cy5 and hspro with Cy3). Following hybridization, slides were washed and scanned using an Affymetrix 428 array scanner at 532 nm (Cy3) and 635 nm (Cy5). Scanned data were quantified using ImaGene® 7.5.0 software (BioDiscovery Incorporated, CA, USA). Microarray experiments were performed using the CATMA (version 3) microarray (Allemeersch *et al.*, 2005), with probe annotations based on the TAIR10 release.

2.4.7.2 Data analysis

The ImaGene® software package was used to extract signal intensities for each slide and the data analysed using the limmaGUI package for the data analysis program, R (Wettenhall & Smyth, 2004). Normalisation of gene expression data was conducted on two separate fronts. Print tip loess within array normalisation was performed and this attempts to normalise the spatial binding biases of a particular dye within the slide. Quantile between array normalisation was used to account for differences across multiple slides and both normalisation steps reduce technical variation in the data. Confirmation of successful normalisation was assessed by looking at MA plots and normalised data should have a mean log ratio of zero and be evenly distributed around this mean. Following normalisation, lists of differentially expressed genes were returned using the linear model tool, along with p and adjusted p-values. Adjusted p-values are the p-values after multiple testing correction (Benjamini and Hochberg false discovery rate) has been applied and were the ones considered for determination of statistical significance. Only genes with adjusted p-values less than 0.05 were considered to be significant.

2.4.7.3 Microarray validation via RT-qPCR

For the RT-qPCR validation the extracted RNA pools (per line) were kept separate and cDNA synthesised from 1 µg total RNA per pool (as previously described in section 2.4.5). Five genes (Table 2.1) displaying differential expression patterns in the experiment were

identified for validation and gene expression was normalised to *ACTIN2* (*ACT2*) expression levels since *ACT2* is routinely used as a reference gene and in this data set there was no observed differential expression of the gene. Gene expression levels in the mutant lines were expressed relative to wild type levels.

2.4.8 Polymerase chain reaction (PCR) amplification of DNA

Unless otherwise stated, all standard PCR and semi-quantitative RT-PCR reactions were conducted with the KAPA Taq ReadyMix PCR Kit (Kapa Biosystems, Cape Town, SA) in 20 µL volumes (1.5 mM $MgCl_2$, 0.2 µM primer) containing the recommended reactant concentrations and reactions were performed using the GeneAmp PCR Systems 2700 PCR machine (Applied Biosystems, Foster City, USA) with an annealing temperature of 55^{o}C. For colony PCR, a sterile toothpick was used to transfer a minute amount of the bacterial colony into the reaction tube instead of using DNA template. The PCR products were visualised according to the gel electrophoresis protocol described below.

2.4.9 High fidelity PCR amplification of DNA

Amplification of DNA for cloning purposes was conducted with the KAPA HiFi HotStart ReadyMix PCR kit (Kapa Biosystems, Cape Town, SA). The following reactants were added to the reaction tube at the recommended final concentrations: 1 x ready mix, 0.3 µM forward and reverse primers each, < 100 ng DNA and made up to the final volume with water. All primer annealing steps were conducted at 55^{o}C.

2.4.10 Restriction endonuclease digestion of DNA

One microgram of total DNA was digested according to the manufacturer‘s guidelines (Thermo Fisher Scientific, Delaware, USA) and separated and visualized using gel electrophoresis as described below.

2.4.11 Visualization of DNA products by gel electrophoresis

The PCR products were mixed with 6 x loading dye (either bromophenol blue: 0.25% (w/v) bromophenol blue in 40% (w/v) sucrose or orange G: 0.15% (w/v) orange G, 10 mM Tris HCl pH 7.5, 60 mM EDTA, pH 8.0 and 60% (v/v) glycerol depending on the expected product size). Products were separated in agarose gels made up in 1 x Tris-Acetate (TAE) buffer (40 mM Tris, 20 mM acetic acid and 1 mM EDTA pH 8.0), containing 0.16 µg/mL EtBr and run in 1 x TAE buffer. An O‘GeneRuler 1 kb DNA Ladder (Fermentas Inc.,

Maryland, UK) was included on each gel to size DNA. Gels were visualized using Gel Doc system and images captured using Quantity One 1D analysis software (both from BioRad Inc., Hercules, CA, USA).

2.4.12 Column purification of PCR amplification products and restriction endonuclease treated DNA

The DNA product was separated on 1 x TAE agarose (0.8 – 2%) gels and visualized under long wavelength UV light (365 nm) and the appropriate bands excised with a sterile scalpel blade and transferred to a 1.5 mL microfuge tube. The DNA was then extracted with the Wizard® SV gel and PCR clean-up system as per manufacturer‘s instructions (Promega Corporation, Madison, USA) and quantified on a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Delaware, USA).

2.4.13 Plasmid DNA purification

Plasmid DNA was extracted and purified from 2-5 mL overnight cultures of *E. coli* or *A. tumefaciens*. Purification was conducted with the PureYield™ plasmid miniprep system according to the manufacturer‘s protocol. Once resuspended, the DNA was quantified with a NanoDrop 2000 spectrophotometer.

2.4.14 DNA ligation

Restriction endonuclease (RE) digested DNA was cloned into RE digested and purified vector backbone. 50 ng of vector backbone were used in the cloning process and the amount of insert was determined according to the following calculation:

$$\frac{\text{amount of vector (ng)} \times \text{size of insert (kb)} \times \text{insert: vector ratio}}{\text{size of vector (kb)}} = \text{amount of insert (ng)}$$

to end with a 3:1 insert to vector ratio in a 10 µL ligation mix (1 x Buffer, T4 DNA ligase). Ligation mixes were incubated at 4^{o}C overnight and then used to transform *E. coli.*

2.4.15 Plasmid DNA recombination

DNA inserts in the pENTR™4D entry vector were sub-cloned into the destination vector via the *att*B/*att*P recombination reaction facilitated by LR Clonase™ II, according to the manufacturer‘s instruction (Life Technologies, California, USA). Briefly, 50-150 ng of the

entry clone was added to 150 ng of the destination vector in a total volume of 8 µL TE buffer (pH 8). 2 µL of LR Clonase™ II enzyme were added to the DNA and the reaction incubated at 25^{o}C for 1 h. Two micrograms of Proteinase K were added to the sample which was then incubated at 37^{o}C for 10 min to inactivate the recombination reaction. Following recombination, the expression vector was transformed into *E. coli*.

2.5 BACTERIAL AND PLANT TRANSFORMATION

2.5.1 E. coli transformation with plasmid DNA

Competent cells were transformed with plasmid DNA according to the heat shock protocol. 10 pg-100 ng of DNA were added to 50 µL of thawed competent cells in a microfuge tube, flicked to gently mix in the DNA and incubated on ice for 30 min. The cells were heat shocked at 42^{o}C for 45 sec and returned to ice for 2 min. 950 µL of *LB* broth were added to the cell/DNA mix and incubated at 37^{o}C for 60 min with shaking. Following incubation 100 µL of the transformation mix were spread-plated onto *LB* agar with the appropriate antibiotic for selection of positive transformants and incubated at 37^{o}C overnight. Glycerol stocks of positive transformants were prepared according to the method below.

2.5.2 A. tumefaciens transformation with plasmid DNA

Plasmid DNA was purified from *E. coli* positive transformants and transformed into competent *A. tumefaciens* GV3101 cells. Twenty-five microlitres of the mini-preparation plasmid DNA was added to 100 µL of competent cells in a microfuge tube. The DNA/cell mix was incubated at 37^{o}C for 5 min to thaw and heat shock the cells. 900 µL of plain *LB* broth were added to the cells and they were incubated at 30^{o}C for 6 h with shaking. After incubation 100 µL of the transformation mix was plated onto *LB* agar containing 150 µg/mL rifampicin and 15 µg/mL gentamicin to select for the *A. tumefaciens* and the plasmid specific antibiotic to select for transformants, and incubated at 30°C for 2-3 days until colonies appeared. Transformants were screened by colony PCR (as described previously) and overnight cultures were prepared from which glycerol stocks of the positive transformants were generated. These were stored at -70°C.

2.5.3 Glycerol stocks

Positive transformants were stored as glycerol stocks, adding 500 µL of log phase bacterial culture to 500 µL of glycerol storage solution (65% v/v glycerol, 0.1 M $MgSO_4.7H_2O$ and 0.025 M Tris HCl pH 8.0) in a 1.5 ml eppendorf tube and snap frozen in liquid nitrogen before storage at -70°C.

2.5.4 A. thaliana transformation with Agrobacterium

Transformation was conducted according to the floral dip method of Clough and Bent (Clough & Bent, 1998) described in further detail below.

2.5.4.1 Plant preparation

To promote secondary bolt formation, the primary bolts that emerged at about 4 weeks were clipped at the base of the leaf rosette. Clipping was performed every other day until all the plants were at a uniform bolting stage. The plants were then allowed to flower for about a week until there were a maximal number of silique-free flowers.

2.5.4.2 Agrobacterium preparation

*A. tumefaciens s*uccessfully transformed with the appropriate vector were streaked onto *LB* agar (1.5% (w/v)) containing the appropriate antibiotic selection (150 µg/mL rifampicin, 15 µg/mL gentamicin and the vector specific antibiotic) and grown at 30°C for 2-3 days. A single colony was then inoculated into 5 mL selective liquid *LB* media and incubated shaking at 30°C for 2 days. Thereafter the entire 5 mL culture was inoculated into a large scale culture of 500 mL *LB* media containing antibiotics and incubated shaking at 30°C overnight. The following day the cells were harvested by centrifugation at 3500 x *g* for 15 min at room temperature. Finally the cells were resuspended in 250 mL 5% (w/v) sucrose containing 0.05% (v/v) Silwet L-77 surfactant.

2.5.4.3 Floral dip

The aerial parts of the *Arabidopsis* plants were submerged in the *A. tumefaciens* solution for approximately 5 s. Dipped plants were laid on their side in trays lined with tissue paper, covered in Clingfilm and left overnight in the plant growth room. The following day the plants were uncovered and placed upright.

2.5.4.4 Screening of primary Arabidopsis transformants

The method used for screening of primary transformants is *Agrobacterium* vector dependent. Kanamycin resistant transformants were identified by sowing seeds on 0.5 x MS media containing 25 µg/mL kanamycin, stratifying for 2 days at 4^{o}C in the dark and cultivating the seedlings for 10 days under normal growth conditions. Kanamycin resistant transformants displayed green and fully expanded dicotyledons while sensitive plants had yellow and closed dicotyledons. Plants that expressed the *Bar* gene were resistant to phosphinothricin and to selection for such transgenics was conducted on plants grown on soil for five days before being sprayed with a 30 mg/L Basta™ solution. Spraying was repeated twice more at day 8 and day 11 and resistant transformants had green leaves while sensitive plants were yellow and pale. Plants transformed with the pFAST-G02

vector had an additional selection marker. Transgenic seeds express GFP protein under the control of the oleosin promoter and transformants were identified by GFP fluorescence under a fluorescent microscope (Shimada *et al.*, 2010)

2.5.5 Arabidopsis protoplast isolation and transfection

Arabidopsis mesophyll protoplasts were isolated and transfected according to the methods described by Yoo and co-workers (Yoo *et al.*, 2007).

2.6 GENERATION AND ISOLATION OF HOMOZYGOUS TRANSGENIC *ARABIDOPSIS*

2.6.1 T-DNA knockout mutants

2.6.1.1 Isolation of homozygous hspro1-2, hspro2 and hspro1-2/hspro2 T-DNA insertion SALK lines by PCR genotyping

Homozygous *hspro1-1*, *hspro1-2* and *hspro2* lines were confirmed via PCR genotyping. The primers for genotyping were obtained from the SALK database (http://signal.salk.edu/tdnaprimers.2.html) and are listed in Table 2.2.

Table 2.2: Primers for screening for homozygous T-DNA insertion lines

Mutant Name	Knockout Line ID	Left Primer	Right Primer	Left Border Primer
hspro1-1	SALK_076686	**5'-GCAGTACATTTCCGCTGTCTC-3' (86F)**	**5'-ACATCGGAGAGTGTTGGACAG-3' (86R)**	5'-ATTTTGCCGATTTCGGAAC-3' (LBb1.3)
hspro1-2	SALK_092737	**5'-TTTTCCGCATAATGACGTCTC-3' (37F)**	**5'-GAACAGCTGGCACTTTCTGTC-3' (37R)**	5'-ATTTTGCCGATTTCGGAAC-3' (LBb1.3)
hspro2	SALK_016065	**5'-AAGTACACGCTCGGACTCGGTG-3' (65F)**	**5'-TACACTAACATCTACGAAGGATAGGTGGTG-3' (65R)**	5'-TGGTTCACGTAGTGGGCCATCG-3'(LBa1)

The primers in bold text are for amplifying the wild type PCR product and the underlined primer sequences for amplification the mutant PCR product. Standard PCR was carried out on genomic DNA as outlined in this chapter.

2.6.1.2 Cross fertilization of hspro1-2 and hspro2 to generate the hspro1-2/hspro2 double knockout mutant

To generate *hspro1-2/hspro2* mutant lines, homozygous *hspro1-2* pollen was the donor for pollinating *hspro2*. *Arabidopsis* plants were grown until the white petals were almost visible on the flowers. Any mature flowers were cut from the inflorescence. All flower parts except the ovaries were removed from the recipient and donor pollen was dusted onto the tip of the exposed ovary. Pollinated plants were then covered in Clingfilm and allowed to develop mature siliques. The fully developed siliques were allowed to dry and the seeds harvested before the siliques burst open. Homozygous double knockout plants were identified via the genotyping PCR methods described above.

2.6.1.3 Expression of HSPRO1 and HSPRO2 in the SALK lines determined by RT-PCR

RT-PCR (primer pairs in Table 2.3) was performed on cDNA from the different mutant lines and Col-O to measure the expression of *HSPRO1* and *HSPRO2* full length CDS in the mutants and the wild type plants. The *ACT2* gene was used as a reference gene.

Table 2.3: Gene expression primers to confirm knockout mutants

Gene Name	Gene ID	Left Primer	Right Primer
HSPRO1	At3g55840	5‘ATGGCTGATTTGGATTTACAGAG3‘	5‘CAATTGGCTCCAGAACTCTCC3‘
HSPRO2	At2g40000	5‘ATGGTTGATATGGATTGGAAGAG3‘	5‘CCTCCCAAATGACTCCAAAACTCT3‘
ACT2	At3g18780	5‘AGTGGTCGTACAACCGGTATTGT3‘	5‘CATGAGGTAATCAGTAAGGTCACGT3‘

2.6.2 Generation of HSPRO1 and HSPRO2 over-expressor transgenic lines

2.6.2.1 PCR amplification of HSPRO1 and HSPRO2 full length CDS

Using high fidelity PCR, the wild type genes were amplified from *Arabidopsis* Col-0 genomic DNA with the primers in Table 2.4. For cloning purposes, *KpnI* and *NotI* restriction endonuclease recognition sites (underlined text) were introduced to the 5‘ ends of the forward and reverse primers respectively.

Table 2.4: *35S::HSPRO* primers for cloning into the pFAST-G02 expression vector

Gene Name	Gene ID	Left Primer	Right Primer
HSPRO1	At3g55840	5‘GCGCGGTACCATGGCTGATTTGGATTTACAGA3‘	5‘GCGCGCGGCCGCTCACAATTGGCTCCAGAACTC3‘
HSPRO2	At2g40000	5‘GCGCGGTACCATGGTTGATATGGATTGGAAGA3‘	5‘GCGCGCGGCCGCTCATCCCAAATGACTCCAAA3‘

The underlined text highlights the *KpnI* and *NotI* RE recognition sites in the left and right primers respectively.

2.6.2.2 Cloning HSPRO1 and HSPRO2 into pFAST-G02

pENTR4-D and the gene amplicons were digested with *KpnI* and *NotI* restriction enzymes, ligated together and transformed into *E. coli*. Positive transformants were identified via RE digestion of purified plasmid DNA with *HindIII* and glycerol stocks were made for

successful transformants. Positive transformant plasmid DNA was sent for sequencing and once the sequence data was confirmed sub-cloned into the pFAST-G02 destination vector via LR clonase recombination. The recombinant DNA was transformed into *E.coli* and positive transformants identified via PCR screening with the cloning primers detailed in Table 2.4. Glycerol stocks of the successful transformants were aliquoted and stored at -70^{o}C. The expression vectors were transformed into *Agrobacterium* and into *Arabidopsis* Col-0 plants.

2.6.2.3 Determining HSPRO1 and HSPRO2 gene expression in 35S::HSPRO1 and 35S::HSPRO2 transgenic Arabidopsis

Total RNA was isolated and purified from 4-week-old transgenic plants and wild type plants, treated with DNase and cDNA synthesised according to the methodologies described in the nucleic acid manipulations section. A 1/10 working dilution of the cDNA was used in the RT-qPCR reactions to determine the gene expression levels. Gene expression was normalised to *ACTIN2* (*ACT2*)

2.6.3 Generation of HSPRO1 and HSPRO2 promoter-luciferase fusion plant lines

2.6.3.1 PCR amplification of HSPRO1 and HSPRO2 promoter regions

Using high fidelity PCR, the 1.5 kb regions upstream of the transcription START codon were amplified from *Arabidopsis* Col-0 genomic DNA with the primers in Table 2.5. For cloning purposes, *SacI* and *EcoRI* restriction endonuclease recognition sites (underlined text) and were introduced to the 5‘ ends of the forward and reverse primers respectively. The amplified PCR products were visualised and purified for downstream processing as described previously.

Table 2.5: Primers for cloning *HSPRO* promoters into the pART27 luciferase expression vector

Gene Name	Gene ID	Left Primer	Right Primer
HSPRO1	At3g55840	GCGCGAGCTCGCTCTCAATCCAAAGTCAAGG	GCGCGAATTCCAGAGATTTTTGTTTATTTGATGAAT
HSPRO2	At2g40000	GCGCGAGCTCACTCGAACCGTTTCCAACTG	GCGCGAATTCTAGAGGATGGGGACGAGAAA

The underlined text highlights the *SacI* and *EcoRI* RE recognition sites in the left and right primers respectively.

2.6.3.2 Cloning HSPRO1 and HSPRO2 promoters into pART27

pART27 and the promoter amplicons were digested with *SacI* and *EcoRI* restriction enzymes, ligated together and transformed into *E. coli*. Positive transformants selected on kanamycin *LB* agar were identified via colony PCR and RE digestion of purified plasmid DNA with *SacI/EcoRI* and glycerol stocks were made for successful transformants. Positive transformants were transformed into *Agrobacterium* and then *Arabidopsis* as described in the methods section.

2.6.4 HSPRO1-YFP and HSPRO2-YFP fusion constructs

2.6.4.1 PCR amplification of HSPRO1 and HSPRO2

The coding regions (from the START codon to penultimate codon) of the genes of interest were amplified via high fidelity PCR using the respective primers in Table 2.6. For cloning into 35S-YFP-NosT, *XbaI* and *KpnI* restriction endonuclease recognition sites (underlined text) were introduced to the 5‘ ends of the forward and reverse primers respectively and additional frame adjusting bases (lowercase text) were introduced into the reverse primers directly after the last codon in the genes.

Table 2.6: Primer sequences for amplifying the *HSPRO* genes for cloning into the 35S-YFP-NosT expression vector

Gene Name	Gene ID	Left Primer	Right Primer
HSPRO1	At3g55840	GCGCTCTAGAATGGCTGATTTGGATTTACAGAG	ATGCGGTACCccCAATTGGCTCCAGAACTCTCC
HSPRO2	At2g40000	GCGCTCTAGAATGGTTGATATGGATTGGAAGAG	ATGCGGTACCccTCCCAAATGACTCCAAAACTCT

The underlined text highlights the *XbaI* and *KpnI* RE recognition sites in the left and right primers respectively. Protein reading frame was adjusted by including ‗cc‘ bases at the end of the gene specific sequence but before the RE sites.

2.6.4.2 Cloning HSPRO1 and HSPRO2 into 35S-YFP-NosT

35S-YFP-NosT and the PCR products were digested with *XbaI* and *KpnI* restriction enzymes, ligated together and transformed into *E. coli*. Positive transformants selected on ampicillin *LB* agar were identified via RE digestion of purified plasmid DNA with *HindIII* and glycerol stocks were made for successful transformants. The reading frame of the constructs was confirmed via DNA sequencing.

2.7 PHENOTYPIC ANALYSIS OF TRANSGENIC *ARABIDOPSIS*

2.7.1 Seed germination assays

Seeds were sterilized and stratified according to the sterilization protocol and single-sown onto 0.5 x MS agar (8% (w/v)) supplemented with the appropriate concentrations of glucose, sucrose, mannitol and NaCl treatments. NaCl and mannitol were added to the MS media prior to autoclaving while glucose and sucrose solutions were filter sterilized prior to addition to the autoclaved MS media. Germination was scored as the emergence of the radicle or the presence of fully expanded and green cotyledons from 24 h after transfer into the growth environment. The number of seeds that germinated was expressed as a percentage of the total number of seeds sown.

2.7.2 Seedling responses

2.7.2.1 Root elongation

Seedlings were germinated on MS agar and grown for 5 days. Plants that germinated successfully and were of similar size and appearance were transferred onto control media or media supplemented with the prescribed mannitol and NaCl concentrations and the position of the root tip marked on the petri dish. After 7 days of vertical growth images of the plants were captured with a hand-held camera and root length increase measured using the ImageJ software (http://imagej.nih.gov/ij/). The data is the mean of at least 40 root measurements per line/treatment.

2.7.2.2 Hypocotyl length

Arabidopsis seeds were germinated on vertical 0.5 x MS agar plates under constant dark, 16 h light/8 h dark (long day) or 8 h light/16 h dark (short day). Seven days following germination the seedlings were mounted in a film of water between a slide and a coverslip to prevent dehydration while images were taken via a Leica EZ4 HD computer-linked stereo microscope (Leica Microsystems Incorporated, Illinois, USA). Hypocotyl length was measured as the distance between the base of the ‗V' made by the petioles of the cotyledons and the top of the root hairs around the collet (Derbyshire *et al.*, 2007). The mean length was derived from at least 25 hypocotyl measurements per line/treatment.

2.7.2.3 Seedling fresh weight (FW) gain

Seedlings were germinated on MS agar and grown for 5 days. Plants that germinated successfully and were of similar size and appearance were transferred onto control media

or media supplemented with the prescribed mannitol and NaCl concentrations and grown for an additional 7 days. The FW of individual shoots was measured, recorded and the average shoot weight calculated. The data is the average of 3-5 plates per treatment, with at least 100 seeds sown per line/plate.

2.7.2.4 Leaf movement assay

Arabidopsis seeds were individually germinated on thick 0.5 x MS agar, grown and entrained horizontally for four days under 12 h light/12h dark conditions. On the 5th day, 2 x 2 cm agar blocks along with the developing seedlings were transferred to 25 well tissue culture plates, with the 1st and 2nd leaves parallel to the lid of the plate. Twenty-four hours after acclimation the light source was switched to a constant state and leaf movements were tracked via automated digital photography with the AVerDiGi Hybrid NV5000 software (AVerMedia®, California, USA) every 20 min over a period of four days. Images were captured via four Panasonic® WV-BP144 CCTV cameras (Panasonic, Midrand, South Africa) (352 x 288 pixels at 96 DPI), with each camera tracking a unique plant line. Sixteen seedlings were imaged for each plant line but only those that displayed non-erratic leaf movement were analysed. Leaf movement position was tagged in ImageJ and overall leaf movement tracking automated in the Image-Pro® Plus software package (Media Cybernetics, Maryland, USA). The BRASS software add-in for Microsoft® Excel was used to analyse the data generated from Image-Pro® Plus.

2.7.3 Mature plant phenotypes

2.7.3.1 Total leaf dry weight

Arabidopsis seeds were germinated on soil and grown for four weeks. Whole leaf rosettes were harvested, collected individually in foil packets and dried in an oven with desiccant at 60^{o}C for two days. Dry weight was measured with an analytical balance and data collected from least 25 plants.

2.7.3.2 Leaf water loss assay

Leaves from 4-week-old soil grown plants were detached from the plants and immediately weighed. The leaves were then placed on filter paper to allow for water loss and the FW of each leaf was measured every h for 6 h. For each time point each leaf weight was expressed as a percentage of its initial FW at the time of detachment ((FW_t/FW_{to}) x 100) (Verslues *et al.*, 2006).

2.7.3.3 Pseudomonas syringae infection

Pseudomonas infections were carried out according to the methods described by Katagiri and colleagues (Katagiri *et al.*, 2002). Briefly, a single colony of *P. syringae* was picked from freshly streaked KB agar plates, inoculated into KB broth with the appropriate antibiotic selection and grown with shaking at 30^{o}C overnight (8-12 h). During mid-log phase, 1.5 mL of the culture was harvested by centrifugation at 10 000 x *g* for 1 min in a bench top microfuge. The pellet was rinsed once in 10 mM $MgCl_2$ before resuspension in 500 µL of 10 mM $MgCl_2$. The OD_{600} of the suspension was quantified with a Beckman DU 650 spectrophotometer and diluted to an OD_{600} of 0.002 in 10 mM $MgCl_2$, equivalent to an infection concentration of 1 x 10^6 cfu/mL. *Arabidopsis* leaves were pressure infiltrated with either 10 mM $MgCl_2$ (control) or the bacterial dilution via the abaxial side of the plant leaf. The leaves were then dried with tissue paper, covered in Clingfilm to maintain a humid environment and returned to the growth chamber. Three leaf discs (0.5 cm^2) were harvested from three separate leaves of the same plant (4 h and 48 h after infection) and homogenized in 1 mL of 10 mM $MgCl_2$. A 1:10 serial dilution (10^0 to 10^{-7}) was created for each sample in a 1 mL total volume and 10 µL spot plated for each dilution on KB agar with the appropriate antibiotics. Colony counts were performed for each plated dilution approximately 2 days after incubation at 30^{o}C.

2.7.3.4 Botrytis cinerea infection

B. cinerea spores were collected 12 days after infection by pipetting 3 mL of sterile water onto the petri dish containing the infected apricot halves. A cloudy spore suspension was created by gently rubbing the spores into the water with a glass rod. A spore count was conducted on the suspension using a haemocytometer and a 5 x 10^4 spores/mL dilution performed in sterile 0.5 x grape juice (Liquifruit, SA). Detached, mature *Arabidopsis* leaves were lined onto 0.8% agar in plastic containers and drop-inoculated with 10 µL of the spore suspension placed onto the middle of the leaf. The container was covered to maintain humidity and kept at room temperature with ambient light for the duration of the experiment at which point lesion size was recorded with a digital camera and lesion area measured in ImageJ.

2.7.3.5 Sugar content analysis

Glucose and sucrose

Extraction of glucose and sucrose was performed in the same procedure. Three hundred mg of plant tissue were homogenised in 1 mL of deionized water and incubated at 60^{o}C for 30 min to facilitate sugar extraction. The homogenate was centrifuged at 16 000 x *g* for 10 min to pellet the cell debris and the supernatant transferred to fresh microfuge tubes and stored at -70^{o}C until needed. Sugar content was measured using the Sigma® glucose (HK) assay kit and the sucrose (SCA20) assay kit.

Starch

Three hundred mg/mL of tissue were harvested, homogenised in 1 mL of water and boiled with gentle shaking for 3 min. The samples were then autoclaved for 1 h at 135^{o}C, allowed to cool down, centrifuged 16 000 x *g* for 10 min and the supernatant transferred to fresh tubes for storage at -70^{o}C. Starch content was measured using the Sigma® starch (SA20) assay kit.

2.7.3.6 Flowering time

Plants were germinated and grown on soil under standard conditions for long day plants and under 8 h light/16 h dark for short day conditions. Flowering time was measured as the number of days it took for the first flower to open and the total rosette leaf count at the day of flower bloom. 16 to 30 plants were used to generate the mean data (Kim *et al.*, 2008)

2.8 ANALYSIS OF GENE EXPRESSION AND REGULATION

2.8.1 Identification of genes co-expressed with HSPRO1 and HSPRO2

The CressExpress tool (http://cressexpress.org/) (Srinivasasainagendra *et al.*, 2008) was used to perform gene co-expression analysis with *HSPRO1* (AT3G55840) and *HSPRO2* (AT2G40000) as the query genes. CressExpress calculates the linear regression between the query gene and the rest of the genes in the expression data extracted from the Nottingham Arabidopsis Stock Centre (NASC) AffyWatch service (Craigon *et al.*, 2004) and also samples from two Affymetrix Arabidopsis array designs: the ATH1 array (22,810 probe sets) and the AG array (approximately 8,000 probe sets). A R^2 is returned together with a p-value that represents the probability of obtaining a particular correlation coefficient assuming a random relationship between the two genes being compared. The returned genes with significant p-values were then ranked according to their R^2 values, in descending order and those genes with a >0.36 R^2 value (equivalent to a Pearson's correlation coefficient (ρ) of 0.6) value were considered to be co-expressed (Srinivasasainagendra *et al.*, 2008) and condensed into an expression correlated gene group (ECGG).

2.8.2 Promoter analysis: Arabidopsis thaliana Expression Network Analysis (ATHENA)

ATHENA (http://www.bioinformatics2.wsu.edu/Athena) was used to identify potential transcription factor binding sites enriched in the promoters of queried gene lists versus the entire genome (O'Connor *et al.*, 2005). The 1000 bp region upstream of the ATG START codon was queried as the promoter sequence and it was chosen to cut-off at adjacent genes. Two different sets of lists were used to conduct motif analysis, namely the ECGGs from the correlation analysis and the lists of genes differentially expressed in the mutants versus the wild type.

2.8.3 Gene ontology analysis: Fast Assignment and Transference of Information using Gene Ontology (FatiGO)

FatiGO (Al-Shahrour *et al.*, 2007) was used in the identification of GO terms functionally enriched within a queried gene list versus the rest of the *Arabidopsis* genome. FatiGO returns a p-value from a Fisher exact test for each GO term and then an adjusted p-value using the False Discover Rate procedure to correct for multiple testing. FatiGO analysis

was run using all three GO categories (biological process, molecular function and cellular component), KEGG pathways and SwissProt filters. GO terms and their adjusted p-values were uploaded into the REVIGO web server (http://revigo.irb.hr/) for visualisation.

2.8.4 Analysis of publicly available microarray data

2.8.4.1 The Bio-Analytic Resource for Plant Biology

The e-Northerns with Expression Browser tool of the Bio-Analytic Resource (BAR) (http://bar.utoronto.ca/welcome.htm) was used to screen for conditions under which *HSPRO1* and *HSPRO2* were differentially expressed in *Arabidopsis* (Toufighi *et al.*, 2005). The analysis tool examined the expression of the two genes across experiments in the Botany Array Resource and the AtGenExpress Consortium (http://www.weigelworld.org/resources/microarray/AtGenExpress/). Biological duplicate expression values were returned by the tool and experimental values expressed relative to the average of the control sample values. Since only two biological repeats were available per time point for each treatment, statistical analysis was not conducted on the available data and a threshold of 2-fold change in expression was utilised in identifying potential gene expression altering conditions.

Abiotic stress

Data generated following treatment with cold, heat, drought, salt, osmotic, oxidative and UV-B stress was downloaded from the BAR tool and normalised to the respective untreated control data. Two biological replicates were available per treatment and all treatments were performed on whole plant tissue (Kilian *et al.*, 2007).

Hormone treatment

Whole plant tissue data was extracted for gene expression in plants treated with exogenous ACC (TAIR accession - ExpressionSet:1007965762), MeJA (TAIR accession - ExpressionSet:1007965964) and ABA (TAIR accession - ExpressionSet:1007964750) and also from seed tissue treated with ABA during imbibing (TAIR accession - ExpressionSet:1007967394). Biological duplicates were analysed and normalised to the appropriate controls. No statistical analysis was conducted as 2 data points are insufficient for analysis.

2.8.4.2 Global transcriptomic changes in response to varying energy levels and comparison to hspro expression profiles

Microarray $\log_2$ expression data for various energy response experiments was obtained for all the genes differentially expressed in at least one of the *hspro* mutants (microarray data). The source of the data is outlined in the table below and clustering analysis was conducted on the data sets in Cluster 3.0 (de Hoon *et al.*, 2004) and visualised in Java Treeview (Saldanha, 2004). Hierarchal gene clustering was conducted using the average linkage clustering method and sub-clusters in which genes behave in opposing patterns in response to high or low sugar treatments were chosen for further analysis.

Table 2.7: Microarray data accessed for clustering analysis

Treatment	Control	Source
100 mM glucose for 3 h	Carbon starved	(Bläsing *et al.*, 2005)
15 mM sucrose for 3 h	Carbon starved	(Osuna *et al.*, 2007)
CO_2 fixation for 4 h after dawn with 350 ppm CO_2	Illumination for 4 h after dawn with < 50 ppm CO_2	(Bläsing *et al.*, 2005)
Carbon starved for 2 days	Sucrose supplemented for 2 days	(Osuna *et al.*, 2007)
Extended dark treatment for 8 h	Normal dark period	(Usadel *et al.*, 2008)

2.8.4.3 The DIURNAL tool

DIURNAL is a web-based tool that allows access to several diurnal and circadian microarray experiments, providing gene expression profiles within different circadian and diurnal conditions (http://diurnal.mocklerlab.org/) (Mockler *et al.*, 2007). For each condition within the dataset there is a predicted model profile that is compared to the profile of the gene(s) of interest and a Pearson's correlation coefficient is returned. *HSPRO1* and *HSPRO2* expression was evaluated under long day (LD) and constant light (LL) conditions, with a $\rho > 0.8$ as the cut-off and the output data plotted and compared to the predicted model for each condition.

2.8.5 Circadian rhythmic oscillation of HSPRO2 promoter activity

Transgenic *HSPRO1::LUC* and *HSPRO2::LUC* lines were sown on 0.5 x MS (0.8%) agar, stratified for three days at 4^{o}C in the dark and grown for two weeks under 12 h light/12 h dark/22^{o}C entrainment conditions. One day before the start of the promoter activity profiling, the seedlings were pre-sprayed twice (dawn and dusk) with 5 mM luciferin dissolved in 0.01% (v/v) Triton X-100 to dissipate accumulated luciferase protein. The

following morning, the luciferin spray was repeated with 1 mM luciferin and luciferase activity measured in the Xenogen® IVIS® 100 Lumina Imaging System (Xenogen Corporation, California, USA). After the initial visualisation control plants were returned to the diurnal conditions and experimental plants moved to constant light conditions and promoter activity was monitored every 4 h thereafter for 56 h.

CHAPTER 3

Generation and initial characterisation of *hspro* transgenic *Arabidopsis* plants

CHAPTER 3: GENERATION AND INITIAL CHARACTERISATION OF *HSPRO* TRANSGENIC *ARABIDOPSIS* PLANTS

3.1 INTRODUCTION

Gene characterisation has traditionally been approached using the forward genetics approach – from phenotype to genotype – but in recent times with the increased access to entire genome datasets, a reverse genetics approach is on the rise.

Reverse genetics involve introducing mutations into a gene or its regulatory region and then screening for phenotypes that can be linked to the mutation. In plant biology, common approaches to generate loss of function mutants include introducing large sequences of T-DNA (ranging from 5 to 35 kb in length) into the gene or its promoter to knockout (or in some cases, knockdown) gene expression, introducing premature STOP codons or altering crucial amino acid sequences required for protein function via treatment with mutagens or site-directed mutagenesis (Bouché & Bouchez, 2001; Parinov & Sundaresan, 2000; Wang, 2008). A parallel approach involves generating gain-of-function mutants through over-expression of the gene of interest (Hilson, 2006; Østergaard & Yanofsky, 2004) and often gain-of-function and loss-of-function produce complementary results (Bolle *et al.*, 2011). In some cases loss-of-function mutations may affect embryogenesis preventing the formation of homozygous mutants (Errampalli *et al.*, 1991). In such cases inducible gene silencing utilising RNA interference or artificial microRNAs can be used to alter gene conditions under the conditions of interest only, or to generate partial knockdown mutants, allowing the transgenic plants to grow and develop normally (Schwab et al., 2010).

It is a frequent occurrence that mutagenesis does not produce an easily identifiable phenotype under normal growth conditions and often loss-of-function mutants do not produce a phenotype due to genetic redundancy (Bouché & Bouchez, 2001; Nakazawa *et al.*, 2003). To increase the likelihood of identifying gene-associated phenotypes, one can identify processes of potential relevance to gene function via several methods and then confirm putative functions through phenotype evaluation under specific conditions.

Inferences into gene function can be made by identifying already characterised homologues within the same organism or across species (Anjos *et al.*, 2007; Levesque *et al.*, 2003; Loewenstein *et al.*, 2009). Molecular function is often conserved across species and this can lead to simple identification of gene functions. Working with the hypothesis that genes are likely to be induced under conditions that require their function, it is also informative to survey public microarray data for conditions that induce expression of the gene of interest. Research is continually providing evidence that supports the idea that genes do not function in absolute isolation but rather function cooperatively to effect a common change (Fraser *et al.*, 2004; Romero-Campero *et al.*, 2013). Genes that function together are often co-ordinately expressed (Horan *et al.*, 2008) and there is evidence that suggests there are evolutionary pressures to maintain this co-expression. The prevailing theory is that the regulation of co-expressed genes is driven by transcription factors that interact with conserved regulatory elements in the promoters of co-expressed genes and so genes co-expressed across multiple and diverse experimental conditions are likely to be involved in the same biological processes (Allocco *et al.*, 2004; Meier & Gehring, 2008). Identification of co-expression gene groups is therefore useful in identifying novel functions for uncharacterised genes.

Once potential processes that require the gene of interest are identified, phenotypic, metabolic and genotypic analyses can be carried out utilising the gene-specific mutant plants to investigate the proposed gene functions. These methods of gene function analysis often have inherent flaws such as the unpredictable pleiotropic effects of T-DNA insertional mutagenesis and often multiple approaches are used in combination to assign functions to uncharacterised genes. Furthermore, due to the nature of T-DNA mutagenesis additional insertions can occur within non-target genes and observed phenotypes and responses may be a result of these unintended insertions. It is therefore necessary to confirm observed results with either multiple alleles of the same mutation or complement the mutation with the wild type gene (Krysan *et al.*, 1999)

In this chapter, several of these approaches were utilised in an attempt to identify novel putative *HSPRO* genes functions. *HSPRO1* is the only homologue of *HSPRO2* (a positive regulator of defence) in *Arabidopsis* and based on gene and protein similarities we hypothesised that *HSPRO1* could similarly be involved in plant defence. Interestingly, *HSPRO1* appeared to be a negative regulator of the defence response to *P. syringae* as

the *hspro1* knockout mutants are less susceptible to virulent *P. syringae* suggesting an antagonistic relationship between the two genes. Consistent with the hypothesis that *HSPRO* genes are defence response regulators, both genes are induced in response to pathogen attack and promoter content and GO analysis of the genes co-expressed with either *HSPRO1* or *HSPRO2* revealed enrichment of W-box in both *HSPRO* expression correlated gene groups (ECGGs) and terms associated with a/biotic stress responses respectively. The W-box is a target of WRKY transcription factors (TFs) and WRKY TFs are strongly associated with stress responses. Phenotypic analysis of the *hspro* knockout mutants grown on mannitol provided evidence for antagonistic roles in response to osmotic stress.

3.2 RESULTS

3.2.1 Generation of genetic screening tools

3.2.1.1 Isolation of homozygous SALK mutant lines

The putative role(s) played by a gene disrupted by a T-DNA insertional mutation can be validated by one of two possible ways. One could complement an insertional mutant with the wild type gene and see if the observed phenotypes are abolished or one could show that multiple mutant alleles of the same gene display the same phenotypes (Krysan *et al.*, 1999). *HSPRO1* T-DNA insertion mutants were identified by querying the SALK database (http://signal.salk.edu/cgi-bin/tdnaexpress) with the *HSPRO1* (At3g55840) gene identifier and two were selected for analysis. Homozygous *hspro1-1* and *hspro1-2* mutant seeds were obtained from the Nottingham Arabidopsis Stock Centre (NASC) (Alonso *et al.*, 2003) and homozygous *hspro2* obtained from Dr Ingle ((Murray *et al.*, 2007)). Homozygosity for the T-DNA insertion was confirmed via PCR with primers obtained from the T-DNA primer design tool (Table 2.2) within the SALK database (http://signal.salk.edu/tdnaprimers.2.html). As represented in Figure 3.1, there are gene specific primers flanking the T-DNA insertion sequence and these should only amplify a PCR product in the wild type gene. In the insertion mutation the length of the insertion sequence (approximately 6000 bp) prevents PCR amplification within the thermocycling conditions used (extension time of 30 secs). A combination of a left border (LB) T-DNA specific primer and a gene specific primer will only yield product when amplifying mutant DNA. To identify homozygous mutants, separate PCRs were performed using either wild type or mutant specific primers and twenty different plants were genotyped per plant line. Results of a representative four plants per mutant line are shown in Figure 3.2.

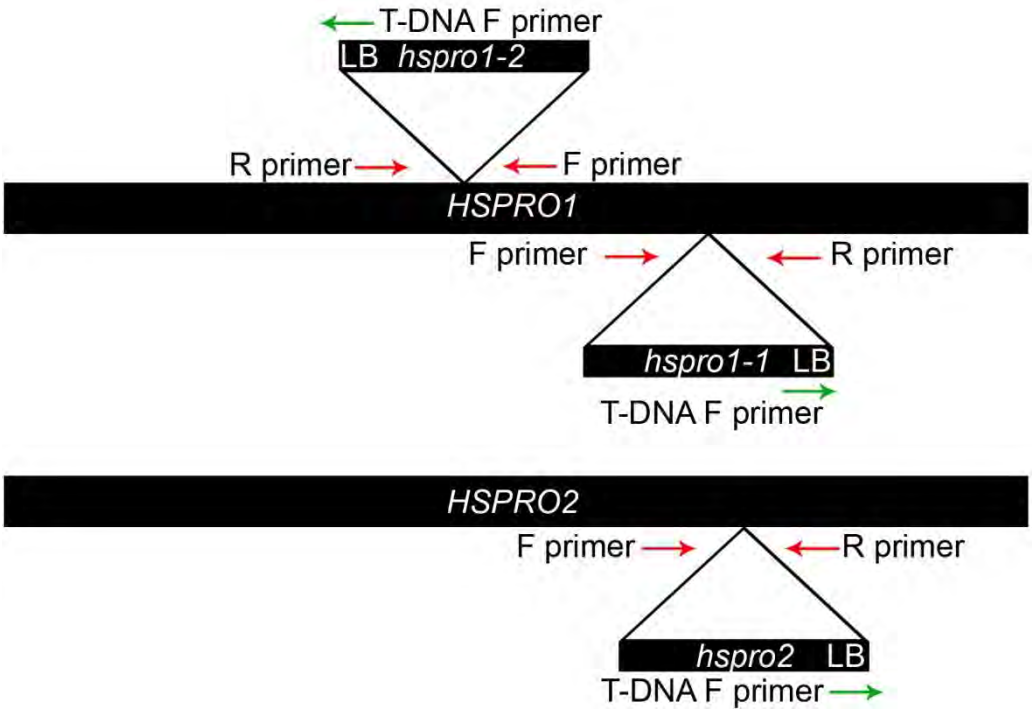


Figure 3.1: Primer design for PCR screening of the segregating SALK lines based on the predicted site of the T-DNA insertions.

Primers are represented by arrows. The presence of an insertion would be reported by PCR product amplification with the T-DNA F (forward, green) primer and the gene-specific R (reverse, red) primer while the wild type product would be amplified by the gene-specific F and R primer (red) combination. LB and RB are the left and right borders of the T-DNA insert respectively.

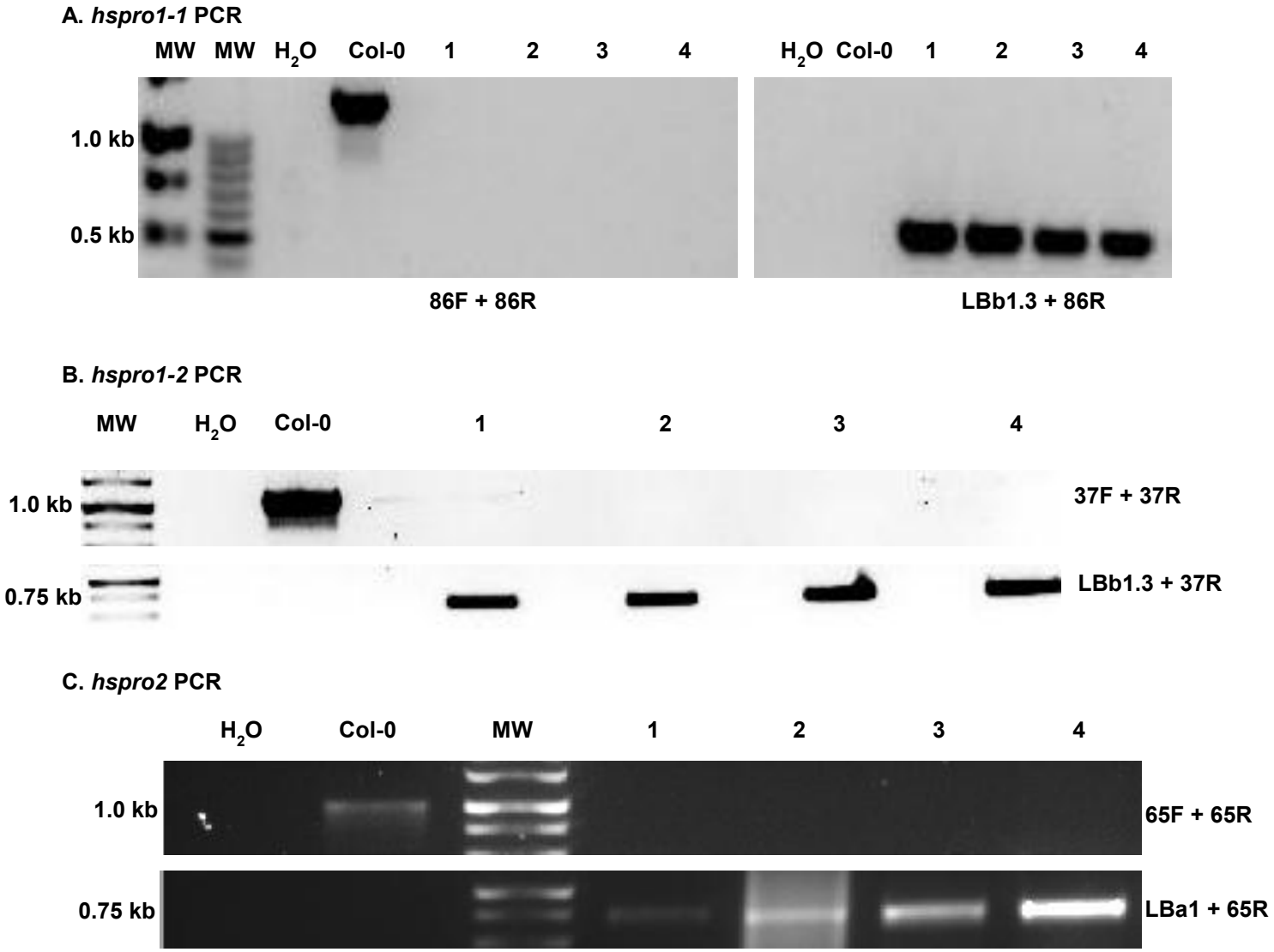


Figure 3.2: PCR genotyping of homozygous T-DNA single knockout insertion lines

Representative images of PCR screening showing four plants (1 - 4) identified as homozygous per T-DNA mutant and the wild type control (Col-0). F and R primer combinations were used to amplify the wild type genes while amplification with the LB and R combinations indicates the presence of the T-DNA insert. MW is molecular weight marker and H_2O is the no template control.

3.2.1.2 Identification and sequencing of hspro T-DNA insertion lines

Through DNA sequencing of the PCR product amplified with the gene specific reverse primer (RP) and the T-DNA specific LB primers, the SALK_076686.50.45.x (*hspro1-1*) insertion site was determined to be 887 base pairs downstream of the ATG START codon (12 bp upstream of the predicted insertion site according to The *Arabidopsis* Information resource (TAIR)), while the predicted insertion site in the SALK_092737.46.75.x (*hspro1-2*) was confirmed to be 582 base pairs downstream of the ATG START codon (as predicted according to TAIR) (Figure 3.3). The *hspro2* insertion line (SALK_016065.56.00.x), had already been characterised and work resulting from its characterisation published (Murray *et al.*, 2007). Sequencing was conducted on mutant specific PCR product amplified with

the mutant specific primers described in Figure 3.1. It should be noted that both the *HSPRO1* and *HSPRO2* genes lack intron sequences and as such all exogenous insertions within the coding sequences would likely prevent the transcription of full mRNA transcript.

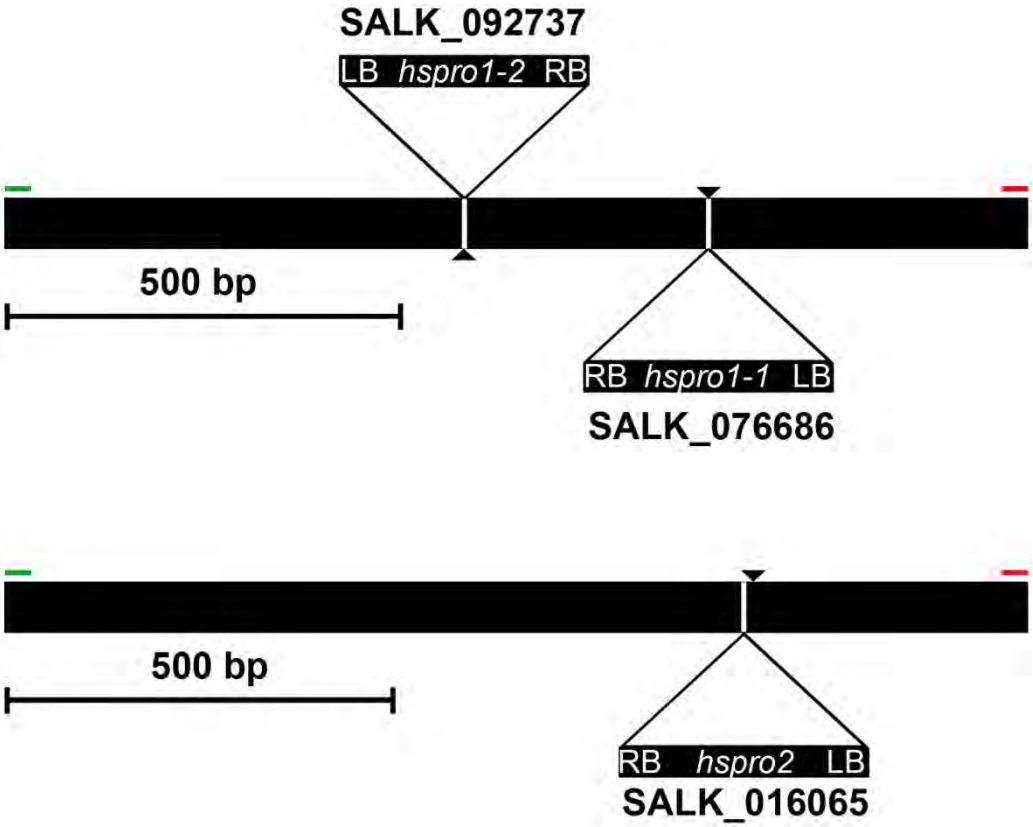


Figure 3.3: *HSPRO1* and *HSPRO2* gene structure displaying the predicted and actual T-DNA insertion sites in the SALK lines.

The black triangles represent the predicted insertion sites, the white triangles(with vertical white bars) the actual insertion sites and the green and red bars represent the START and STOP codons respectively.

3.2.1.3 Generation of a hspro1-2/hspro2 double knockout mutant

Homozygous *hspro1-2* and *hspro2* mutants were cross-fertilised to generate heterozygous *hspro1-2/hspro2* F_1 seeds. The F_1 plants were allowed to self-fertilise and F_2 seed was collected. Homozygous double knockout mutants were identified in the F_2 generation via PCR with the same primers sets used to identify the parental homozygous lines. Ten F_3 plants from one of putative homozygous F_2 lines were genotyped and results from five of those plants are shown in Figure 3.4

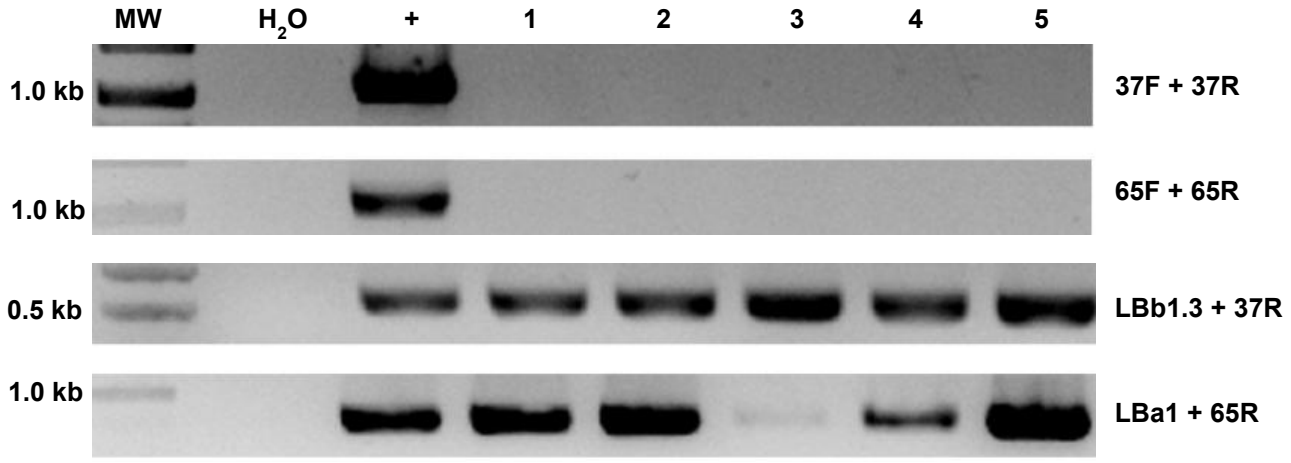


Figure 3.4: PCR genotyping of the homozygous *hspro1-2/hspro2* double knockout T-DNA insertion mutant.

Homozygous *hspro1-2* and *hspro2* single knockout lines were cross-fertilised and plants homozygous for both mutations were isolated via PCR screening of the F_3 generation. H_2O is the no template control, + is the positive control from Col-0 for the wild type PCRs (37F+37R for *HSPRO1* and 65F+65R for *HSPRO2*) and the respective parents for the mutant PCRs (LBb1.3+37R for *hspro1-2* and LBa1+65R for *hspro2*). MW represents the molecular weight marker. PCR Screening was conducted on five biological repeats (1-5).

3.2.1.4 HSPRO1 and HSPRO2 expression in the hspro knockout mutants

An absolute necessity when working with T-DNA insertion lines is confirmation of mRNA expression levels (or lack thereof) of the genes of interest in the mutant lines. This confirms whether one is working with a null knockout mutant or a knockdown mutant and in some cases even an over-expressor line (Ülker *et al.*, 2008). *HSPRO1* and *HSPRO2* full length CDS amplicons were not detected in reverse transcriptase PCR experiments from *hspro1-2* and *hspro2* mutants respectively (Figure 3.5), nor were full length amplicons of either *HSPRO1* or *HSPRO2* detected in the homozygous double knockout but were indeed detected in the wild type positive control, providing evidence that the T-DNA insertions had abolished the generation of full-length *HSPRO* transcripts and that the mutants are true knockouts.

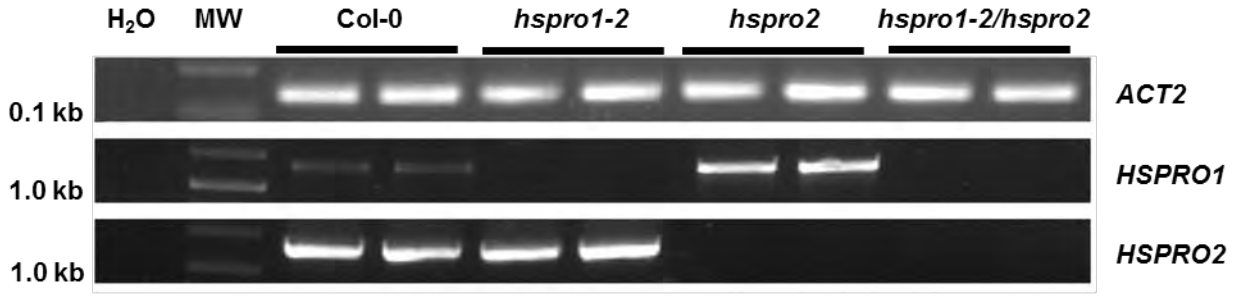


Figure 3.5: Expression of *HSPRO1* and *HSPRO2* in the *hspro* knockout mutants

Two-week-old seedlings were harvested and total RNA extracted and used to synthesise cDNA. PCR was used to determine whether *HSPRO1* and *HSPRO2* were expressed in the *hspro* mutants. *ACT2* was the reference gene used to confirm successful cDNA synthesis. H_2O is the no template control and MW is the molecular weight marker.

3.2.1.5 hspro knockout mutants do not have obvious morphological phenotypes

The *hspro* mutants do not appear to have any observable morphological growth phenotypes (Figure 3.6) and generate biomass in a manner comparable to wild type plants (Figure 3.7). On an overall scale it appears *HSPRO* genes are not crucial for normal vegetative growth in adult shoot tissue of *Arabidopsis* plants.

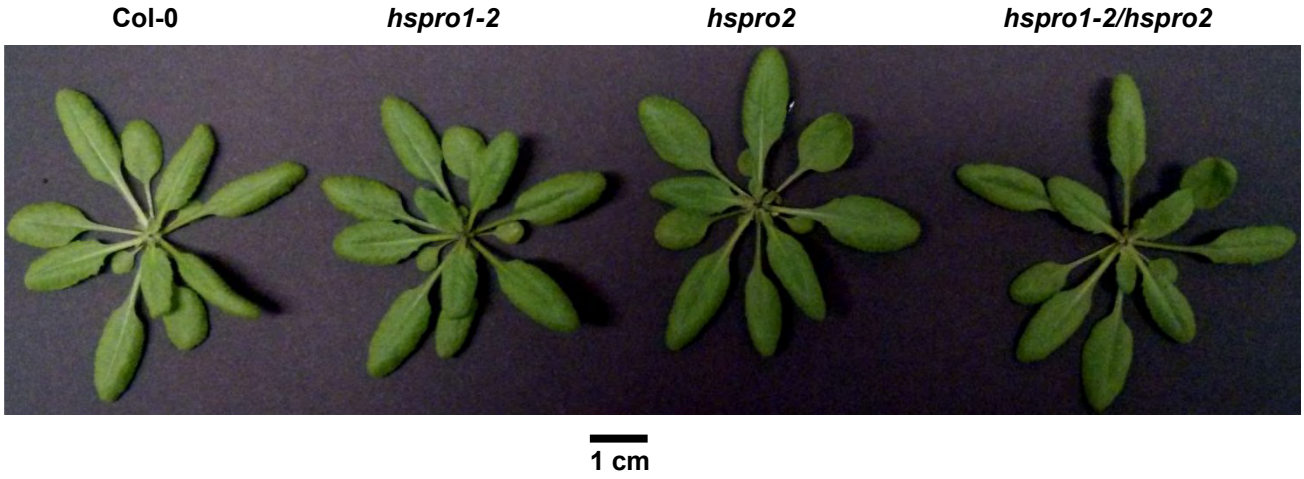


Figure 3.6: Gross morphology of adult mutant plants

Plants were grown for 4 weeks on soil under standard light and temperature conditions.

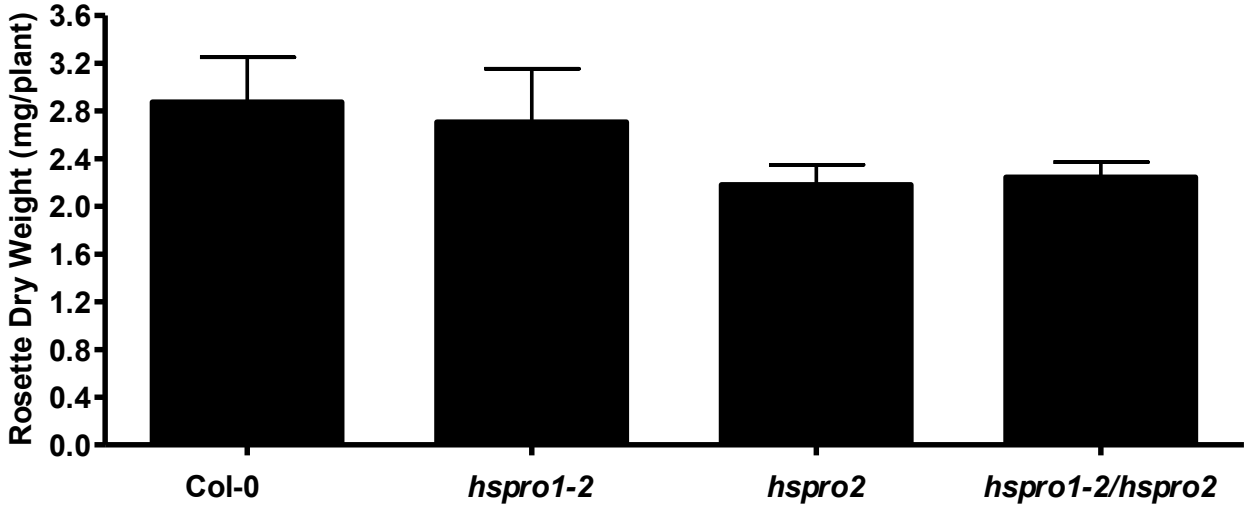


Figure 3.7: Dry weight of 28-day-old plants.

The average rosette dry weight per plant line was measured 28 days after germination. Each column represents the mean dry weight and the S.E.M of at least 25 plants. Statistically significant deviation from the wild type was evaluated via the Student's t-test and no significant differences were observed. This data is representative of two independent experiments.

3.2.1.6 HSPRO1 and HSPRO2 localise to the cytoplasm under normal growth conditions

The two genes of interest do not display any known protein localisation sequences and have been previously shown to interact with the SnRK1 complex in the cytosol of *N. benthamiana* (Gissot *et al.*, 2006), which normally functions under stress conditions (Baena-González *et al.*, 2007; Baena-González & Sheen, 2008). To determine if cytosol localisation is indeterminate of stress and occurs under normal growth conditions (including endogenous levels of *AKIN*βγ*)* in *Arabidopsis*, *GENE-YFP* constructs were generated. The two genes were cloned into the 35S-YFP-NosT over-expression vector (Seidel *et al.*, 2005) , directly upstream of the YFP tag sequence and transformed into *E. coli*. Plasmid DNA from successful transformants was isolated, purified and the gene fusions transiently expressed in *Arabidopsis* mesophyll protoplasts isolated from 4-week-old plants. Transfection reactions were incubated overnight and localisation was visualised via YFP fluorescence using confocal microscopy. It was determined that both proteins localise to the cytoplasm of *Arabidopsis* protoplasts as is seen in Figure 3.8. This supported the idea that there are no known localisation sequences in the protein sequences and HSPRO proteins function in the cytoplasm.

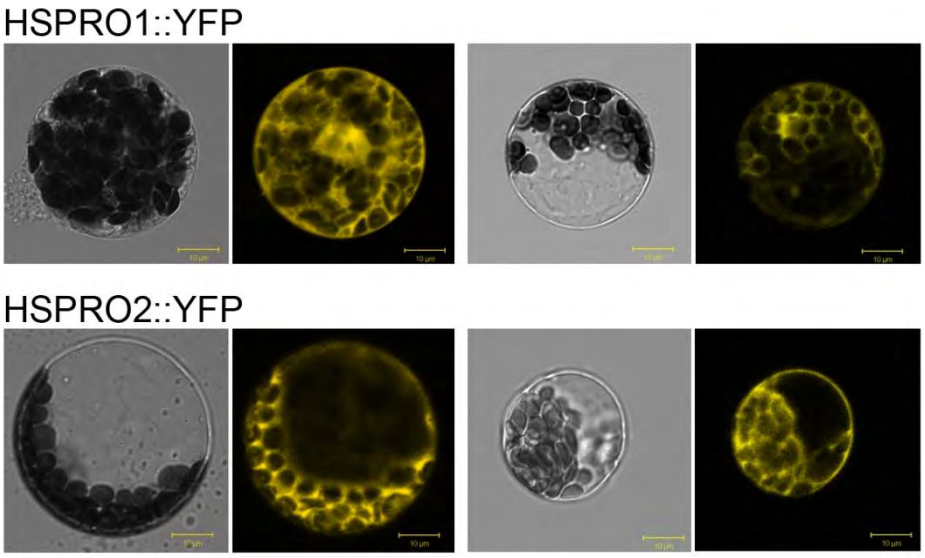


Figure 3.8: HSPRO1 and HSPRO2 localise to the cytoplasm of *Arabidopsis* protoplasts.

HSPRO1-YFP and HSPRO2-YFP fusion constructs were transfected into the mesophyll protoplasts of mature *Arabidopsis* leaves overnight and transient protein expression was monitored for 6 h on the following day. Two representative protoplasts per protein fusion, from two independent transfections are shown above with the bright-field view images on the left and the corresponding fluorescence images on the right. The scale bar represents 10µm.

3.2.1.7 Generation of HSPRO1 and HSPRO2 over-expressor lines

Over-expression of wild type gene products has been long used to complement conclusions derived from loss-of-function experimental analysis (Prelich, 2012) and a similar approach was attempted in this research project with limited success. *HSPRO1* and *HSPRO2* coding sequences were cloned into the pENTR4-D entry vector via the *KpnI* and *NotI* restriction enzyme recognition sites (see chapter 2). Plasmid DNA was isolated from successful transformants and sequenced before proceeding to sub-cloning into the pFAST-G02 destination vector (Shimada *et al.*, 2010) via DNA recombination. The vector contains a p35S promoter that drives the over- expression of any gene cloned immediately downstream to it. Successful recombinants were then used to stably transform wild type (Col-0) plants, via *Agrobacterium tumefaciens* mediated transformation. *35S::HSPRO1* and *35S::HSPRO2* plants were identified via selection by Basta™. Successive generations of resistant progeny were allowed to self-fertilise and screened for Basta™ resistance until individual T_3 lines displaying 100% resistance to the herbicide were identified, indicating homozygosity.

3.2.1.8 Gene expression of HSPRO1 and HSPRO2 in the over-expressor lines

Expression of *HSPRO1* and *HSPRO2* was assessed in several independent transgenic lines in order to determine if the lines were indeed over-expressing these genes at higher

levels. *HSPRO1* levels were undetectable in the wild type plants in this experiment (Figure 3.9) but a range of expression levels was detected in the independently transformed *35S::H1* lines. *35S::H1-7.1.5* (*35S::H1-7*) and *35S::H1-4.3.1* (*35S::H1-4*) were chosen for downstream research as these lines had the highest expression of *HSPRO1*. Conversely, all of the *35S::HSPRO2* lines showed expression profiles either similar to wild type plants or significantly less than the normal levels of expression (suggesting that silencing of the native gene may have occurred) and consequentially no further work was conducted with the *35S::HSPRO2* lines.

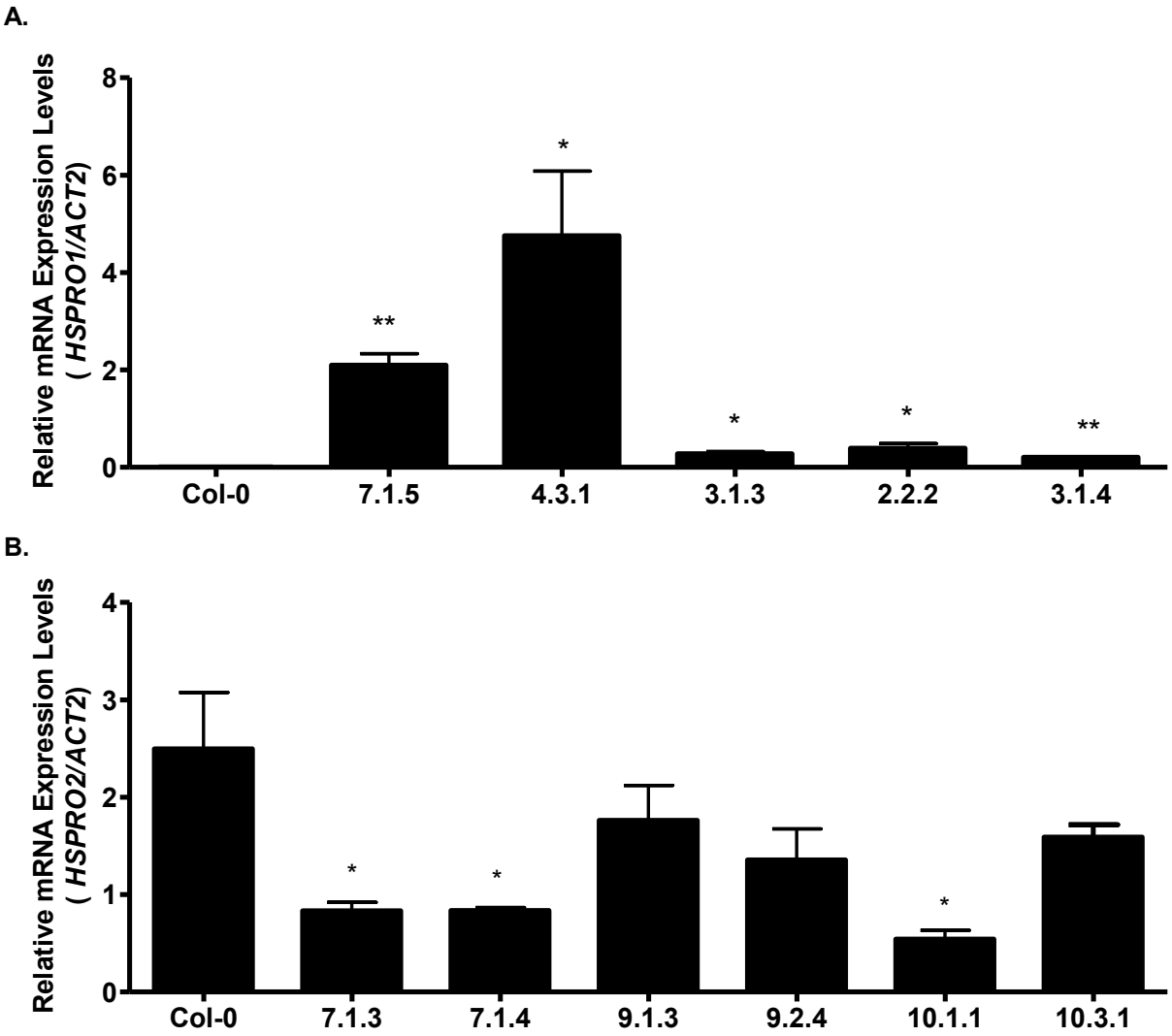


Figure 3.9: Gene expression in plants over-expressing *HSPRO1* (A) and *HSPRO2* (B).

Gene expression of *HSPRO1* and *HSPRO2* was examined using RT-qPCR and normalised relative to ACT2. Error bars represent the S.E.M (n = 3). Statistical significance was tested using the Student's t-test analysis whereby * denotes a significant difference from expression levels in Col-0 plants at $p < 0.05$ (*) and $p < 0.01$ (**). There was only one experiment conducted for over-expression analysis.

3.2.2 Dissecting potential roles for HSPRO1 and HSPRO2

3.2.2.1 The HSPRO genes are co-expressed with genes involved in defence responses and abiotic stress responses

Identification of genes co-expressed with HSPRO1 and HSPRO2

A common approach for identifying putative biological roles for genes with unknown functions is to examine correlated expression patterns between the gene of interest and the rest of the genome. Genes in related biological processes are often cooperatively expressed and functional information regarding an unknown gene can be extrapolated from the behaviour of a group of correlated genes (Kinoshita & Obayashi, 2009; Wei *et al.*, 2006). Genes co-expressed with either *HSPRO1* or *HSPRO2*, across multiple experiments were identified in order to determine potential biological roles of the two genes in *Arabidopsis.* Expression correlation analysis was performed using the CressExpress tool (http://cressexpress.org/) (Srinivasasainagendra *et al.*, 2008) and a list of 90 genes for *HSPRO1* and 254 genes for *HSPRO2* were returned as having a positive expression correlation with the genes in question (Appendix Table 1 and Appendix Table 2). Only genes with R^2 values greater than 0.36 - equivalent to a Pearson's correlation coefficient (ρ) greater than 0.6 - were considered to be co-expressed and interestingly, *HSPRO1* and *HSPRO2* shared a ρ value of 0.63 (Appendix Table 1 and Appendix Table 2) suggesting both genes are expressed under similar conditions and therefore potentially involved in the same biological processes. Genes co-expressed together form an expression correlated gene group (ECGG) and from here on, *HSPRO1* ECGG and *HSPRO2* ECGG refer to the lists of genes co-expressed with *HSPRO1* and *HSPRO2* respectively.

Functional enrichment of GO terms in the HSPRO ECGGs

To investigate if there was any functional enrichment of GO terms in the two ECGGs, GO analysis was conducted with the FatiGO tool (Al-Shahrour *et al.*, 2007). FatiGO returned terms associated with biological processes (level 3 to 9) with an adjusted p-value less than 0.05. Several of the most significant GO terms were enriched for in both ECGGs (Figure 3.10 and Figure 3.11). These included the following terms: ~~r~~esponse to chitin", ~~r~~esponse to carbohydrate stimulus" ~~d~~efence response", ~~i~~mmune response", ~~i~~nnate immune response", ~~r~~esponse to bacterium" and several other disease related terms. It appears both genes are co-regulated with disease response genes suggesting potential roles in plant immunity. In addition to enrichment of a multitude of biotic stress biological terms, the *HSPRO2* ECGG also has an enrichment of abiotic stress response terms such as

–~~a~~sponse to water", –~~r~~esponse to heat", –~~r~~esponse to water deprivation", –~~r~~esponse to salt stress", –~~r~~esponse to osmotic stress" implicating *HSPRO2* in abiotic stress responses. Regarding abiotic stress *HSPRO1* may respond to ozone stress and wounding.

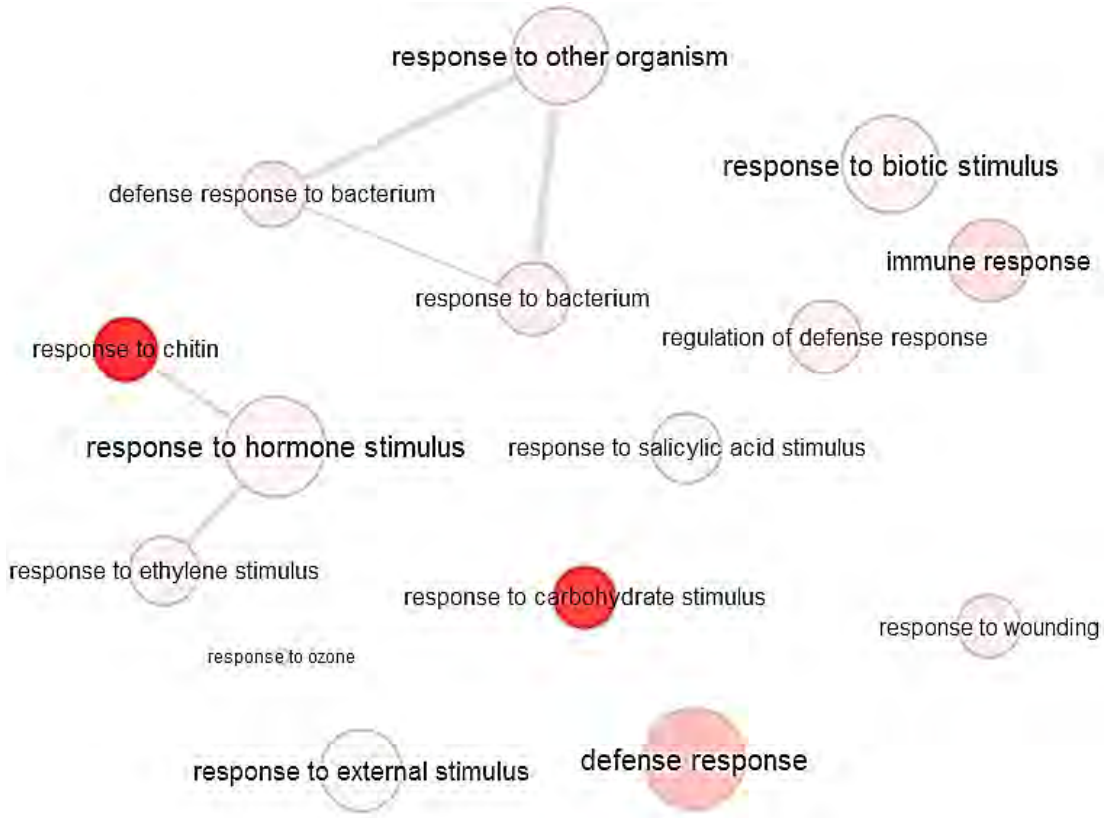


Figure 3.10: Functional categories of genes co-expressed with *HSPRO1*

GO terms returned from the FatiGO tool were uploaded into the REVIGO GO term visualisation tool (http://revigo.irb.hr/). Bubble colour indicates the p-value associated with the GO term, with the darkest colour being the most significant. Bubble size represents the frequency of the GO term in the *Arabidopsis* GOA database (bubbles of more general terms are bigger). Similar GO terms are linked by the four edges of the image and the line width of connecting lines represents the percentage of similarity.

Figure 3.11: Functional categories of genes co-expressed with *HSPRO2*

GO terms returned from the FatiGO tool were uploaded into the REVIGO GO term visualisation tool (http://revigo.irb.hr/). Bubble colour indicates the p-value associated with the GO term, with the darkest colour being the most significant. Bubble size represents the frequency of the GO term in the *Arabidopsis* GOA database (bubbles of more general terms are bigger). Similar GO terms are linked by the four edges of the image and the line width of connecting lines represents the percentage of similarity.

Promoter analysis of the HSPRO ECGGs

If a group of genes are being co-expressed together it is possible that they may be co-regulated via the same transcription factor(s)-transcription factor binding site (TFBS) interactions. To determine potential regulatory motifs conserved in the *HSPRO* genes and co-expressed genes, the *HSPRO1* and *HSPRO2* ECGGs were analysed for enrichment of known TFBS via the promoter analysis package ATHENA (http://www.bioinformatics2.wsu.edu/Athena).

There is significant functional enrichment of the W-box promoter motif in the *HSPRO1* ECGG ($p < 10^{-7}$) (Table 3.1) with 84.6% of the ECGG containing this motif. Not only is this motif conserved in 77/91 different promoters, it is present a total of 191 times. Having multiple TFBS within a single promoter leads to cooperative TF binding and more potent transcriptional activation (Maleck *et al.*, 2000). 68% of the HSPRO1 ECGG promoters have two or more repeats of the W-box motif and of this 68%, 87% have multiple repeats in close proximity to each other. W-box binding TFs are the most prominent TFs in defence response research and so perhaps the HSPRO1 ECGG is involved in defence responses. Although the Z-box motif (light responsive gene regulation) is also significantly enriched it is unlikely to be regulating the co-expression of the ECGG since it is only conserved in 9.9% of the ECGG and occurs just 10 times.

Table 3.1: ATHENA analysis of *HSPRO1* ECGG

Motif	p value	# P	# S	consensus sequence	percentage of list with motif - %	associated process
Enriched TF sites						
TATA-box motif	**$< 10^{-3}$**	**82**	**255**	**TATAAA**	**90.1**	**Core promoter element**
W-box motif	**$< 10^{-7}$**	**77**	**191**	**TTGACY**	**84.6**	**Defence response**
Z-box promoter motif	**$< 10^{-3}$**	9	10	ATACGTGT	10.8	Light responsive gene regulation

P is the number of promoters with the particular TFBS and # S that is the number of times that the TFBS occurs in the *HSPRO1* ECGG Y = C/T. Motifs highlighted in bold are also present in the *HSPRO1* promoter.

Looking at the *HSPRO2* ECGG there is significant enrichment of several motifs, besides the TATA-box. The second most enriched motif was the defence response W-box with 74.3% of the ECGG containing this motif. This motif occurs 416 times in 188 genes and may be a potential co-regulatory element for the *HSPRO2* ECGG (Table 3.2). Other motifs worth mentioning are the I-box and G-box motifs, which have well characterised roles in mediating responses to light (Spensley *et al.*, 2009) and the ABRE-like binding site motif which is a subset of G-box motifs. I-box and G-box motifs have also been described as top-weighted classifiers of glucose repression by (Li *et al.*, 2006) and are associated with starch metabolism genes. The salt and drought responsive DRE core motif is also enriched in 28% of the ECGG genes but within the 73 genes shown to have it, there are only 86 copies of the motif suggesting a less crucial role in the regulation of the ECGG.

Table 3.2: ATHENA analysis of *HSPRO2* ECGG

Motif	p value	# P	# S	consensus sequence	percentage of list with motif - %	associated process
Enriched TF sites						
TATA-box motif	**$< 10^{-6}$**	**223**	**672**	**TATAAA**	**88.1**	**Core promoter element**
W-box motif	**$< 10^{-7}$**	**188**	**416**	**TTGACY**	**74.3**	**Defence response**
I-box promoter motif	**$< 10^{-6}$**	**120**	**186**	**GATAAG**	**47.4**	**Light responsive gene regulation**
ABRE-like binding site motif	$< 10^{-6}$	78	128	BACGTGKM	30.8	ABA-mediated stress signalling
DRE core motif	**$< 10^{-4}$**	**73**	**86**	**RCCGAC**	**28.9**	**Salt and drought responsive element**
G-box motif	**$< 10^{-4}$**	59	158	CACGTG	32.3	Light responsive gene regulation
ACGTABREMOTIFA2OSEM	**$< 10^{-4}$**	55	81	ACGTGKC	21.7	ABA-mediated stress signalling
GADOWNAT	**$< 10^{-4}$**	39	52	ACGTGTC	15.4	GA gene regulation
Z-box promoter motif	**$< 10^{-3}$**	16	18	ATACGTGT	6.3	Light responsive gene regulation

P is the number of promoters with the particular TFBS and # S that is the number of times that the TFBS occurs in the *HSPRO2* ECGG. R = A/G; Y = C/T; K = G/T; M = A/C; B = C/G/T. Motifs highlighted in bold are also present in the *HSPRO2* promoter.

3.2.2.2 HSPRO1 and HSPRO2 are involved in PAMP triggered immunity

Disease phenotype

Different strains of *P. syringae* can be either virulent or avirulent on *Arabidopsis* depending on the plant ecotype. Strain DC3000 is known to be virulent on Col-0 plants and DC3000 *AvrB* is avirulent (Katagiri *et al.*, 2002). PTI is induced upon the detection of PAMPs highly conserved across both virulent and avirulent strains. Virulent bacteria can successfully repress PTI whilst attempts by avirulent strains are recognised by the plant via the detection of effectors leading to the induction of ETI which prevents disease progression. The *hspro2* mutant had already been shown to be more susceptible to virulent *P. syringae* than Col-0 plants (Murray *et al.*, 2007) and given the sequence homology between *HSPRO1* and *HSPRO2* and enrichment in both *HSPRO* ECGGs for the W-box, the disease responses of *hspro1* and *hspro1-2/hspro2* were also evaluated. The mutants and Col-0 plants were infected with both virulent and avirulent *P. syringae* to investigate the potential involvement of the two genes in PTI and ETI. Bacterial suspensions of virulent DC3000 and avirulent DC3000 *AvrB* were pressure-inoculated into the leaves of 4-week-old plants and the disease progression monitored over the following 2 days. The disease progressions followed similar profiles to those expected in such experiments as described by (Glazebrook, 2005). Four hours after inoculation similar bacterial titres were seen in all plant lines regardless of the strain used and within 48 h there was an approximately 100-fold difference between bacterial titres in the virulent versus avirulent infected wild type plants (Figure 3.12). As expected the *hspro2* mutant showed increased susceptibility to *P. syringae* DC3000. However, both *hspro1-1* and *hspro1-2* were significantly less susceptible to DC3000 compared to Col-0 implying that *HSPRO1* may be involved in the negative regulation of PTI, and that HSPRO1 and HSRPO2 may act antagonistically in the regulation of PTI. This hypothesis is supported by the double mutant which displayed bacterial titres comparable to wild type plants. In contrast, all lines displayed similar titres of Pst DC3000 *AvrB* 48 h post infection (hpi), suggesting that neither HSPRO1 nor HSRPO2 is involved in ETI. Since the two *hspro1* alleles behaved identically in response to *P. syringae* infection all further *hspro1* analysis was conducted solely on the *hspro1-2* allele.

Differential gene expression in response to P. syringae infection.

The disease phenotypes of *hspro* mutants pointed to potential roles of *HSPRO* genes in PTI and RT-qPCR analysis revealed both genes are significantly up-regulated in response

to virulent, *P. syringae* 24 hpi in Col-0 plants (Figure 3.13A and B). This supports the hypothesis that *HSPRO* genes are involved in the *Arabidopsis* disease response to *P. syringae* infection. *HSPRO2* has been shown to be SA-responsive and PR1, a typical SA-mediated defence signalling protein is seen to be negatively regulated by HSPRO2 at the protein level (Murray *et al.*, 2007) and so it was investigated whether HSPRO1 also imposes regulatory control on *PR1* expression. Four-week-old plants were infected with *P. syringae* DC3000 and tissue harvested at the time of infection and 24 hpi. *PR1* expression was induced in all the plant lines following infection but surprisingly in all three mutants, the fold-change in *PR1* expression was lower than that observed in Col-0 plants. The antagonism in *HSPRO* gene function suggested in the disease resistance phenotypes did not translate to opposing regulatory functions in *PR1* expression (Figure 3.13). *PR1* expression in *hspro2* had already been described as comparable to wild type levels (Murray *et al.*, 2007) but this research had been conducted with the less sensitive northern blot method. The more sensitive RT-qPCR analysis showed that *PR1* expression is perhaps dependent on functional HSPRO proteins.

A.

B.

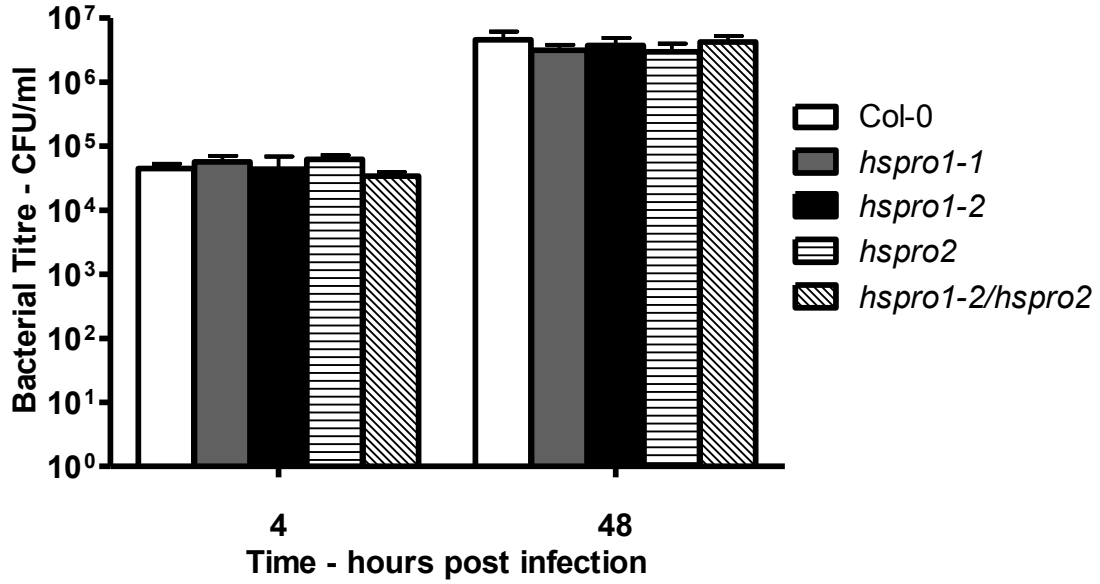


Figure 3.12: Infection of the mutants and wild type plants with *P. syringae*.

Four-week-old plants were pressure-infiltrated with a 1 x 10^6 cfu/mL suspension of virulent *P. syringae* DC3000 (A) or avirulent AvrB strain (B) and disease progression quantified 4 and 48 h after infection. Single leaf discs were harvested from 3 separate leaves per plant, pooled and homogenised in 1 mL of 10 mM MgCl2 and bacterial titre was measured as the number of CFUs/mL of the homogenised leaf tissue. Each bar represents the mean of at least 3 biological repeats and the error bars represent the standard error of the mean. Statistical significance was evaluated via the Student‘s t-test with * representing a p-value less than 0.05. This data is representative of at least three experiments.

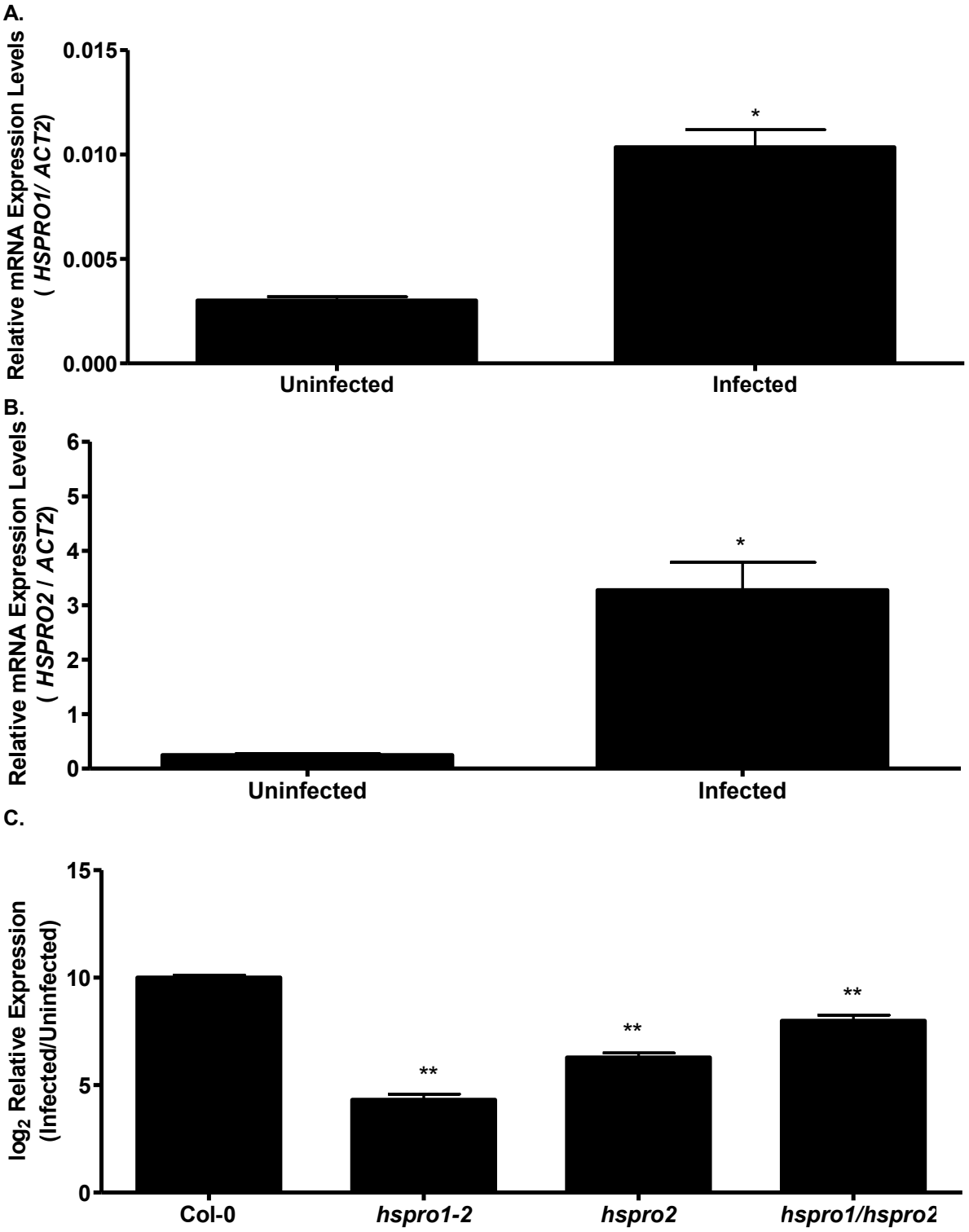


Figure 3.13: Gene expression following infection with *P. syringae*.

Four-week-old plants were pressure-inoculated with virulent *P. syringae* DC3000. Gene expression was examined using RT-qPCR and normalised relative to *ACT2* expression. A and B represent *HSPRO1* and *HSPRO2* expression in Col-0, respectively 24 h after infection. C shows relative *PR1* expression 24 h after infection normalised to uninfected control plants. Error bars represent the S.E.M (n = 3). Statistical significance was tested using the Student's t-test. * represents a p-value < 0.05 and ** represent a p-value < 0.01. This data is representative of only one experiment.

3.2.2.3 hspro knockout mutations do not affect the Arabidopsis:Botrytis cinerea interaction

Given the disease phenotypes in *hspro* mutants in response to biotrophic *P. syringae* and the enrichment of fungal pathogenesis associated terms in the *HSPRO* ECGGs (Figure 3.10 and Figure 3.11), such as ‗response to chitin', ‗ethylene', ‗fungus associated' and ‗jasmonic acid' it was decided to investigate the potential role of *HSPRO1* and *HSPRO2* in disease resistance against the necrotrophic fungal pathogen, *Botrytis cinerea* (Pepper). 4-week-old rosette leaves were detached from whole plants and inoculated with 10 µL droplets of mature fungal spore suspension. Disease development was assessed as increase in lesion size surrounding the infection site 4 dpi. There were no significant differences in susceptibility between the mutants and Col-0 (Figure 3.14). This led to the conclusion that although the microarray data suggests involvement of these two genes in fungal pathogen disease responses, loss of HSPRO1 and/or HSPRO2 has no impact on the outcome of this plant-pathogen interaction.

Figure 3.14: Infection of the mutants and wild type plants with *Botrytis cinerea*.

Ten microlitre drops of *B. cinerea* suspensions, at 5 x 10^4 spores/mL were used to infect the leaves of 4-week-old plants. Disease progression was quantified by determining lesion area 4 days after infection. Each bar represents the mean of 45 individual leaves and the error bars represent the S.E.M. This data is representative of at least three experiments.

3.2.2.4 HSPRO1 and HSPRO2 are differentially expressed during plant stress responses

HSPRO1 and HSPRO2 are known interacting partners of the SnRK1 complex, a central regulator of metabolic reprogramming in response to various environmental and internal cues (Baena-González *et al.*, 2007; Gissot *et al.*, 2006). It follows then that HSPRO1 and HSPRO2 may be involved in other biological processes through this SnRK1 interaction. To identify such potential processes, *HSPRO* gene expression was evaluated under various conditions through analysis of publicly available microarray data curated within the Bio-Analytic Resource for Plant Biology (BAR) database. *At3g55840* and *At2g40000* gene IDs were queried into the Expression Browser tool for *HSPRO1* and *HSPRO2* respectively and the returned data analysed for differential expression. A $\log_2$ value of 1 equivalent to a two-fold change in expression when compared to untreated plants was set as the threshold for differential expression. Statistical analysis was not utilised to identify significant gene differential expression since only two biological repeats were available per time point for each treatment.

Abiotic responses

Bio-Array Resource data revealed that in addition to induction in response to virulent DC3000, both *HSPRO1* and *HSPRO2* are induced in response to a diverse range of abiotic stresses in both root and shoot tissue (Figure 3.15). In root tissue, cold, salt and osmotic (mannitol) stress were the most potent inducers/repressors of *HSPRO1* and *HSPRO2* differential gene expression, with *HSPRO1* also responding to drought stress (Figure 3.15A). Heat, oxidation and UV-B treatment also caused differential gene expression of both genes but to a lesser extent. In shoot tissue *HSPRO1* expression most strongly responsive to cold, salt and osmotic stress while *HSPRO2* expression is responsive to cold, heat, drought and osmotic stress (Figure 3.15B). Differential expression of *HSPRO* genes in response to several abiotic stresses prompted investigation into the phenotypic responses of *hspro* mutants to abiotic stress.

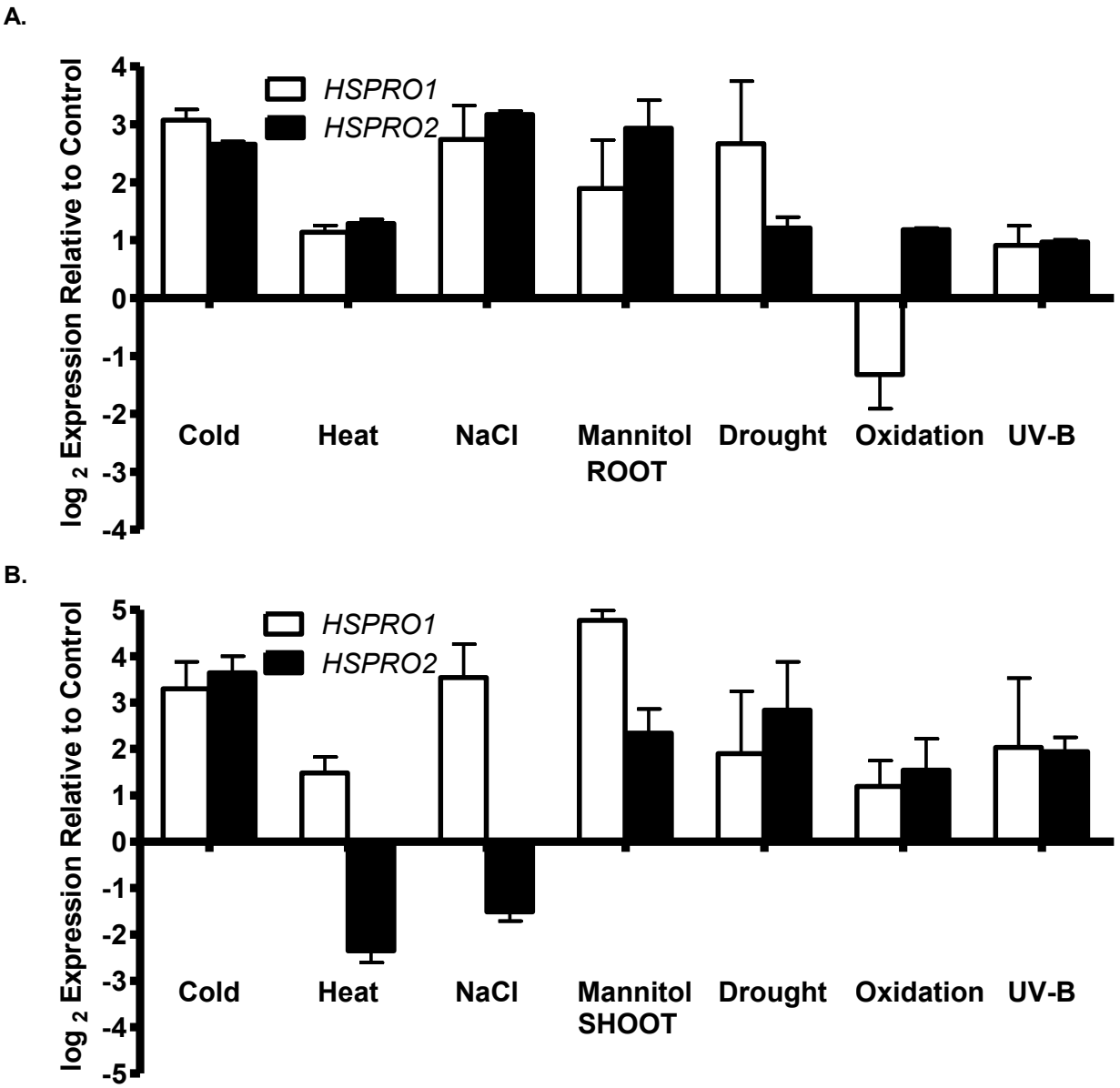


Figure 3.15: *HSPRO1* and *HSPRO2* expression in response to abiotic stress treatments

Gene expression in Col-0 roots (A) and shoots (B) expressed relative to mock treated plants. Plants were treated with cold, heat, salt, mannitol, drought, oxidative and UV-B stress with n = 2 and the error bars represent the S.E.M. The highest measurement of induction or repression within a time course is displayed for each treatment.

Hormone treatment

Both *HSPRO1* and *HSPRO2* are responsive to ACC and ABA treatment in whole plants while only *HSPRO1* is MeJA-responsive and neither was differentially expressed in response to ABA treatment of seeds (Figure 3.16). ACC (precursor to ET), MeJA and ABA are all hormones involved in disease resistance and defence gene expression in *Arabidopsis (Anderson et al., 2004)*. Additionally, ABA is also an integral molecule in abiotic stress responses including several of the stresses thought to cause differential expression of *HSPRO1* and *HSPRO2* (Figure 3.15).

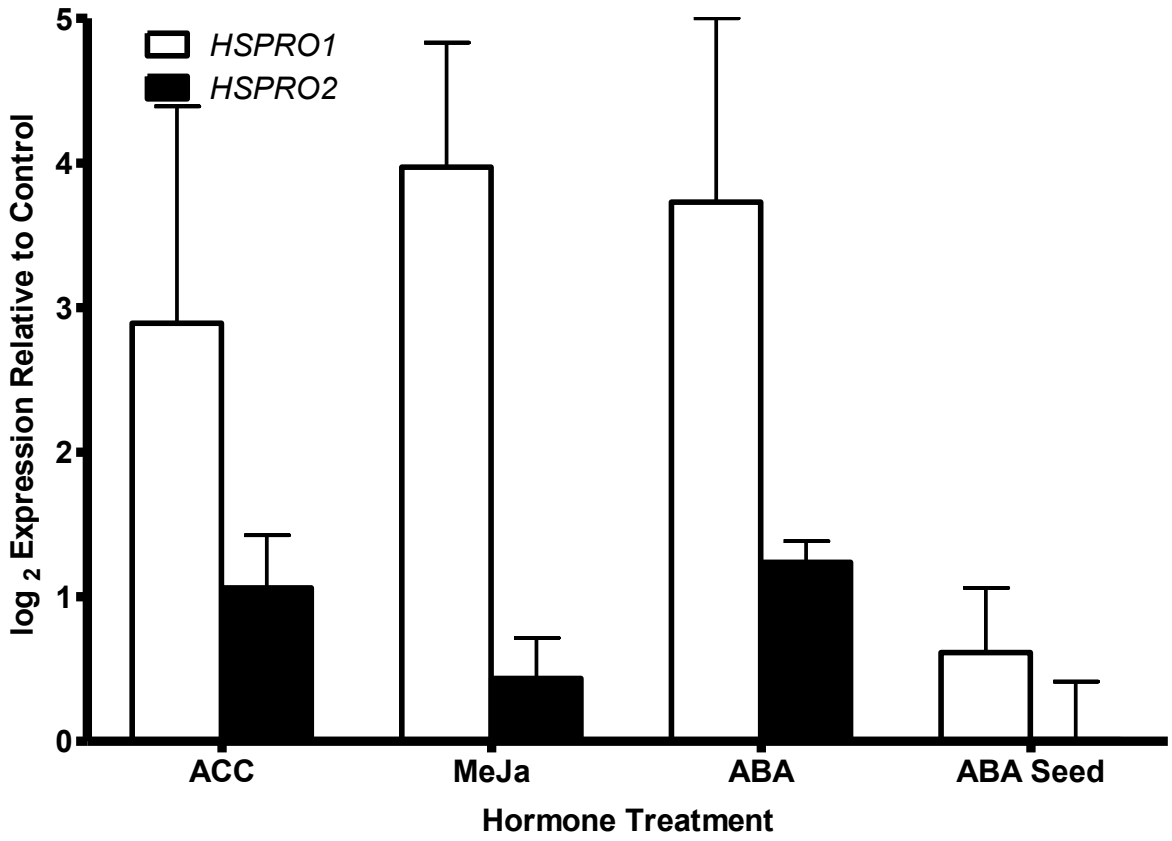


Figure 3.16: Gene responses to exogenous treatment with plant hormones

Gene expression in whole plants and seeds (in the case of ABA) was monitored after treatment with the plant hormones ACC, MeJA and ABA. Gene expression was monitored over a time course and only the strongest response time-point is illustrated in the figure per treatment. N=2 and the error bars represent the S.E.M.

3.2.2.5 Abiotic stress phenotypes

The microarray data described above hinted at the possibility of the involvement of *HSPRO1* and *HSPRO2* in abiotic stress responses including but not limited to response to salt and osmotic stress. Additionally, abiotic stress terms are enriched in the *HSPRO2* ECGG and promoter elements associated with abiotic stress responses are present in a sub-set of genes co-expressed with and including *HSPRO2* as well. For the scope of this research abiotic stress was limited to salt and osmotic stress only.

hspro2 seeds are hypersensitive to osmotic stress

Wild type and mutant seeds were germinated on various concentrations of NaCl and mannitol to investigate any involvement of *HSPRO1* and *HSPRO2* in salt and osmotic stress responses within the seed and developing seedlings. Germination was monitored at the developmental stages of radicle emergence and cotyledon expansion. Germination efficiency was uniform in the untreated control plants except for *35S::H1-7* where approximately only 65% of the seeds germinated successfully (Figure 3.17). It is evident

that the *hspro2* mutation results in a hypersensitivity to both mannitol and NaCl (Figure 3.17). Since the changes seen in response to mannitol and NaCl are very similar, it seems there is no additional ionic effect of NaCl on the mutant plants. Except for germination on 75 mM NaCl (radicle emergence), no significant difference in germination efficiency was observed between the *hspro1-2* mutants and the wild type plants, and the double knockout lines exhibited the same phenotype as the single *hspro2* knockout line. Interestingly, the *HSPRO1* over-expressor line showed lower germination efficiency than Col-0 and *hspro1-2* not only on control media but also in response to salt and mannitol treatment. It is therefore unclear whether the observed abiotic stress phenotypes are treatment specific or a result of altered germination responses caused by gene over-expression. In conclusion, it appears that *HSPRO2* is involved in perception or signalling responses to osmotic stress at the seed germination level.

Seedling responses to salt and osmotic stress

Often, abiotic stress responses are specific to particular tissue (Kiegle *et al.*, 2000; Kim *et al.*, 2007) and/or growth stage (Gao *et al.*, 2003) and it is often the case that there is no correlation between phenotypes seen in seeds and later growth stages (Quesada *et al.*, 2000; Werner & Finkelstein, 1995). To investigate the effects of osmotic and salt stress on seedlings, root length (Figure 3.18) and rosette fresh weight gain (Figure 3.19) were measured as indicators of altered stress susceptibility within root and leaf tissue. Seeds were specifically germinated on control media and transferred to the stress treatments to control for the observed altered germination rates under stress seen in Figure 3.17. Wild type plant roots were sensitive to the abiotic treatments as expected but the *hspro2* mutant displayed a root growth phenotype and grew to a lesser extent compared to Col-0, under control conditions and as such root growth in the stress-treated plants was normalised to the control plants (Figure 3.18 B). Interestingly though, *hspro2* was more tolerant of the osmotic stress imposed by mannitol and *hspro1-2* had a contrasting phenotype to that of *hspro2* and is more sensitive to osmotic stress. Once again the double knockout behaves as *hspro2* reinforcing the idea that *hspro2* is epistatic to *hspro1-2* and it would appear that the *HSPRO* genes potentially have antagonistic roles in osmotic stress responses. To determine if *HSPRO* genes are involved in salt and osmotic stress responses in shoot tissue, tissue FW gains were recorded for plants grown on salt and osmotic stress media. This analysis revealed that *hspro* mutant growth is comparable to wild type plants under

both salt and osmotic stress (Figure 3.19) suggesting the stress factors do not impact vegetative shoot growth rate in the mutants in a manner different to wild type plants.

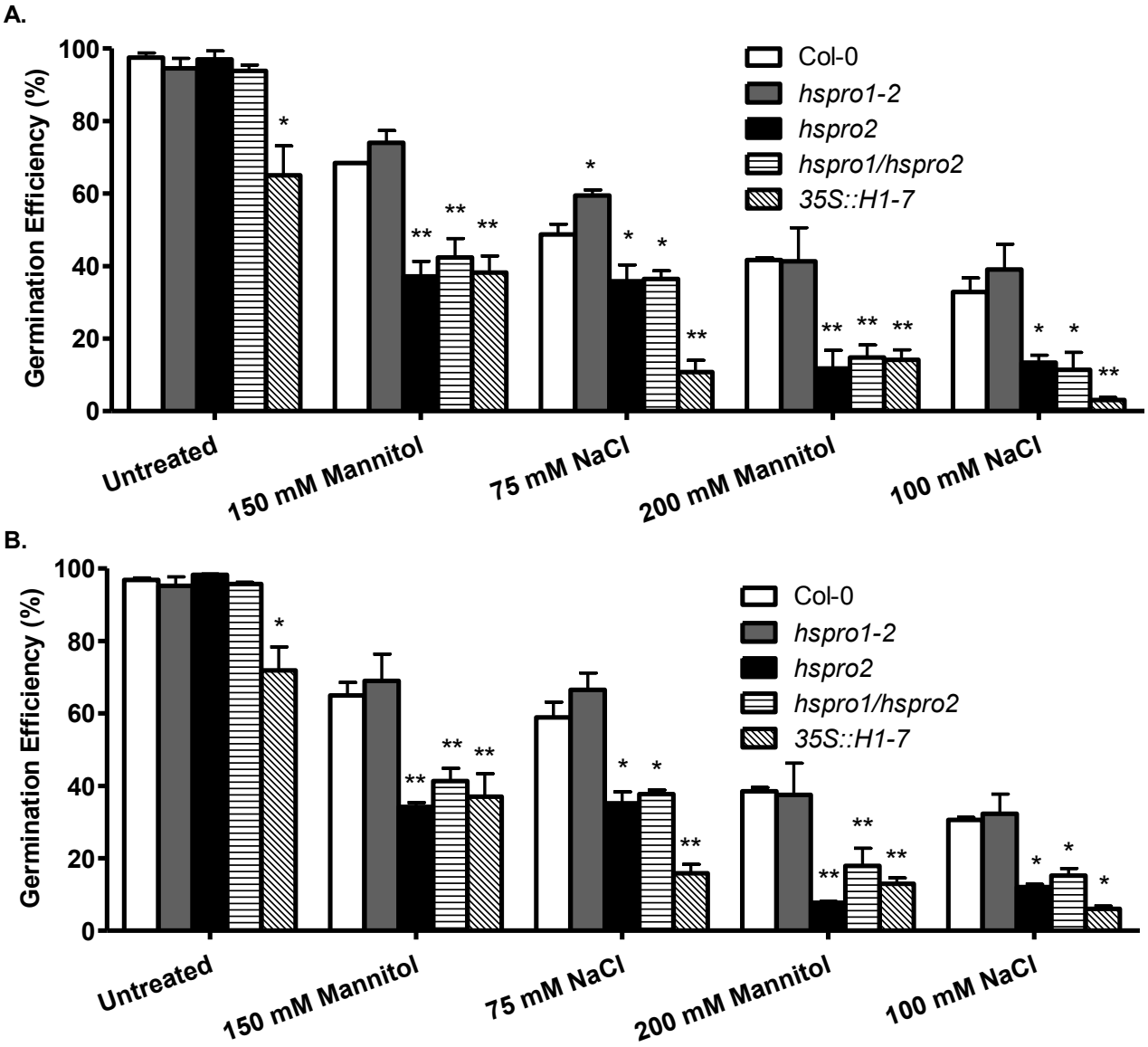
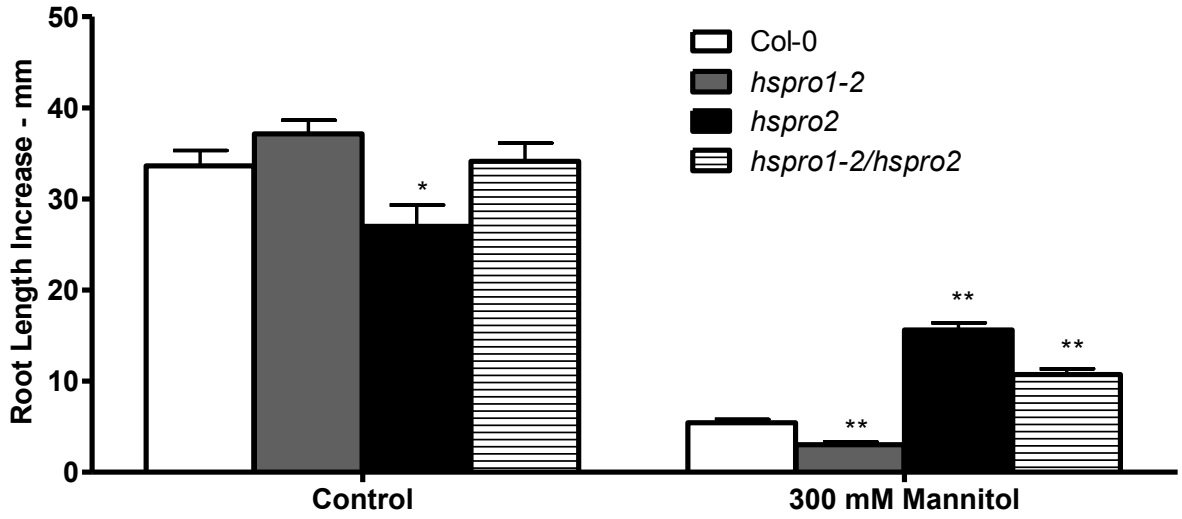


Figure 3.17: Germination response of seeds sown on NaCl and mannitol.

Seeds were sterilised and stratified in 0.1% agar, in the dark for three days before being sown onto 0.5 x MS agar containing the specific stress compound. Germination counts were performed 24 h (A - radicle emergence) and 48 h (B – cotyledon expansion) after transfer to standard growth conditions. N = 3 plates with 100 seeds per line and significance was assessed for using the Student's t-test. '*' = $p < 0.05$ and '**' = $p < 0.01$. This data is representative of at least three experiments.

A.



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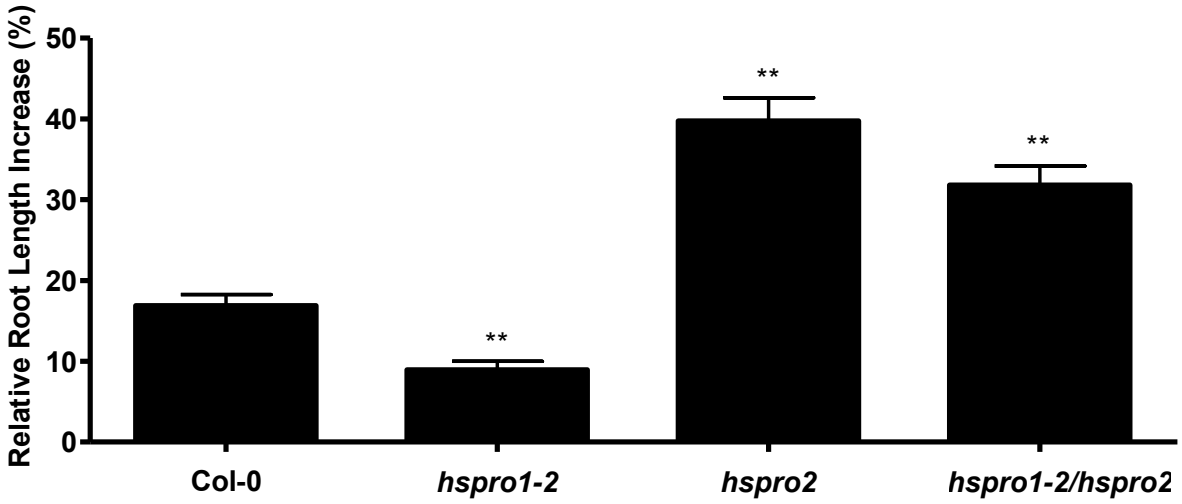


Figure 3.18: Root elongation of seedlings grown on mannitol.

Plants were germinated and grown for 5 days on 0.5 x MS agar and then transferred to fresh control media and 300 mM mannitol for 7 days. Root length increase was measured post treatment (A) and root length increases on stress media expressed relative to the growth of control plants (B). The data represents the mean and S.E.M of at least 40 plants. Statistical significance was tested using the Student's t-test. '*' represents a p-value < 0.05 and '**' represents p < 0.01. This data is representative of at least three experiments.

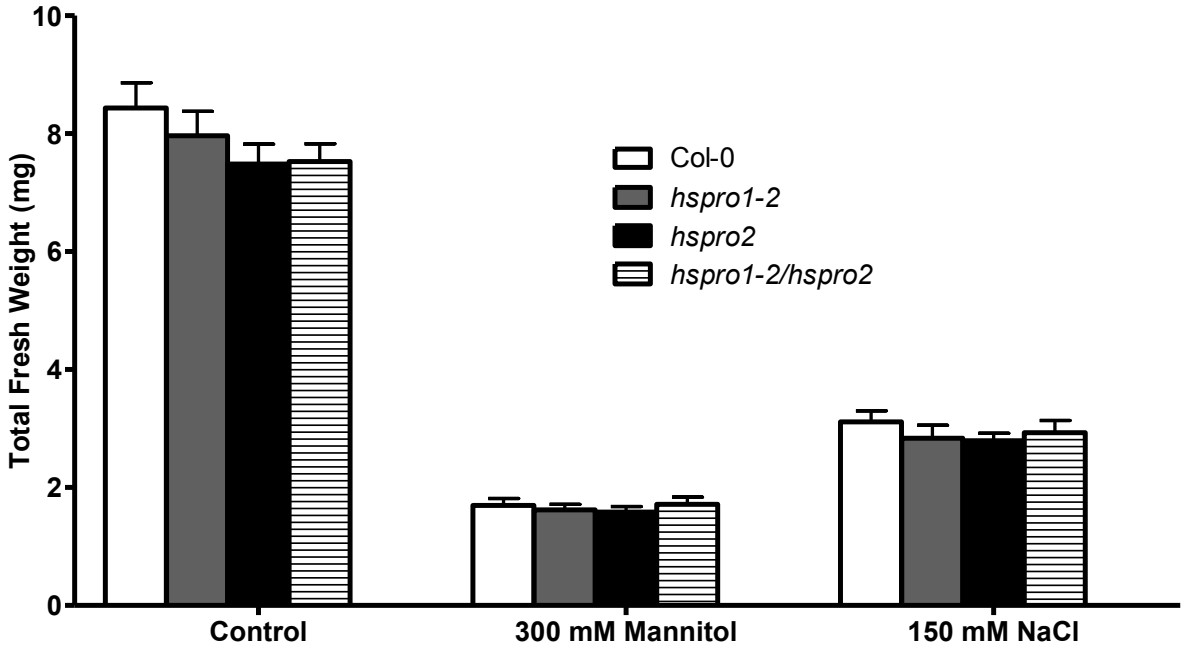


Figure 3.19: Fresh weight gain of seedlings grown on mannitol and NaCl.

Plants were germinated and grown for 5 days on 0.5 x MS agar and then transferred to stress media for 7 days. Fresh weight gain was measured after growth on stress. The data represents the mean and S.E.M of at least 30 plants. This data is representative of at two experiments.

Water loss assay

Drought stress imposes osmotic stress on plants and leads to changes in membrane permeability and alters leaf water status (Bai *et al.*, 2006; Xiong & Zhu, 2002). ABA-mediated stomatal leaf water loss is linked to drought tolerance (Li *et al.*, 2008) and so to investigate the potential involvement of *HSPRO* in drought tolerance in mature plants, leaf water loss assays were conducted on the *hspro* mutants. Leaves were detached from 4-week-old, soil grown plants and water loss measured through changes in tissue weight over several hours. The initial weights of Col-0 and mutant plants were comparable (Figure 3.20A) but over time the *hspro1-2/hspro2* mutants were significantly more resistant to stomatal water loss and perhaps this is due to a cooperative effect of loss of both *HSPRO1* and *HSPRO2* (Figure 3.20B) and potentially implicates HSPRO genes in drought tolerance.

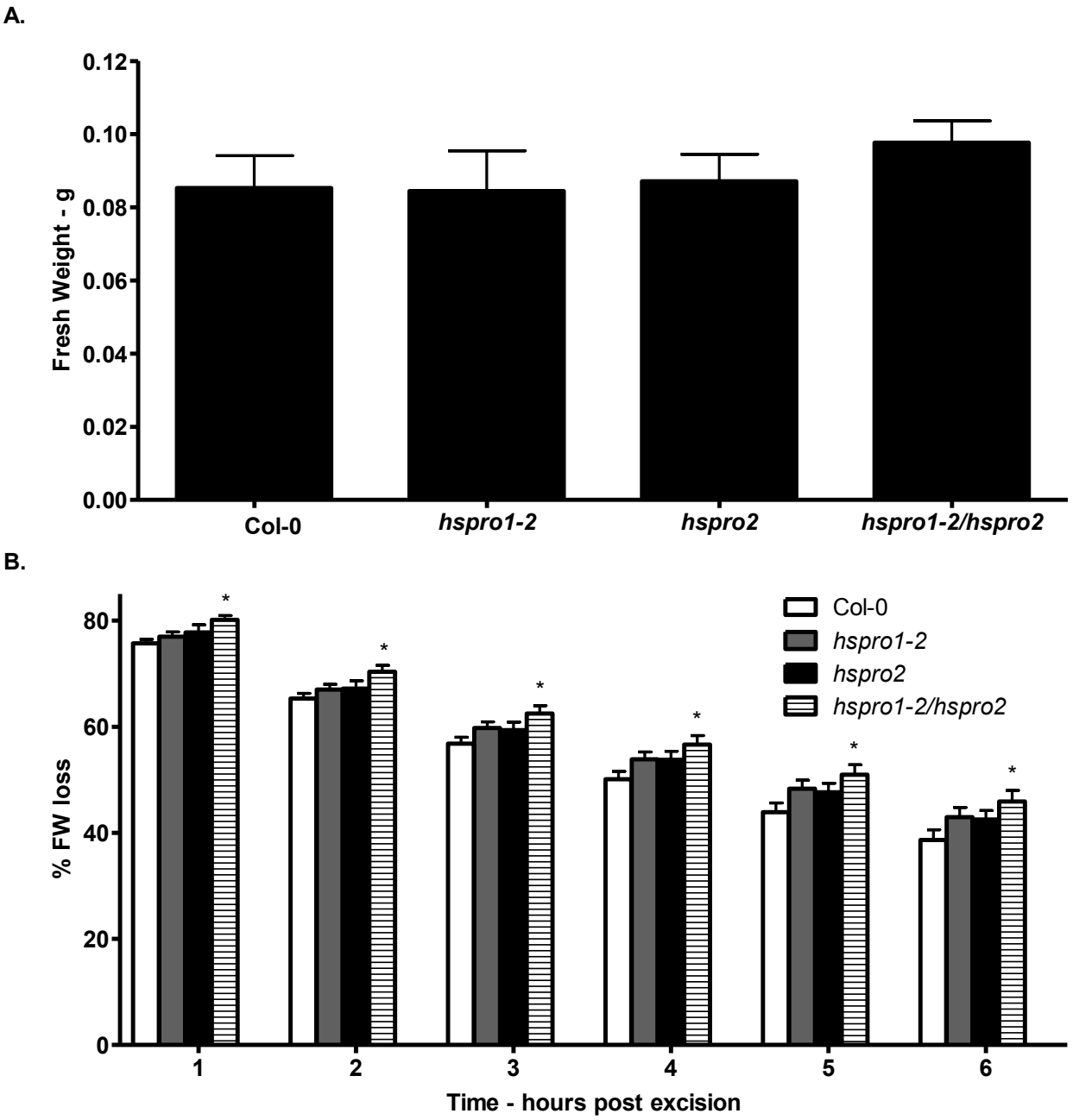


Figure 3.20: Adult leaf water loss assay to determine the ABA-mediated stomatal response to water deficit stress.

Four-week-old leaves were harvested from *Arabidopsis* plants grown on soil under standard growth conditions and were immediately weighed (A). Water loss was measured as loss in weight over a 6 h time period and expressed relative to the initial mean weight for each plant line (B). N = 15 plants per line and error bars represent the S.E.M. Significant deviation from the wild type was determined via the Student's t-test and '*' represents a p-value less than 0.05. This data is representative of only one experiment.

3.3 DISCUSSION

In this chapter both computational and experimental analyses were conducted in an attempt to identify biological processes in which *HSPRO* genes may play a role. In an effort to achieve this, homozygous *hspro1* knockout lines were successfully isolated and a *hspro1-2/hspro2* double knockout mutant (which lacks expression of both *HSPRO* genes) generated for combinatorial analysis with the *hspro2* mutant. Fluorescent microscopy-based visualisation of *Arabidopsis* mesophyll protoplasts transfected with *HSPRO-YFP* fusion constructs, confirmed the cytoplasmic localisation of HSPRO proteins as previously reported during their interaction with the SnRK1 complex (Gissot *et al.*, 2006). Expression correlation analysis generated lists of genes most strongly and consistently expressed with the *HSPRO* genes, termed *HSPRO1* and *HSPRO2* ECGGs. GO analysis revealed that both *HSPRO* genes are co-expressed with defence related genes and *HSPRO2* specifically, is associated with abiotic stress genes. Additionally, promoter analysis revealed enrichment of the defence related cis element, the W-box, in the promoters of the genes in both ECGGs. Gene expression analysis and public microarray data revealed that *HSPRO* gene expression is responsive to both abiotic and biotic stresses, and phenotypic analysis supports the involvement of *HSPRO* genes in stress responses.

3.3.1 HSPRO1 and HSPRO2 may function antagonistically in PTI

Published data already ascribed a role for HSPRO2 as a positive regulator of the plant defence response in *Arabidopsis (Murray et al., 2007). hspro2* mutant plants were shown to display an increased susceptibility to infection with virulent *P. syringae* DC3000 implicating *HSPRO2* in the positive regulation of PTI. This result was confirmed in the research described in this thesis and a previously undiscovered role of the only known *Arabidopsis* homologue of *HSPRO2*, *HSPRO1* in plant defence response was also uncovered. Transgenic *hspro1* knockout mutants were seen to be more resistant to infection with virulent DC3000 compared to the wild type and *hspro2* plants. Since *hspro* mutants (and the other *hspro* mutants) did not display an altered susceptibility to avirulent *P. syringae AvrB*, a potential role for *HSPRO1* as a negative regulator of PAMP triggered immunity is proposed. This data suggested that the HSPRO proteins behave in an antagonistic manner and are not redundant in function during defence response, despite the fact that they are both induced by *Pst* infection. The work by Murray also suggested that HSPRO2 function negatively regulates expression of a hallmark protein of defence response, PR1 at a protein level and our findings suggest that *PR1* gene induction may be

positively regulated by both *HSPRO* genes during infection responses to virulent *P. syringae*. The regulation of *PR1* by HSPRO1 and HSPRO2 does not display the opposing pattern observed in the disease resistance phenotypes so one could argue it is not the misregulation of *PR1* that results in the proposed antagonism. It would be interesting and more informative to investigate global gene expression responses in the *hspro* mutants following infection with *P. syringae* and future research could tackle this.

3.3.1.2 WRKY transcription factors are possible regulators of HSPRO expression correlated gene groups

Interpretation of correlation values is dependent on the context of the biological system under review but generally speaking ρ values ranging from 0.5 to 0.8 are considered to show an intermediate correlation between two genes. ρ values greater than 0.9 have been associated with genes in a single pathway, those greater than 0.84 associated with genes that share *cis* regulatory elements and those greater than 0.7 associated with enrichment of functional annotations or keywords (Allocco *et al.*, 2004; Manfield *et al.*, 2006). The minimum for gene expression correlation was set at a Pearson‘s correlation coefficient of 0.6 and reached a maximum of 0.75 and 0.8 for *HSPRO1* and *HSPRO2* respectively. The intermediate correlations associated with the *HSPRO* ECGGs could be attributed to regulation of the ECGGs at multiple *cis* elements by multiple and possibly antagonistic transcription factors and pathways (Meier & Gehring, 2008). Correlation analysis revealed groups of genes co-expressed with *HSPRO* genes and suggested potential roles in defence response. *HSPRO* genes are both co-expressed with *WRKY* transcription factors and the W-box (WRKY factor binding site) is the most enriched promoter element in the promoters of genes in the ECGGs. There are 6 WRKY factors associated with the *HSPRO* genes with *WRKY15*, *WRKY33*, *WRKY40* and *WRKY48* being common to both ECGGS. This is of significance because WRKY proteins are the most characterised and important TFs in plant defence in response to microorganisms and can negatively and positively regulate defence gene responses (Xing *et al.*, 2008; Zheng *et al.*, 2006). Furthermore, both *HSPRO1* and *HSPRO2* have W-box motifs in their promoter sequences and are co-expressed together with a ρ value of 0.63 suggesting regulation in part through a common *cis* regulatory motif and function in similar processes. ECGG analysis also revealed functional enrichment of defence response terms and stress signalling hormone terms associated with both *HSPRO1* and *HSPRO2*. In light of this evidence we propose that

WRKY factors may be driving the co-regulation of the *HSPRO* associated genes in the ECGGs.

3.3.2 Roles for HSPRO1 and HSPRO2 in abiotic stress responses

3.3.2.1 Functions of HSPRO *proteins in osmotic stress*

As seen in public microarray data *HSPRO* genes are differentially expressed in response to several abiotic stressed including drought, salt, osmotic, heat and cold stress, in both root and shoot tissue. All these stresses create a water deficit in the plants and phenotypic analysis revealed that *hspro2* and *hspro1-2/hspro2* seeds are hypersensitive to both osmotic and salt stress. Furthermore, *hspro1-2* and *hspro2* mutants have opposing altered sensitivities in root responses to osmotic stress. Unlike in the disease response it seems *HSPRO2* is a negative regulator of osmotic stress tolerance while *HSPRO1* positively regulates stress tolerance in root tissue reinforcing the hypothesis that the two proteins are antagonistic in function. However, the *hspro1-2/hspro2* mutant displays a phenotype similar to *hspro2* suggesting that during osmotic stress, the *hspro2* mutation is epistatic to *hspro1-2* unlike in the disease response to *P. syringae*. It was interesting to observe different *hspro* phenotypes that were developmental and tissue specific. *HSPRO1* does not appear to be involved in osmotic responses in seed germination and shoot tissue growth but appears to be a positive regulator of osmotic responses in the root. *hspro2* seeds are sensitive to osmotic stress but the root growth rate in *hspro2* is better than Col-0 during osmotic stress treatment. Mutants with tissue-specific, opposing phenotypic responses to abiotic stress are less prolific in literature and one such mutant is *hkt1*. The *hkt1* mutant is more sensitive to NaCl in the shoot tissue but NaCl tolerant in roots when compared to wild type plants (Mäser *et al.*, 2002)

3.3.2.2 HSPRO2 expression may be regulated by abiotic stress responsive transcription factors

In addition to biotic stress, *HSPRO2* is also co-expressed with genes involved in multiple abiotic stress responses. Water deprivation, heat, salt and osmotic stress are enriched terms in the *HSPRO2* ECGG and generally speaking these stresses all have an effect on water availability in the plant. Additionally, a subset of *HSPRO2* ECGG genes have enrichment of regulatory motifs in their promoters that are associated with abiotic stress. The ABRE-like binding site motif is enriched in 30% of the *HSPRO2* ECGG and ABRE motifs are known to be a target site for ABA-dependent regulation of drought, cold and

salinity stress responses (Huang *et al.*, 2008). The promoters of drought, salt and cold response genes that contain ABRE motifs typically contain the DRE core motif as well and it is thought that the two motifs function in a cooperative manner (Knight *et al.*, 2004; Narusaka *et al.*, 2003). This trend is also observed in the *HSPRO2* ECGG with 29% of the gene list seen to be DRE dependent. It is possible that this subset of abiotic stress genes is regulated through ABRE-like/DRE cis elements during altered water availability responses. *HSPRO2* is co-expressed with numerous TFs including several involved in abiotic stress responses. These include SALT-INDUCIBLE ZINC FINGER1 (SZF1)(Sun *et al.*, 2007), MYB73 (salt stress responsive) (Kim *et al.*, 2013; Rasmussen *et al.*, 2013), DREB2A (binds to the DRE core motif and functions in water deficit regulation during cold, drought, heat and salt stress) (Sakuma *et al.*, 2006a; Sakuma *et al.*, 2006b), bZIP28 (heat responsive) (Gao *et al.*, 2008a), RAV1 (drought and salt tolerance response regulation) (Sohn *et al.*, 2006) and it is possible that *HSPRO2* expression is regulated by some of these DNA-binding proteins.

3.3.3 Summary

Characterisation of the *hspro* mutants and gene expression analysis confirmed the role of *HSPRO2* in plant defence while also revealing the novel negative (and antagonistic to *HSPRO2*) regulatory role of *HSPRO1* in PTI. Correlation analysis also suggested that WRKY TFs may be integral in the regulation of *HSPRO* gene expression but this needs to be experimentally determined. Furthermore, *hspro* mutant phenotypic analysis also revealed additional roles for *HSPRO* genes in osmotic stress responses to mannitol, with *hspro2* mutants displaying increased stress tolerance while *hspro1-2* mutants are more sensitive to osmotic stress than wild type plants. Interestingly, in abiotic stress responses the *hspro2* mutation appears to be epistatic to the *hspro1-2* mutation as the double knockout plants behave as the *hspro2* single knockout. An overview of the results suggests that HSPRO proteins play important and antagonistic roles in response to both biotic and abiotic stresses.

CHAPTER 4

Identification of HSPRO downstream targets

CHAPTER 4: IDENTIFICATION OF HSPRO DOWNSTREAM TARGETS

4.1 INTRODUCTION

Global transcriptome analysis is another invaluable tool utilised in characterising genes of unknown function or identifying genes involved in a biological process of interest (Al-Shahrour *et al.*, 2007; Allemeersch *et al.*, 2005; Covington *et al.*, 2008; Wren, 2009). Microarray analysis is often performed on plants of the same genotype undergoing the test conditions and the gene expression profiles compared to those of control plants growing under standard growth conditions. This approach is particularly useful in identifying groups of genes involved in a particular process but is not as useful in the characterisation of genes with unknown functions or associated biological processes.

To identify potential targets of a gene of interest or biological processes it may be associated with, a different approach is necessary. The transcriptome expression profiles of gain/loss-of-function mutants grown under standard conditions can be compared to the wild type expression profiles and identify genes misregulated in the mutants. These misregulated genes can be potential targets of the gene of interest and if they have function annotations, inferences can be made about the gene of interest potentially regulating them. Once again FatiGO analysis can be utilised to highlight potential biological processes, molecular functions and cellular components associated with the gene of interest (Al-Shahrour *et al.*, 2007), while promoter content analysis can assist in identifying potential *cis* regulatory motifs (Molina & Grotewold, 2005; O'Connor *et al.*, 2005).

In this chapter the expression profiles of the *hspro* mutants growing under standard conditions were compared to that of the wild type and patterns of expression of misregulated genes in the absence of the *HSPRO* genes revealed. FatiGO analysis and promoter content analysis of the differentially expressed gene lists revealed additional potential processes the *HSPRO* genes may be involved in, besides the pre-established a/biotic stress responses detailed in chapter 3.

Combining the knowledge that HSPRO proteins interact with the SnRK1 complex and the observation that carbohydrate response and transport terms are enriched for in the FatiGO

analysis of ECGG and *hspro* microarray data, we hypothesised that *HSPRO* may be involved in sugar sensing and signalling. *HSPRO2* was identified as a KIN10 target during energy perception and signalling, and clustering analysis revealed four particular clusters that show overlaps between *hspro* transcriptomes and several energy rich or depleted conditions. Glucose germination assays revealed an altered germination response in *hspro2* plants and gene expression analysis revealed that *HSPRO2* is glucose repressed and gene responses in *hspro2* during starvation may be compromised.

Microarray analysis also revealed the potential for *HSPRO* gene function in circadian clock based processes. Using reporter gene analysis we discovered that *HSPRO2* expression appeared to be regulated by the circadian clock in *Arabidopsis*, which drives its rhythmic expression. However, although *HSPRO2* expression appears to be circadian clock dependent, *HSPRO2* expression is not required for the normal function of several clock regulated biological processes.

4.2 RESULTS

4.2.1 Microarray analysis of the hspro mutants

To identify potential downstream targets of *HSPRO1* and *HSPRO2*, the expression profiles of rosette tissue from 4-week-old *hspro1-2*, *hspro2* and *hspro1-2/hspro2* were compared to that of wild type plants using CATMA microarrays. M box plots indicated the need to normalise the data sets as the M values were not centred around a mean $\log_2$ value of zero (Figure 4.1, left panel). Print tip loess normalisation was performed to correct for spatial effects that can occur during hybridisation, whereby one dye hybridises more in particular areas of the slide. Following this quantile normalisation was performed to normalise for technical error across slides. M plots of the normalised expression data confirmed that values were centred around zero (Figure 4.1, right panel). Differential expression was determined by testing for a statistically significant deviation from a $\log_2$ ratio of zero. The Linear Model statistical test (a form of the t-test) with Benjamini and Hochberg (BH) correction was used to establish significance and returned an adjusted p-value after multiple testing correction (MTC). MTC attempts to limit the number of false positives associated with multiple t-testing as is the case when testing tens of thousands of genes in a microarray experiment.

4.2.1.1 Microarray validation

Several genes identified as significantly differentially regulated in at least one of the mutants (when compared to Col-0) were used to independently validate the microarray data via RT-qPCR. Validation was necessary for meaningful interpretation of the microarray data, especially since it was generated from technical repeats of pooled samples and not independent biological repeats. One µg aliquots of the same RNA used for the microarrays were used to make cDNA for the validation experiments and unlike in the microarray, the biological repeats were treated separately. Five genes were chosen for validation and were found to have similar patterns of expression across the mutants in both the microarray and RT-qPCR data sets (Figure 4.2). The validation of the microarray was thus considered to be successful and useful deductions could be made from the data set with a high degree of confidence.

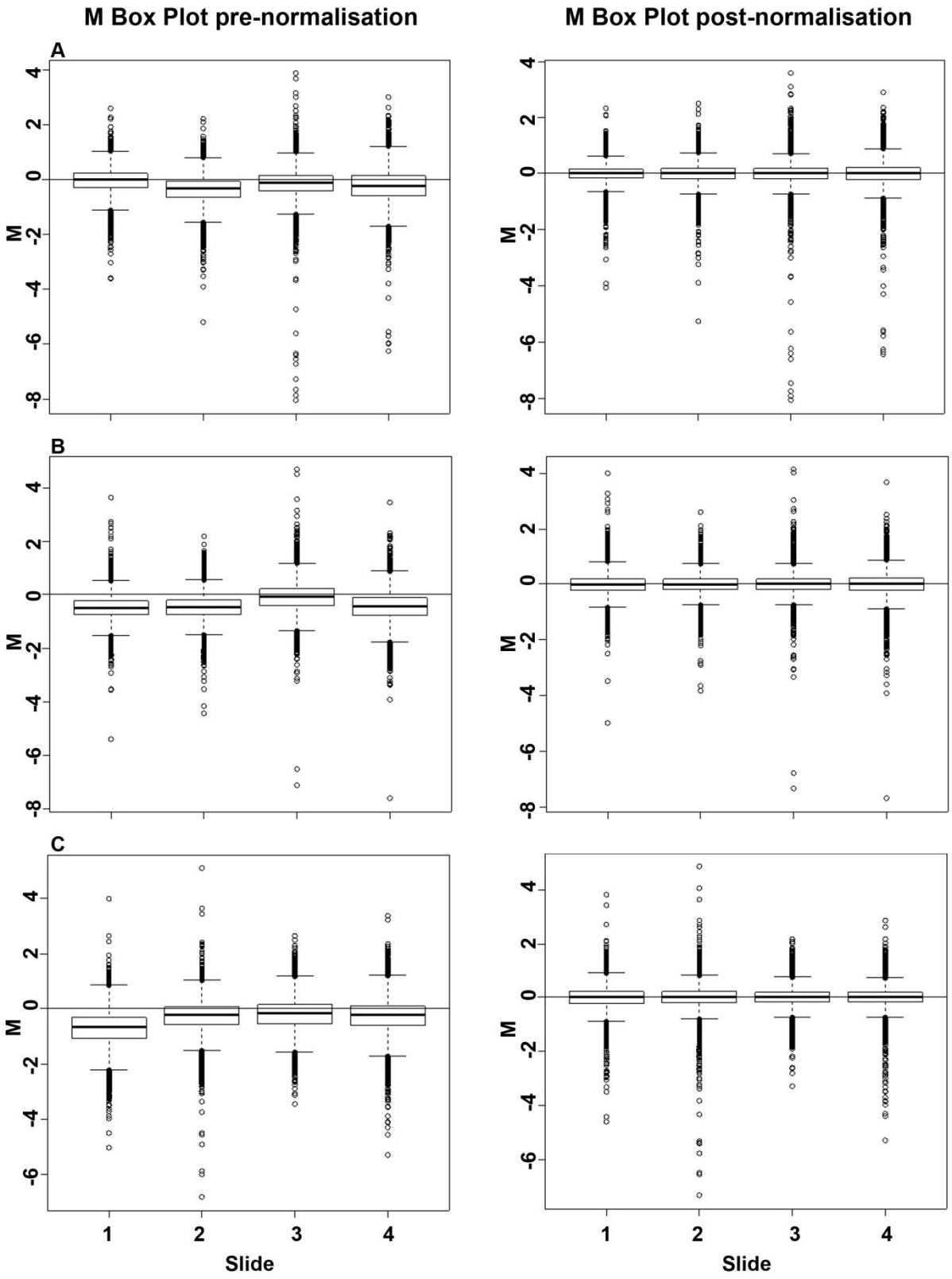


Figure 4.1: Microarray normalisation within and across slides

The left hand panel shows the M plot before normalisation and the right panel shows the data after print tip loess (within slide) and quantile (between slides) normalisation for *hspro1-2* (A), *hspro2* (B) and *hspro1-2/hspro2* (C), all relative to Col-0.

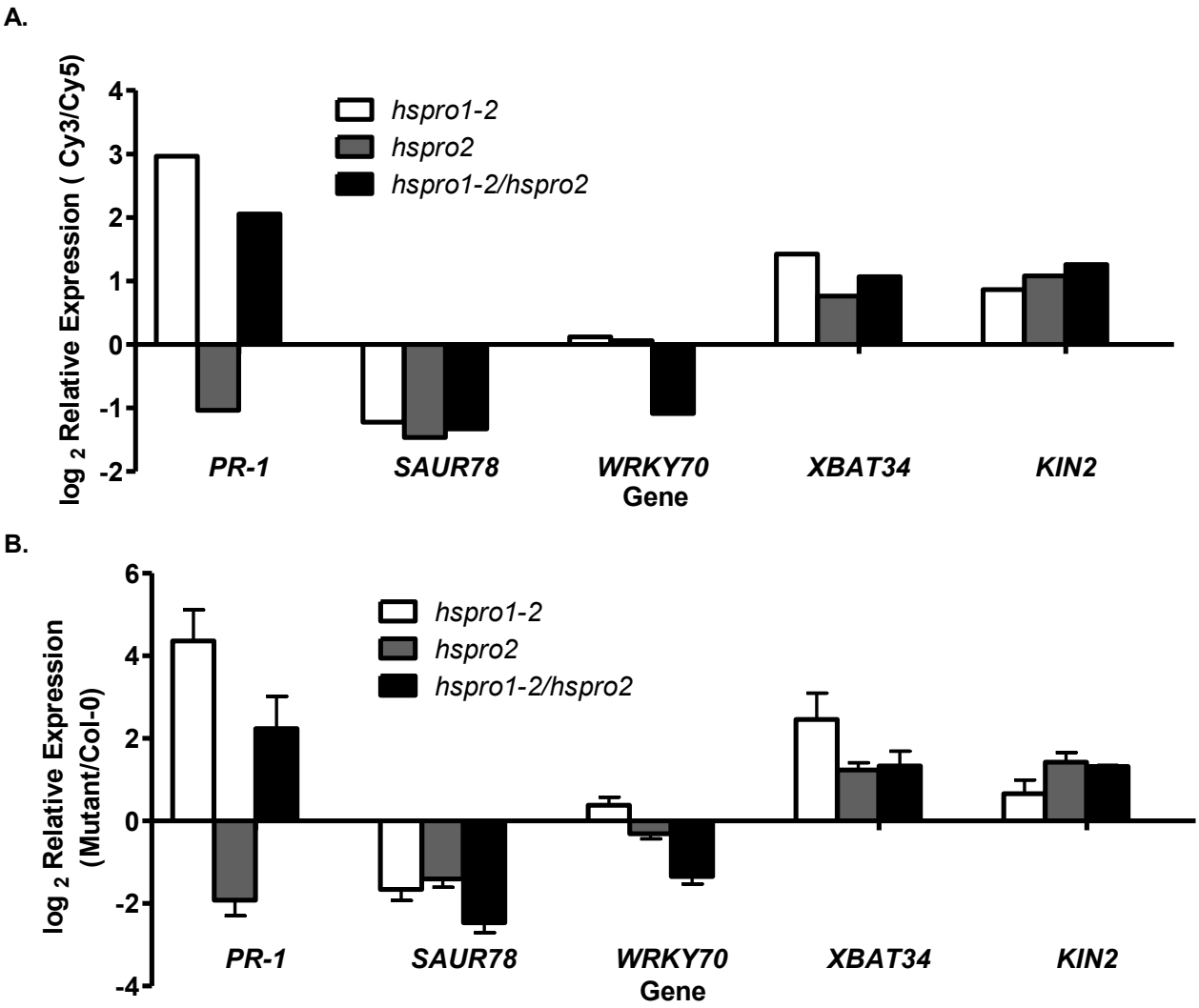


Figure 4.2: Microarray data validation.

Five genes identified to be differentially expressed in the microarray data were used to validate the data set. RNA was extracted from 4 pools of leaves (with 4 plants per pool) for wild type and *hspro* mutants. For microarray analysis (A), the 4 RNA samples per plant line were pooled and 1 µg total RNA amplified for downstream use. Log_2 expression ratios for each gene in the three mutant lines relative to wild type plants are shown. For the RT-qPCR validation (B) plant RNA pools were kept separate and cDNA synthesised from 1 µg total RNA per pool. Gene expression was normalised to *ACT2* expression levels and expression levels in the mutant lines was expressed relative to wild type levels. Error bars represent the S.E.M and n=3. The validation experiment was conducted once with the same RNA source as the microarray experiment.

4.2.1.2 Differential gene expression in hspro knockout mutants

From the list of differentially expressed genes it was observed that *hspro2* had the highest number of differentially expressed genes (382) compared to *hspro1-2* (205) and *hspro1-2/hspro2* (256) (Table 4.1). Many of the genes are differentially expressed in at least two of the mutants but the number of *hspro2* specific, differentially expressed genes (183) is greater than double the number of *hspro1-2* (72) or *hspro1-2/hspro2* (79) specific genes (Figure 4.3). Furthermore, genes unique to *hspro2* make up 47.9% of all genes differentially expressed in *hspro2* while only 35.1% and 30.9% of genes differentially

expressed in *hspro1-2* and *hspro1-2/hspro2* are unique to the two mutants respectively. This suggests the *hspro2* mutation may lead to a greater amount of gene misregulation when compared to *hspro1-2* and *hspro1-2/hspro2*. 95.8% of the genes uniquely responsive to the *hspro1-2* mutation are induced while 61.7% of the *hspro2* specific genes are repressed and approximately 50% of the *hspro1-2/hspro2* specific genes are either induced or repressed. It was also observed that it was more likely that if a gene was differentially expressed in more than one mutant, the expression pattern was similar across all the respective mutants. This can be seen by comparing the sum of Figure 4.3B&C intersection points with the Figure 4.3A intersection points. The sum of B and C intersection points are approximately equal to A but a small number of genes are indeed present that have contrasting expression profiles across mutants. This suggests that the *HSPRO* genes may be involved in the regulation of similar genes in addition to their regulation of unique gene groups.

Table 4.1: Summary of genes differentially expressed in the *hspro* mutants

	hspro1-2		***hspro2***		***hspro1-2/hpro2***	
	Total	%	Total	%	Total	%
Expression Pattern	**205**		**382**		**256**	
Down-regulated	36	18	204	53	108	42
Up-regulated	169	82	178	47	148	58

4.2.1.3 Functional enrichment of GO terms in differentially expressed gene lists of the hspro mutants

To extract information about potential functional roles of *HSPRO1* and *HSPRO2* in *Arabidopsis* the lists of differentially expressed genes within each mutant line were uploaded into the Babelomics database (babelomics.bioinfo.cipf.es) and analysed for enrichment of GO terms. It should be noted there was no functional enrichment of GO terms in the list of genes repressed in the *hspro1-2* mutation and this could be attributed to the fact that the gene list comprised of only 36 genes, possibly involved in different processes.

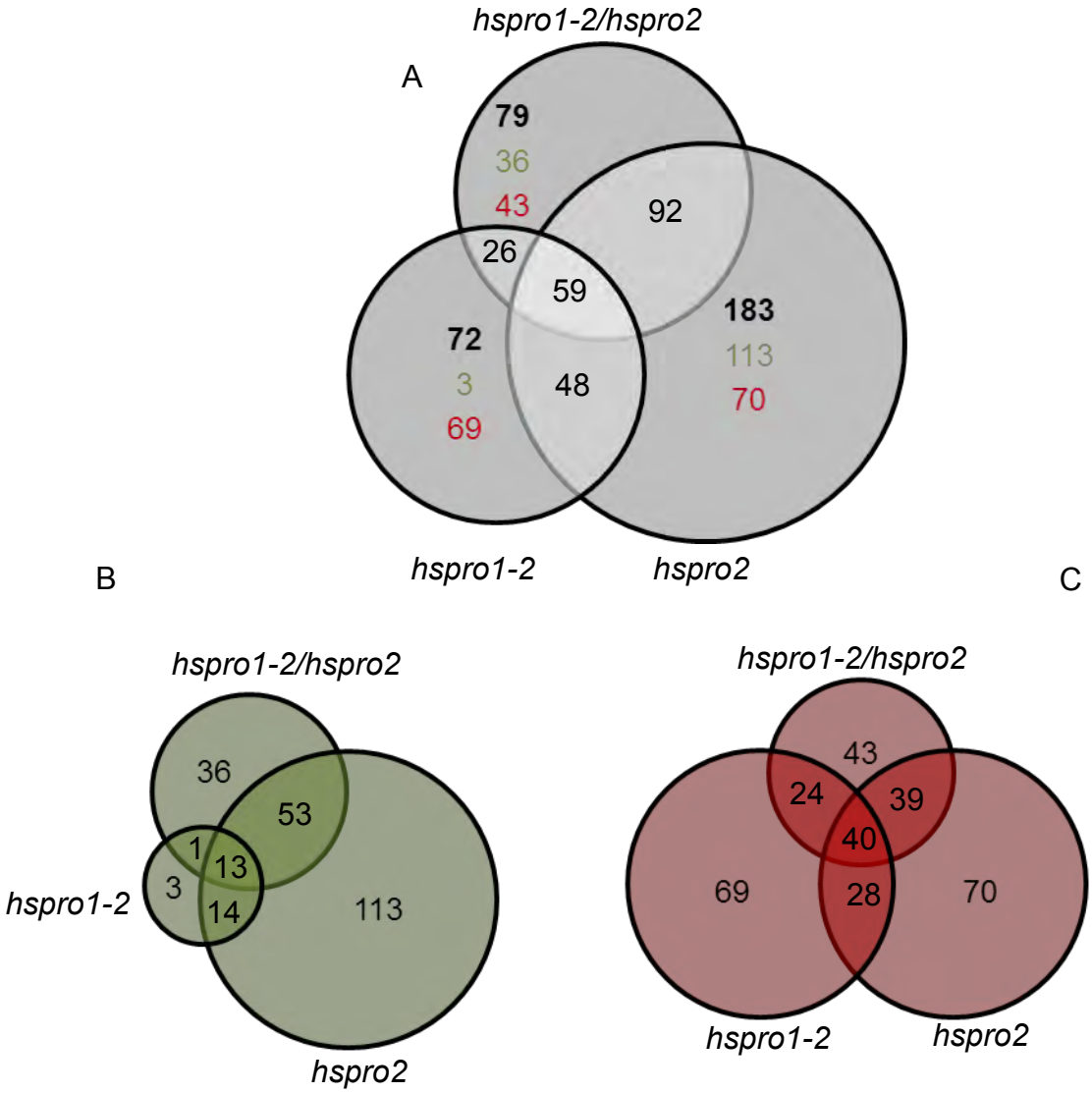


Figure 4.3: Distribution of mutant-specific and shared gene responses.

A is the overall distribution with black text representing total number of responsive genes, green text signifies repression and red text representing induction. B and C are the genes that display a consistent expression pattern regardless of mutant line. Green = repression and red = induction.

GO analysis of genes differentially expressed in hspro1-2

The list of genes induced in *hspro1-2* had enrichment of terms associated with several themes. The most significantly enriched GO terms were associated with detection or response to other living organisms (Figure 4.4). Many of the returned terms are associated with the plant defence response including terms such as "plant immune response", "incompatible interaction", "defence response to bacteria", "defence response to fungus", "defence response by callose deposition", "hypersensitive response", "host programmed cell death", "systemic acquired resistance", "cell wall thickening", "response to chitin", "response to salicylic acid and jasmonic acid". To a lesser extent, there was enrichment of abiotic stress associated terms including response to oxidative, ozone, osmotic, salt, and cold stress. The enrichment of a/biotic stress–associated terms was not entirely surprising as *hspro1-2* plants had already been shown to have altered responses to disease and abiotic stress in chapter 3. GO analysis also revealed enrichment of terms associated with carbohydrates including 'response to carbohydrate stimulus', 'response to fructose stimulus' and 'carbohydrate transport' hinting at potential involvement of *HSPRO1* in sugar-based responses.

GO analysis of genes differentially expressed in hspro2

In chapter 3, *hspro2* was described as having both biotic and abiotic stress phenotypes and so it was not surprising that there was enrichment of a/biotic stress functional categories in the lists of genes induced or repressed in the *hspro2* mutant (Figure 4.5 and Figure 4.6). There appears to be a more complex and diverse list of GO categories associated with the genes induced versus the genes repressed in *hspro2* and besides a/biotic stress response terms, carbohydrate response and transport terms are also enriched in both gene lists. Other terms also associated with *hspro2* were those associated with light and circadian regulation including "circadian rhythm", "response to absence of light", "regulation of long-day photoperiodism, flowering", "response to light stimulus", "response to light intensity" and "vegetative to reproductive phase transition of meristem", implying additional roles in light and circadian based gene regulation and developmental transitions from vegetative growth to reproductive growth. In this instance GO analysis revealed that *HSPRO2* may be potentially involved in carbohydrate perception and transport and the regulation of light and circadian clock associated genes in addition to function in a/biotic stress responses.

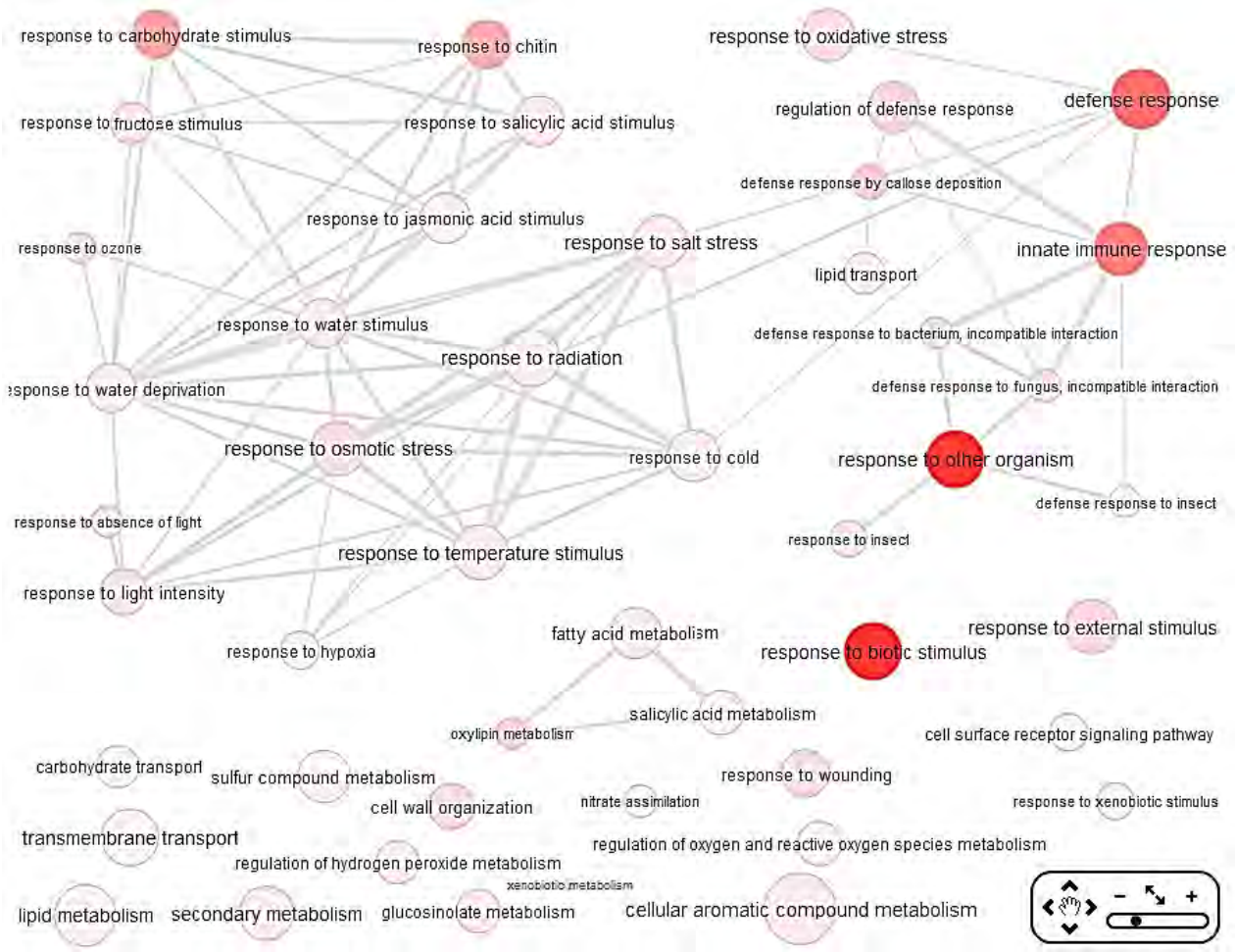


Figure 4.4: Functional categories enriched in *hspro1-2*-dependent gene induction

GO terms returned from the FatiGO tool were uploaded into the REVIGO GO term visualisation tool (http://revigo.irb.hr/). Bubble colour indicates the p-value associated with the GO term, with the darkest colour being the most significant. Bubble size represents the frequency of the GO term in the *Arabidopsis* GOA database (bubbles of more general terms are bigger). Similar GO terms are linked by the four edges of the image and the line width of connecting lines represents the percentage of similarity.

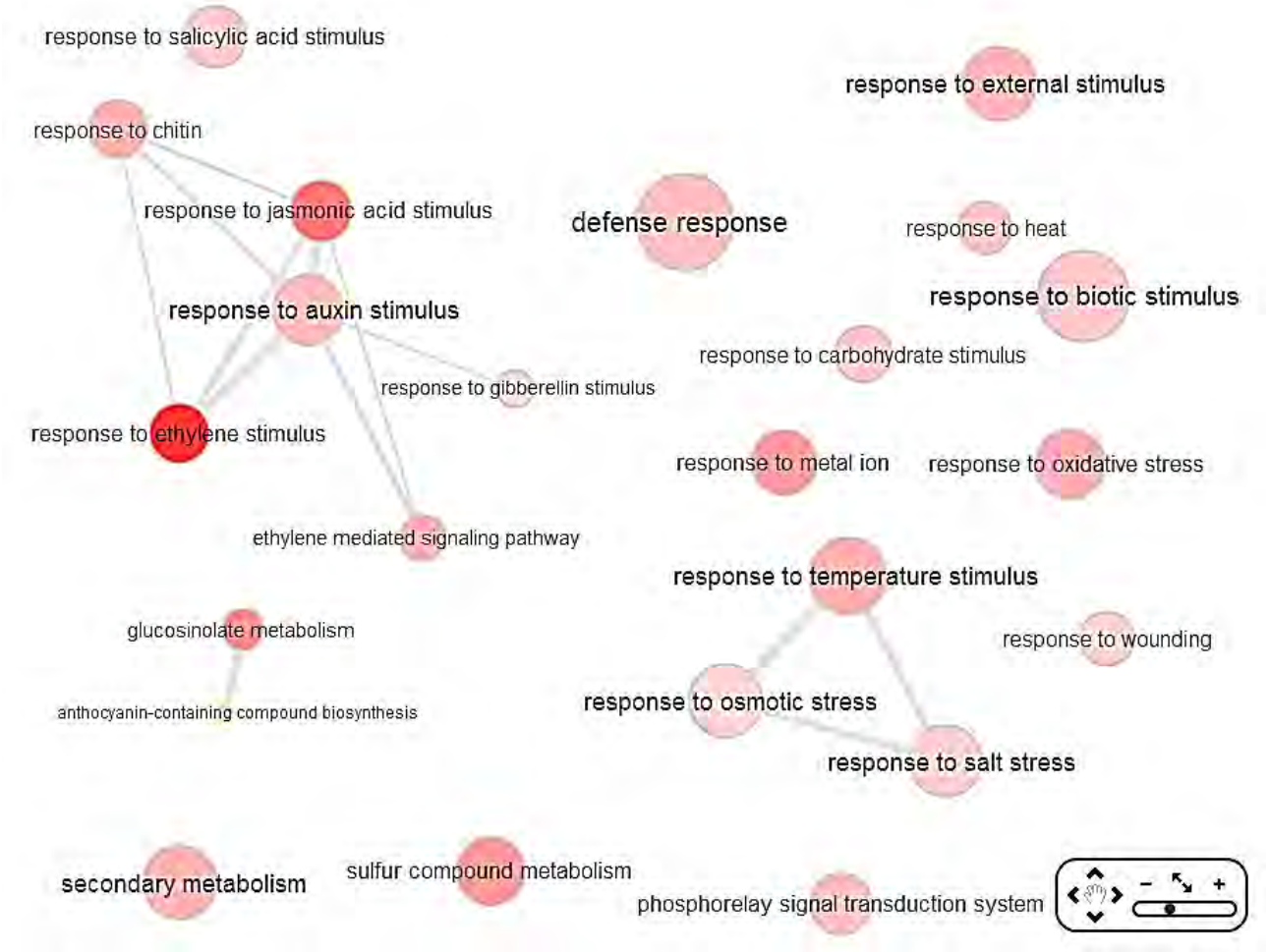


Figure 4.5: Functional categories enriched in *hspro2*-dependent gene repression

GO terms returned from the FatiGO tool were uploaded into the REVIGO GO term visualisation tool (http://revigo.irb.hr/). Bubble colour indicates the p-value associated with the GO term, with the darkest colour being the most significant. Bubble size represents the frequency of the GO term in the *Arabidopsis* GOA database (bubbles of more general terms are bigger). Similar GO terms are linked by the four edges of the image and the line width of connecting lines represents the percentage of similarity.

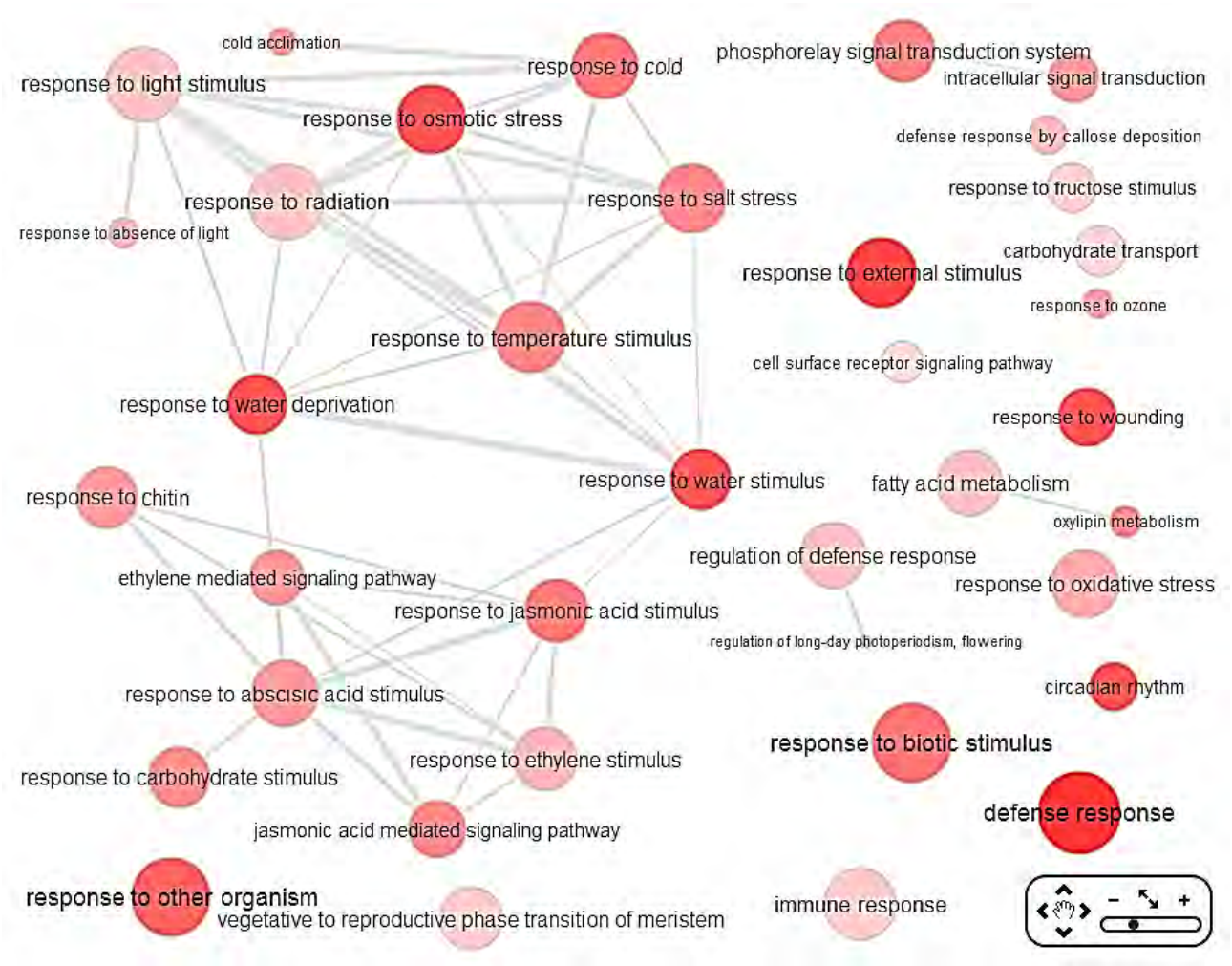


Figure 4.6: Functional categories enriched in *hspro2*-dependent gene induction

GO terms returned from the FatiGO tool were uploaded into the REVIGO GO term visualisation tool (http://revigo.irb.hr/). Bubble colour indicates the p-value associated with the GO term, with the darkest colour being the most significant. Bubble size represents the frequency of the GO term in the *Arabidopsis* GOA database (bubbles of more general terms are bigger). Similar GO terms are linked by the four edges of the image and the line width of connecting lines represents the percentage of similarity.

GO analysis of genes differentially expressed in hspro1-2/hspro2

There are overlaps in genes differentially expressed in either *hspro1-2* or *hspro2* and the double knockout mutant and unsurprisingly there is conservation of the same GO terms identified in the single knockout mutant data when compared to the *hspro1-2/hspro2* data set (Figure 4.7 and Figure 4.8). Common themes consistent across all three mutants include defence response, immune response, response to other organisms, various abiotic stress associated stress terms, response to and mediation of stress-associated phytohormone signalling and carbohydrate associated terms. Circadian clock associated terms are unique to the *HSPRO2* mutants with an increased enrichment for such terms in the *hspro2* single knockout mutant. Considering the overlap across gene expression and

GO terms, an investigation into potential overlaps in *cis* regulatory elements was conducted via promoter content analysis.

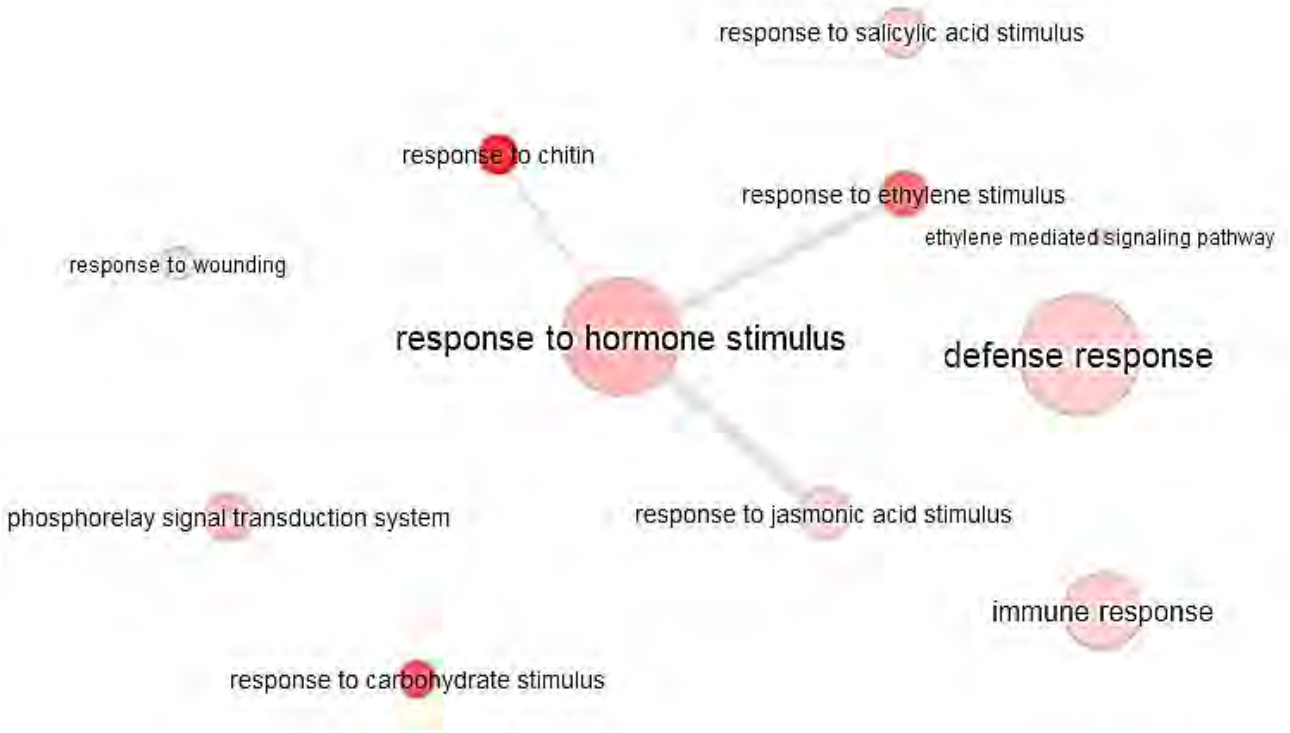


Figure 4.7: Functional categories enriched in *hspro1-2/hspro2*-dependent gene repression

GO terms returned from the FatiGO tool were uploaded into the REVIGO GO term visualisation tool (http://revigo.irb.hr/). Bubble colour indicates the p-value associated with the GO term, with the darkest colour being the most significant. Bubble size represents the frequency of the GO term in the *Arabidopsis* GOA database (bubbles of more general terms are bigger). Similar GO terms are linked by the four edges of the image and the line width of connecting lines represents the percentage of similarity.

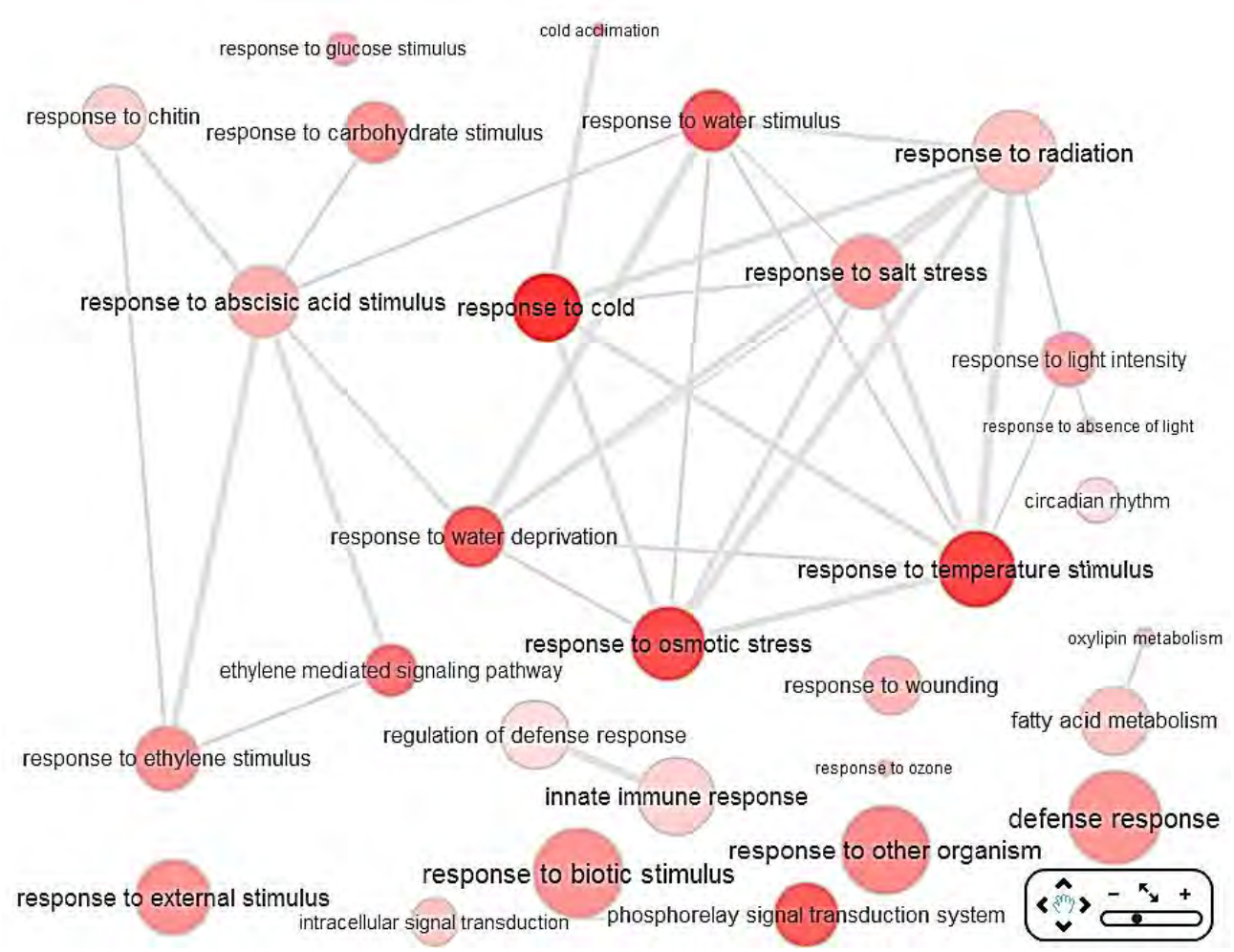


Figure 4.8: Functional categories enriched in *hspro1-2/hspro2*-dependent gene induction

GO terms returned from the FatiGO tool were uploaded into the REVIGO GO term visualisation tool (http://revigo.irb.hr/). Bubble colour indicates the p-value associated with the GO term, with the darkest colour being the most significant. Bubble size represents the frequency of the GO term in the *Arabidopsis* GOA database (bubbles of more general terms are bigger). Similar GO terms are linked by the four edges of the image and the line width of connecting lines represents the percentage of similarity.

4.2.1.4 Promoter content analysis of differentially expressed gene lists

The regulatory roles of HSPRO1 and HSPRO2 in *Arabidopsis* can be indirectly assessed by examining the enrichment of specific TFBS in genes differentially expressed in the *hspro1-2* and *hspro2* null mutants, during normal growth. Enrichment was considered in the lists for genes either up-regulated or down-regulated in *hspro1-2*, *hspro2* and *hspro1-2/hspro2* and it should be noted that there was no enrichment of TF sites in the *hspro1-2* repressed genes list. The T-box and Evening Element (EE) promoter motifs are enriched for in 64% and 17% of the genes up-regulated in *hspro1-2* respectively (Table 4.2). The T-box motif is important in light-activated gene transcription (Chan *et al.*, 2001) and the EE motif important in circadian regulation of evening-expressed genes (Gendron *et al.*, 2012; Harmer, 2000). The ABRE-like binding site (modified G-box) and G-box motifs are

implicated in both *hspro2* induced/repressed gene lists although less than half the promoters contain either one of these motifs so it is unlikely regulation through these sites drives expression of the entire list but may rather drive the expression of a subset of genes. G-box based regulation has been linked to sugar responses and it is possible that this subset of genes is involved in sugar responses. *hspro2* and *hspro1-2/hspro2* induction lists have highly similar enrichment of TF sites, suggesting an epistatic condition of *hspro2* to *hspro1-2*. There is significant enrichment of several motifs associated with response to abiotic stress (ABA signalling, cold, drought and salt stress) and light and circadian regulated gene expression potentially implicating HSPRO2 in the regulation of genes associated with the respective motifs.

Table 4.2: Enrichment of transcription factor sites in the promoters of genes differentially expressed in the *hspro* mutants

		Repressed			Induced		
		% of list with motif			% of list with motif		
Motif	**Associated Process**	*hspro1-2*	*hspro2*	*hspro1-2 /hspro2*	*hspro1-2*	*hspro2*	*hspro1-2 /hspro2*
ABRE-like binding site motif	ABA-mediated stress signalling	-	40.4	-	-	38.9	39.7
CBF1 BS in cor15a	Cold and drought stress response	-			-	5.6	9.5
DRE core motif	Salt and drought responsive element	-	-	-		44.4	46
DREB1A/CBF3	Cold, drought and salinity stress response	-	-	-	-	18.9	30.2
Evening Element promoter motif	Circadian control of gene expression	-	-	-	16.9	24.4	33.3
G-box motif	Light responsive gene regulation	-	31.9	-	-	30	30.2
LTRE promoter motif	Low temperature responsive element	-	-	-	-	13.3	17.5
MRE motif in CHS	Water stress response	-	-	3.6	-	-	-
T-box promoter motif	Light activated gene regulation	-	-	-	64	-	-
UPRMOTIFIIAT	Light responsive element	-	-	-	-	-	12.7
Z-box promoter motif	Light responsive gene regulation		-	-	-	8.9	-

4.2.1.5 Refined identification of potential downstream targets of the HSPRO genes

Genes that are co-expressed with the *HSPRO* genes can assist in identifying biological processes the *HSPRO* genes are potentially involved in but by applying an additional filtering step and comparing the ECGG lists with the *hspro* microarray data we can resolve this data further and identify downstream targets of *HSPRO1* and *HSPRO2* with more confidence. Using this overlap analysis 9 genes co-expressed with *HSPRO1* were also observed to be differentially expressed in *hspro1-2* (Table 4.3) while 31 genes were identified in the comparison between the *HSPRO2* ECGG and the *hspro2* array gene list (Table 4.4). There was enrichment of the I-box and the DREB1A/CBF3 promoter motifs - that may be of significance in downstream target regulation - in the refined *HSPRO2* list

and no enrichment in the *HSPRO1* list (Table 4.5). These genes may be interesting for further *HSPRO* gene function analysis as they may be important *HSPRO* targets.

Table 4.3: Overlap between *HSPRO1* ECGG and *hspro1-2* microarray differential expression gene list

Gene	Description	ρ	Log_2 expression
At3g55980	SALT-INDUCIBLE ZINC FINGER 1 (SZF1); FUNCTIONS IN: transcription factor activity	0.61	0.617932
At4g34390	extra-large GTP-binding protein 2 (XLG2)	0.6	0.692964
At1g02660	lipase class 3 family protein	0.64	0.753638
At5g22690	disease resistance protein (TIR-NBS-LRR class) putative	0.6	0.758547
At1g65390	ARABIDOPSIS THALIANA PHLOEM PROTEIN 2 A5 (ATPP2-A5); FUNCTIONS IN: carbohydrate binding;	0.61	0.783842
At4g23190	CYSTEINE-RICH RLK11 (CRK11)	0.62	0.904706
At4g17230	Scarecrow-like 13 (SCL13)	0.62	1.199275
At1g72520	lipoxygenase putative	0.63	1.418655
At5g66210	CPK28; FUNCTIONS IN: protein serine/threonine kinase activity calmodulin-dependent protein kinase activity	0.61	1.563465

Table 4.4: Overlap between *HSPRO2* ECGG and *hspro2* microarray differential expression gene list

Gene	Description	ρ	Log_2 expression
At1g73540	Arabidopsis thaliana Nudix hydrolase homolog 21 (atnudt21); FUNCTIONS IN: hydrolase activity	0.66	-1.75558
At1g22190	AP2 domain-containing transcription factor putative	0.73	-1.53703
At1g32920	unknown protein; FUNCTIONS IN: molecular_function unknown; INVOLVED IN: response to wounding	0.75	-1.35472
At4g36040	DNAJ heat shock N-terminal domain-containing protein (J11);	0.65	-1.31664
At5g61600	ethylene-responsive element-binding family protein	0.7	-1.14457
At5g57560	Touch 4 (TCH4); FUNCTIONS IN: hydrolase activity acting on glycosyl bonds xyloglucan	0.69	-0.98295
At5g51390	unknown protein	0.66	-0.97354
At1g76600	unknown protein	0.73	-0.92127
At1g78080	related to AP2 4 (RAP2.4); FUNCTIONS IN: transcription factor activity DNA binding	0.61	-0.88537
At1g13260	RAV1; FUNCTIONS IN: transcription repressor activity transcription factor activity DNA binding	0.62	-0.86524
At3g10930	unknown protein	0.68	-0.81252
At3g61060	Arabidopsis thaliana phloem protein 2-A13 (AtPP2-A13); FUNCTIONS IN: carbohydrate binding	0.63	-0.81054
At3g46620	zinc finger (C3HC4-type RING finger) family protein; FUNCTIONS IN: protein binding zinc ion binding	0.73	-0.77919
At1g25400	unknown protein	0.75	-0.77495
At1g23710	unknown protein	0.82	-0.7528
At4g27280	calcium-binding EF hand family protein; FUNCTIONS IN: calcium ion binding	0.76	-0.73838
At5g54490	PINOID-BINDING PROTEIN 1 (PBP1); FUNCTIONS IN: protein binding calcium ion binding; INVOLVED IN: res..//	0.62	-0.71677
At1g80920	J8; FUNCTIONS IN: unfolded protein binding heat shock protein binding	0.7	-0.70572
At2g26530	AR781	0.68	-0.70082
At1g69490	NAC-like activated by AP3/PI (NAP); FUNCTIONS IN: transcription factor activity	0.66	-0.69202
At3g19580	ARABIDOPSIS ZINC-FINGER PROTEIN 2 (AZF2)	0.66	-0.63626
At5g04080	unknown protein	0.6	-0.56725
At4g29900	AUTOINHIBITED CA(2+)-ATPASE 10 (ACA10); FUNCTIONS IN: calmodulin binding calcium-transporting ATPase..//	0.6	0.592003

At1g09940	HEMA2; FUNCTIONS IN: glutamyl-tRNA reductase activity;	0.64	0.666373
At5g62570	calmodulin-binding protein; FUNCTIONS IN: calmodulin binding	0.71	0.683285
At1g28380	necrotic spotted lesions 1 (NSL1)	0.62	0.736556
At3g10020	unknown protein; INVOLVED IN: response to oxidative stress	0.67	0.885977
At5g02020	unknown protein;	0.61	0.887153
At2g18700	ATTPS11; FUNCTIONS IN: transferase activity transferring glycosyl groups	0.75	1.225239
At4g17230	Scarecrow-like 13 (SCL13); FUNCTIONS IN: transcription factor activity;	0.65	1.31789
At5g66210	CPK28	0.69	1.544055

Table 4.5: Enrichment of transcription factor sites in the promoters of genes that are co-expressed with either one of *HSPRO* genes and are differentially expressed in the *hspro* single mutants

		HSPRO1/hspro1-2	***HSPRO2/hspro2***
Motif	**Associated Process**	% of list with motif	% of list with motif
DREB1A/CBF3	Cold, drought and salinity stress response	-	25.8
I-box promoter motif	Light responsive gene regulation	-	64.5

4.2.2 Integration of HSPRO with energy sensing and signalling

Analysis of both publically available array data (chapter 3) and the array data generated here suggest that *HSPRO* genes are potentially involved in carbohydrate perception and/or signalling. FatiGO analysis of genes co-expressed with either *HSPRO* genes revealed that both genes are co-expressed with genes that respond to carbohydrate stimulus. Additionally, FatiGO analysis of microarray data from *hspro* mutants revealed that genes involved in carbohydrate perception and transport, and ABA signalling are differentially expressed in the absence of *HSPRO* genes. Furthermore, the G-box promoter motif and the ABRE-like binding site motif– that have been implicated in sugar responsive gene regulation (Baena-González *et al.*, 2007; Kang *et al.*, 2010b; Li *et al.*, 2006; Lu *et al.*, 2002) – were enriched for in the *HSPRO2* ECGG and the lists of genes differentially expressed in *hspro2* and *hspro1-2/hspro2* mutants hinting at the potential involvement of *HSPRO2* in cellular responses during detection of carbohydrate stimuli. The I-box motif which has been reported to be a classifier of glucose repression (Li *et al.*, 2006) was also identified in the promoter regions of both *HSPRO* genes and enriched for in the *HSPRO2* ECGG. Interestingly, both HSPRO1 and HSPRO2 have been shown to interact with the SnRK1 complex (Gissot *et al.*, 2006), a regulatory complex of significance in energy and stress signalling (Baena-González *et al.*, 2007; Baena-González & Sheen, 2008) and perhaps it is this interaction with SnRK1 that drives these observed patterns.

4.2.2.1 A subset of KIN10 *target genes are misregulated in the hspro mutants*

Within the context of energy signalling the SnRK1 complex and particularly the KIN10 subunit have been shown to be responsive to energy limiting conditions such as light and nutrient deprivation (Baena-González *et al.*, 2007). Since HSPRO1 and HSPRO2 are known interacting partners of the SnRK1 complex it is possible that the two proteins could be involved in the modulation of SNRK1 activity in response to plant energy status. The potential targets of SnRK1 have been identified by comparing the transcriptome profiles of *Arabidopsis* protoplasts with or without the transient expression of *KIN10* (the SnRK1 α subunit) and microarray expression profiles from several energy limiting or energy rich conditions (Baena-González *et al.*, 2007). Genes were considered to be KIN10 targets if their expression pattern in response to KIN10 was similar to the response under starvation conditions and additionally displayed an opposing expression pattern under energy rich conditions. The effects of *KIN10* transient expression are akin to those triggered by energy depleting conditions e.g. extended night and result in the induction of genes typically repressed by glucose or sucrose and induced during starvation, including *HSPRO2* (Figure 4.9). However, although *HSPRO1* expression was induced in response to *KIN10* expression, the gene did not behave strictly as other identified KIN10 targets as it was only induced under low energy conditions but was not sugar responsive. To determine whether HSPRO1 and HSPRO2 may be involved in the regulation of KIN10 target genes, the Baena-González data set was compared to the data from the *hspro* mutant transcriptome profiling. Thirty-four of the 599 KIN10 target genes were determined to be differentially expressed in at least one of the *hspro1-2*, *hspro2* and *hspro1-2/hspro2* mutants, suggesting potential involvement of the *HSPRO* genes in the regulation of a subset of KIN10 target genes. Notably, all 34 of these genes are up-regulated during *KIN10* expression and down-regulated in response to sugar: none of the 321 genes repressed by KIN10 were differentially expressed in the *hspro* mutants. Within each individual *hspro* mutant line most of the KIN10 targets are up-regulated in expression, much like during *KIN10* expression. This data suggests *HSPRO1* and *HSPRO2* may be involved in the regulation of a subset of carbon starvation induced genes via KIN10.

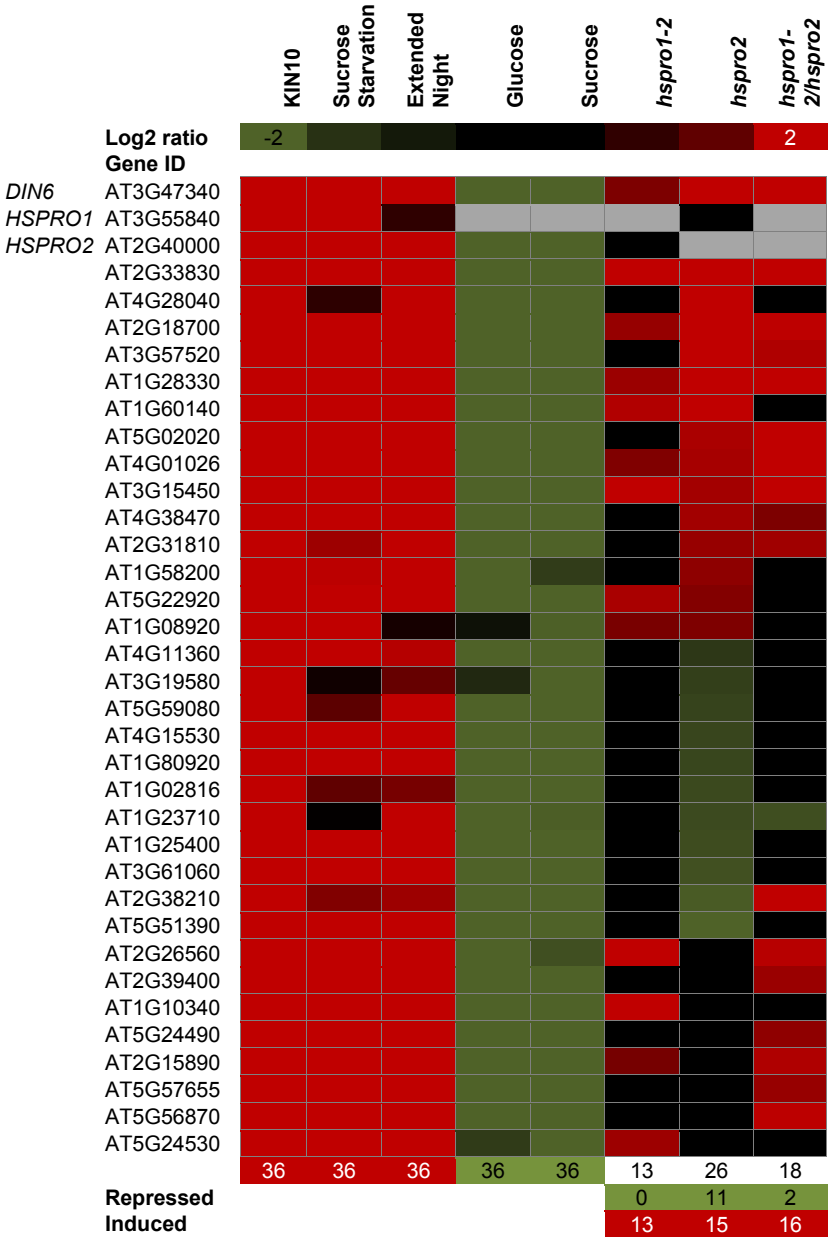


Figure 4.9: A subset of KIN10 targets are misregulated in *hspro* mutants.

The expression of KIN10 targets identified from the Baena-González data set was evaluated in the *hspro* mutant array data and KIN10 target genes that showed differential expression in at least one mutant are shown.

In agreement with the results described above, only *HSPRO2* (and not *HSPRO1*) expression was shown to be responsive to altered sugar levels during actual experimental analysis (Figure 4.10). Two-week-old seedlings acclimated to growth in liquid MS media were treated with glucose over a 25 h period and harvested 1 h after dawn. RT-qPCR analysis revealed that *HSPRO1* expression is not glucose responsive while *HSPRO2* is repressed following glucose treatment supporting the hypothesis that *HSPRO2* is involved in energy signalling, possibly as a KIN10 target.

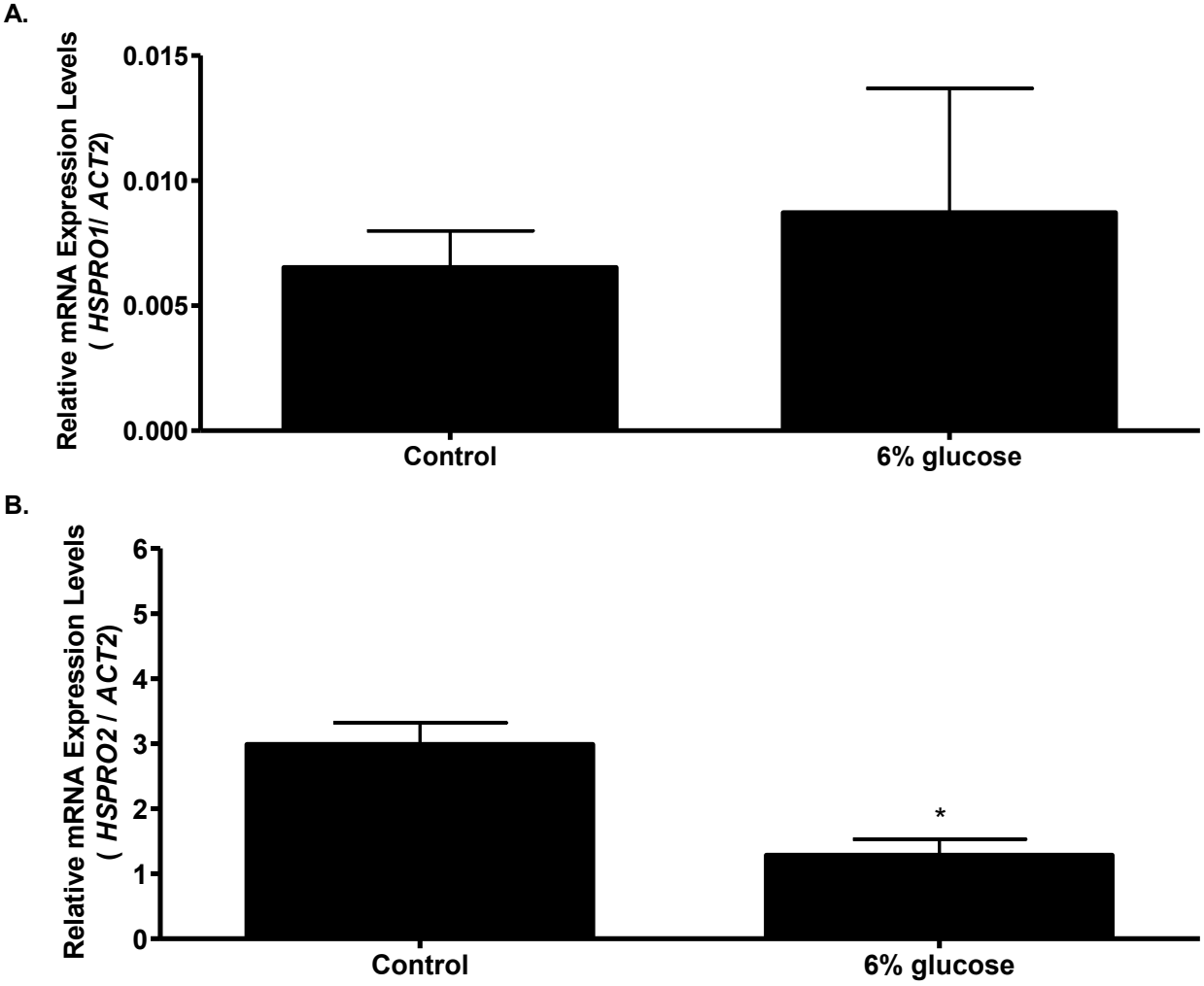


Figure 4.10: *HSPRO2* expression is glucose repressed.

Seeds were germinated on solid 0.5 x MS medium with 0.2% glucose and grown for two weeks under normal conditions. The two-week-old seedlings were transferred onto liquid 0.5 x MS (0.2% glucose) and allowed to acclimate. 24 h later the plants were treated with 0.5 x MS media containing either 0.2% or 6% glucose and incubated for 25 h. Leaf tissue was collected after 1 h of light. *HSPRO1* (A) and *HSPRO2* (B) gene expression was examined using RT-qPCR and normalised to *ACT2* expression. Error bars represent the S.E.M (n = 3 biological pools of 10 seedlings each). Statistically significant deviation from the control plants was tested using the Student‘s t-test. * = $p < 0.05$.

4.2.2.2 hspro mutants have altered expression of energy responsive genes during normal growth

Given that *hspro* mutants displayed altered regulation of a subset of KIN10 targets which are induced in response to energy depletion, we decided to investigate if the two genes are involved in the regulation of expression of other energy-responsive genes in *Arabidopsis*. To achieve this microarray data from several energy-limiting and energy-rich experiments was downloaded from publicly available publications and compared to the genes differentially expressed in the *hspro* mutants (Table 2.7).

Table 4.6: Summary of gene overlap between *hspro* transcriptomes and energy responsive gene lists

	hspro1-2		*hspro2*		*hspro1-2/hpro2*	
	Total	%	Total	%	Total	%
MUTANT GENE LIST	**205**		**382**		**256**	
SUGAR AND MUTANT OVERLAP	**34**	**17**	**101**	**26**	**59**	**23**

On a more global scale, 26% and 23% of all the genes differentially expressed in *hspro2* and *hspro1-2/hspro2* respectively are also energy responsive while 17% of the *hspro1-2* mutant genes are energy responsive implicating *HSPRO* genes in the regulation of a subset of energy responsive genes (Table 4.6). Cluster analysis revealed four interesting patterns of expression within the data set that suggested the *hspro* mutants are potentially defective in energy status perception or signalling (Figure 4.10). Energy-responsive gene expression in clusters A and B showed the *hspro* mutants had gene expression profiles similar to those observed under energy limiting conditions such as the extended night treatment and sucrose starvation, while clusters C and D showed a reversal of this pattern with the mutants behaving more like plants growing during high energy conditions. Thirty of the 35 KIN10 target genes identified as differentially expressed in the *hspro* mutants were also found in clusters B and C which display genes induced under starvation conditions. Although *HSPRO1* and *HSPRO2* appeared to be involved in energy perception or signalling it was not easily apparent what overall regulatory effects they had on energy responses given the opposing expression profiles observed in clusters A and B versus C and D. Interestingly though, the *hspro2* mutant consistently had a higher (than *hspro1-2* and *hspro1-2/hspro2*) percentage of differentially expressed genes within the clusters (except for cluster B) implicating *HSPRO2* more in the regulation of these energy responsive genes (Table 4.7). The *hspro1-2/hspro2* mutant does not behave like either *hspro1-2* or *hspro2* in this regard and perhaps this is due to the unpredictable combinatorial effects of knocking out both genes.

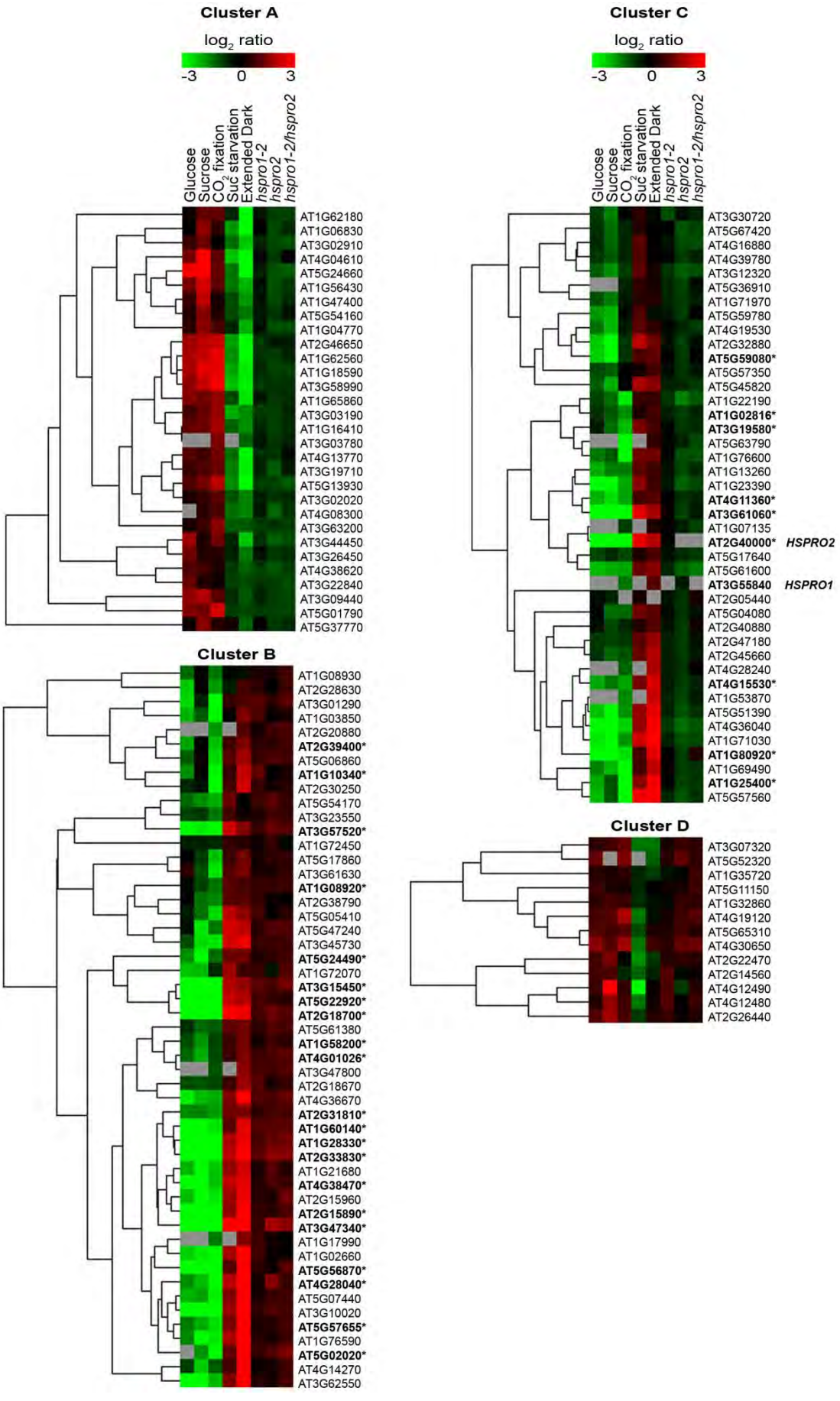

Cluster A
log2 ratio
-3
0
3
Glucose
Sucrose
CO2 fixation
Suc starvation
Extended Dark
hspro1-2
hspro2
hspro1-2/hspro2
AT1G62180
AT1G06830
AT3G02910
AT4G04610
AT5G24660
AT1G56430
AT1G47400
AT5G54160
AT1G04770
AT2G46650
AT1G62560
AT1G18590
AT3G58990
AT1G65860
AT3G03190
AT1G16410
AT3G03780
AT4G13770
AT3G19710
AT5G13930
AT3G02020
AT4G08300
AT3G63200
AT3G44450
AT3G26450
AT4G38620
AT3G22840
AT3G09440
AT5G01790
AT5G37770
Cluster B
AT1G08930
AT2G28630
AT3G01290
AT1G03850
AT2G20880
AT2G39400*
AT5G06860
AT1G10340*
AT2G30250
AT5G54170
AT3G23550
AT3G57520*
AT1G72450
AT5G17860
AT3G61630
AT1G08920*
AT2G38790
AT5G05410
AT5G47240
AT3G45730
AT5G24490*
AT1G72070
AT3G15450*
AT5G22920*
AT2G18700*
AT5G61380
AT1G58200*
AT4G01026*
AT3G47800
AT2G18670
AT4G36670
AT2G31810*
AT1G60140*
AT1G28330*
AT2G33830*
AT1G21680
AT4G38470*
AT2G15960
AT2G15890*
AT3G47340*
AT1G17990
AT1G02660
AT5G56870*
AT4G28040*
AT5G07440
AT3G10020
AT5G57655*
AT1G76590
AT5G02020*
AT4G14270
AT3G62550
Cluster C
log2 ratio
-3
0
3
Glucose
Sucrose
CO2 fixation
Suc starvation
Extended Dark
hspro1-2
hspro2
hspro1-2/hspro2
AT3G30720
AT5G67420
AT4G16880
AT4G39780
AT3G12320
AT5G36910
AT1G71970
AT5G59780
AT4G19530
AT2G32880
AT5G59080*
AT5G57350
AT5G45820
AT1G22190
AT1G02816*
AT3G19580*
AT5G63790
AT1G76600
AT1G13260
AT1G23390
AT4G11360*
AT3G61060*
AT1G07135
AT2G40000* HSPRO2
AT5G17640
AT5G61600
AT3G55840 HSPRO1
AT2G05440
AT5G04080
AT2G40880
AT2G47180
AT2G45660
AT4G28240
AT4G15530*
AT1G53870
AT5G51390
AT4G36040
AT1G71030
AT1G80920*
AT1G69490
AT1G25400*
AT5G57560
Cluster D
AT3G07320
AT5G52320
AT1G35720
AT5G11150
AT1G32860
AT4G19120
AT5G65310
AT4G30650
AT2G22470
AT2G14560
AT4G12490
AT4G12480
AT2G26440

Figure 4.11: Global gene expression of energy responsive genes in *hspro* mutants

A subset of genes differentially expressed in at least 1 of the 3 *hspro* mutants (but not necessarily all of them) was compared to genes differentially expressed under varying energy status. Cluster A genes are repressed in *hspro* mutants and sugar induced/starvation repressed, cluster B genes are induced in *hspro* mutants and sugar repressed/starvation induced, while cluster C genes are repressed in the mutants but sugar repressed/starvation induced and cluster D genes are induced in the mutants and sugar induced/starvation repressed. KIN10 target genes are in bold with an asterisk at the end of gene IDs.

Table 4.7: Summary of gene differential expression in the cluster data

	Percentage of cluster gene list significantly differentially expressed in *hspro* mutants			
Mutant	**A**	**B**	**C**	**D**
hspro1-2	23	43	2	31
hspro2	97	57	83	62
hspro1-2/hspro2	30	60	33	38

Interestingly, promoter content analysis revealed that both I-box and G-box promoter motifs were enriched for in both clusters B and C while the ABRE-like binding site motif was enriched for in cluster B (Table 4.8) This enrichment of sugar-responsive promoter elements was not unexpected in a subset of energy related genes and highlights the likelihood of *HSPRO* genes being involved in energy status response or regulation. It should be noted that no promoter motifs were enriched for in clusters A and D.

Table 4.8: Enrichment of transcription factor sites in the promoters of the genes in clusters B and C

		Cluster B			Cluster C		
Motif	**Associated Process**	% of list with motif	#P	#S	% of list with motif	#P	#S
I-box promoter motif	Light responsive gene regulation	73	37	62	62	26	40
ABRE-like binding site motif	ABA-mediated stress signalling	49	25	37	-	-	-
G-box motif	Light responsive gene regulation	35	18	50	33	14	40
Evening Element promoter motif	Circadian control of gene expression	33	17	22	-	-	-
DREB1A/CBF3	Cold, drought and salinity stress response	-	-	-	24	10	10

P is the number of promoters with the particular TFBS and # S that is the number of times that the TFBS occurs in the cluster.

4.2.2.3 Glucose sensing, signalling and metabolism

The expression profiles of the *hspro* mutants suggested that the mutants may be defective in energy perception or signalling and so we decided to evaluate two hypotheses that are not necessarily mutually exclusive. Mutant plants might have altered expression of energy response genes either because of an inability to perceive and/or respond to energy levels or they may be compromised in carbohydrate metabolism, and the gene expression patterns observed may in fact reflect altered carbohydrate status of the plant.

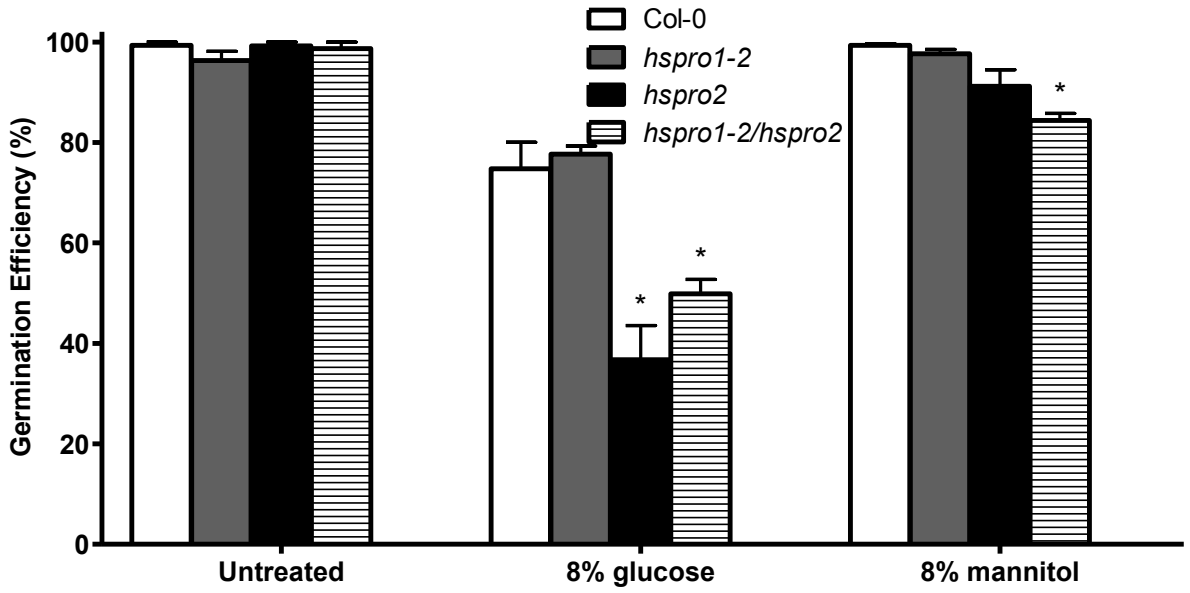
hspro2 and hspro1-2/hspro2 mutants are hypersensitive to exogenous glucose

Glucose is known to inhibit seed germination in *Arabidopsis* and mutants defective in glucose perception or signalling have altered germination responses to exogenous glucose (Dekkers *et al.*, 2004; Rognoni *et al.*, 2007; To *et al.*, 2002; Ullah *et al.*, 2002). In order to test whether the *hspro* mutants were defective in carbohydrate signalling, germination assays were performed on glucose, with mannitol as the osmotic control (Morita-Yamamuro *et al.*, 2004; Rognoni *et al.*, 2007). *hspro2* and *hspro1-2/hspro2* plants appear to be glucose hypersensitive and germinate at a delayed rate compared to the wild type and *hspro1-2* (Figure 4.12). At this concentration and number of hours after germination the mannitol grown plants behaved as the wild type except for *hspro1-2/hspro2* at the radicle stage (Figure 4.12A). Lack of functional *HSPRO2* appears to cause an increased sensitivity to glucose and perhaps HSPRO2 is important in regulating the response to glucose during germination.

PR1 and DIN6 expression in hspro mutants under high and low energy conditions

Given that the germination experiments suggested that *hspro2* mutants may be hypersensitive to glucose, we decided to test for any evidence that glucose signalling is altered in mature plants. There are two established branches of glucose signalling in plants, HXK1-dependent or HXK1- independent and gene expression analysis was utilised to determine if there were any perturbations in these signalling pathways in the *hspro* mutants. *PR1* is not only a hallmark gene for SA-mediated plant defence but it has also been shown to be glucose inducible in a HXK1-dependent manner (Jossier *et al.*, 2009; Xiao *et al.*, 2000). As expected *PR1* was induced in the wild type plants following glucose treatment (Figure 4.13). However, there was no statistically significant misregulation of *PR1* expression in any of the mutants and it appears knocking out either or both of the *HSPRO* genes does not result in the misregulation of glucose-driven *PR1* expression.

A.



B.

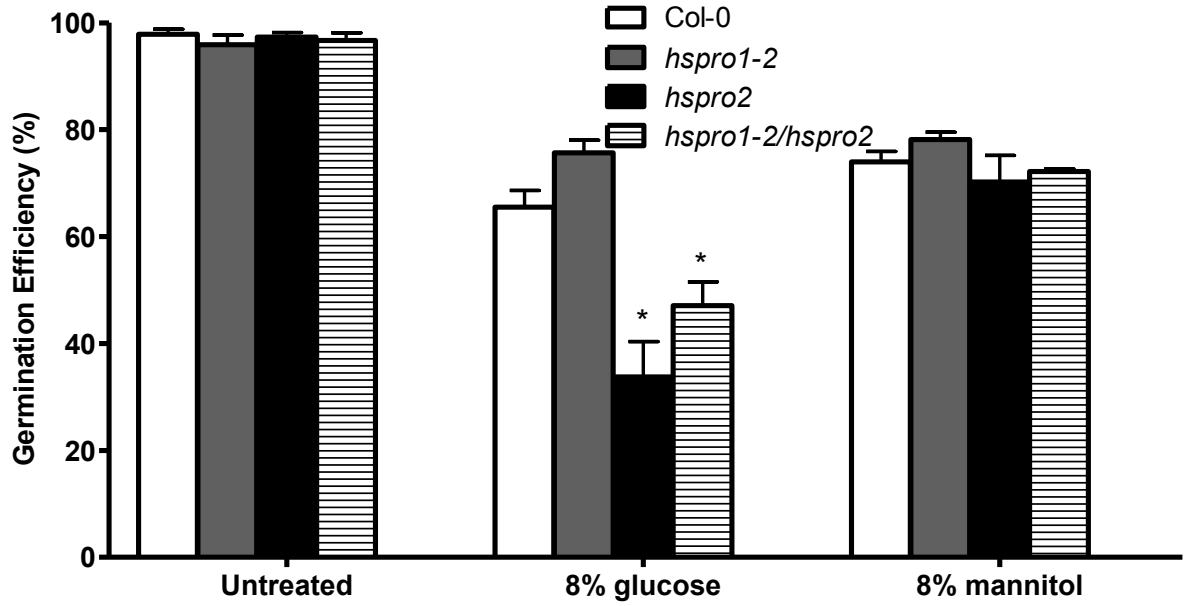


Figure 4.12: Germination response of seeds sown on sugars and mannitol.

Seeds were sterilised and resuspended in 0.1% agar and stratified at 4°C, in the dark for 3 days, before being sown onto standard 0.5 x MS agar or agar containing the specific sugar or osmotic control. Germination counts were performed 192 h (A - radicle emergence) and 240 h (B – cotyledon expansion) after transfer to normal growth conditions. N = 3 plates with 100 seeds per line and significance was assessed for using the Student's t-test. '*' = $p < 0.05$. This data is representative of two experiments.

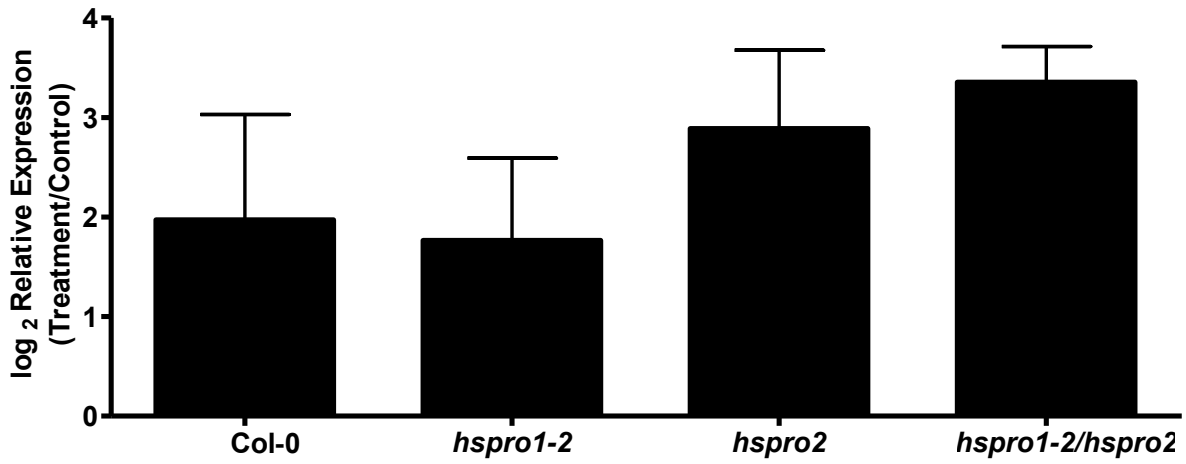


Figure 4.13: *PR1* gene expression following treatment with glucose.

Seeds were germinated on solid 0.5 x MS medium with 0.2% glucose and grown for two weeks under standard conditions. The 2-week-old seedlings were transferred into liquid 0.5 x MS (0.2% glucose) and allowed to acclimate. 24 h later the plants were treated with 0.5 x MS media containing either 0.2% or 6% glucose and incubated for 25 h. Leaf tissue was collected after 1 h after dawn. Gene expression was examined using RT-qPCR and normalised to *ACT2* expression. Error bars represent the S.E.M (n = 3 biological pools of 10 seedlings each). Statistically significant deviation from wild type expression levels was tested using the Student's t-test. This data is from one experiment only.

Dark inducible (*DIN*) genes are activated under sugar and energy limiting conditions such as darkness and flooding and are repressed by exogenous sugars and light in a HXK1-independent manner. *DIN6* is a target of KIN10 that has been shown to be up-regulated in plants over-expressing *KIN10* versus the wild type and repressed under energy-rich conditions (Baena-González *et al.*, 2007). To evaluate if the *hspro* mutants were defective in KIN10-driven energy signalling, *DIN6* expression in the mutants was examined under both high and low energy conditions. Two-week-old plants grown under long day conditions were kept in standard conditions until dawn whereby the controls were exposed to normal light conditions and 3 different populations were transferred to either light/glucose, extended dark/glucose or extended dark/no glucose conditions for 6 hs. Gene expression was expressed relative to the expression in the control plants and as previously reported *DIN6* was up-regulated under extended dark conditions while the presence of 0.45% glucose abolished the dark induction of expression (Figure 4.14) (Baena-González *et al.*, 2007). While *hspro1-2* mutants behaved in a manner similar to Col-0, in both *hspro2* and *hspro1-2/hspro2*, *DIN6* induction under extended dark conditions was significantly lower than in Col-0, and a combination of both extended dark and 0.45% glucose resulted in a strong repression of *DIN6* expression (relative to control plants under light minus glucose conditions) not observed in *hspro1-2* and wild type plants (Figure 4.14). *DIN6* was strongly repressed in response to a combination of both light and 0.45% glucose in all 4 genotypes, suggesting that *hspro2* and *hspro1-2/hspro2* are not defective

in glucose signalling *per se*. Instead the altered expression pattern of *DIN6* observed in *hspro2* and *hspro1-2/hspro2* is consistent with a failure of these mutants to perceive or respond normally to extended night.

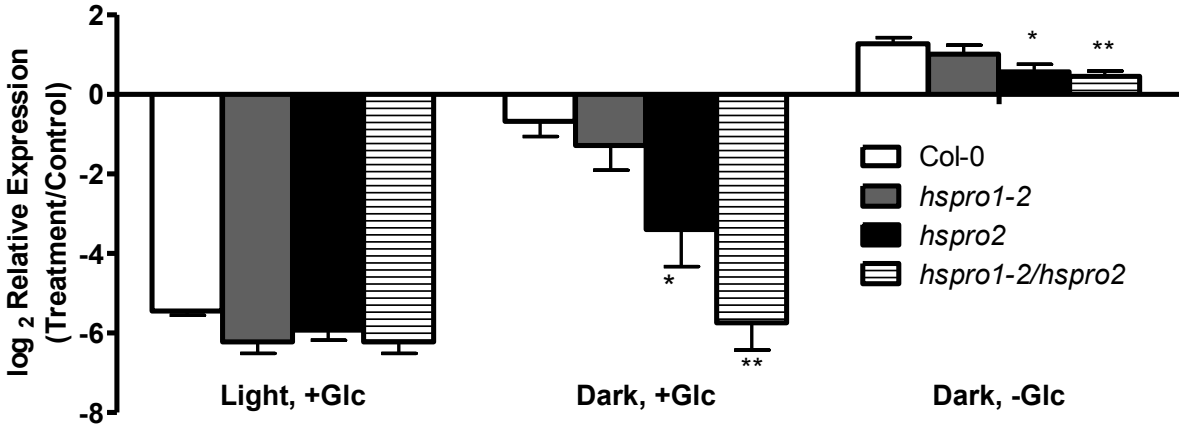


Figure 4.14: *DIN6* gene expression under energy-rich and -limiting conditions.

Two-week-old seedlings grown in liquid 0.5 x MS media (after stratification) under long day light conditions were either kept in normal conditions or treated with extended dark for 6 h, with or without 0.45% (25 mM) glucose (Glc). RNA was extracted from leaf tissue and *DIN6* gene expression was examined using RT-qPCR and normalised relative to *ACT2* expression. Gene expression levels following treatment were normalised to expression levels in the untreated control (light, - glucose) plants per line. Error bars represent the S.E.M (n = 3). Statistically significant deviation from wild type expression levels was tested using the Student's t-test. * represents $p < 0.05$ and ** = $p<0.01$. This data is representative of two experiments.

hspro2 may have altered carbohydrate content

It is possible that the altered regulation of sugar responsive genes in *hspro1-2*, *hspro2* and *hspro1-2/hspro2* was a result of altered endogenous sugar levels in the mutant plants caused by the absence of *HSPRO1* and *HSPRO2* rather than defectiveness in sugar signalling. To test this alternative hypothesis, glucose, sucrose and starch levels were measured at dawn and at dusk. Sugar levels were consistently lower at the end of the night period compared to the end of the day as was expected under normal growth of *Arabidopsis*. While average levels of glucose, sucrose and starch appeared to be lower in the *hspro2* mutants (particularly at dawn), these differences were not statistically significant. In addition, this trend was not seen in the *hspro1-2/hspro2* mutant, which might be expected to show the same phenotype, given the high percentage of energy-status responsive genes which are differentially expressed in both *hspro2* and *hspro1-2/hspro2* (Figure 4.10 and Table 4.7). However, a high degree of variation in carbohydrate levels was observed between the small number of biological repeats in this experiment (see S.D. in Figure 4.15) and so this data must be regarded as preliminary. Re-analysis of

carbohydrate content of a large number of samples using gas chromatography–mass spectrometry (GC-MS) would potentially generate more reliable data.

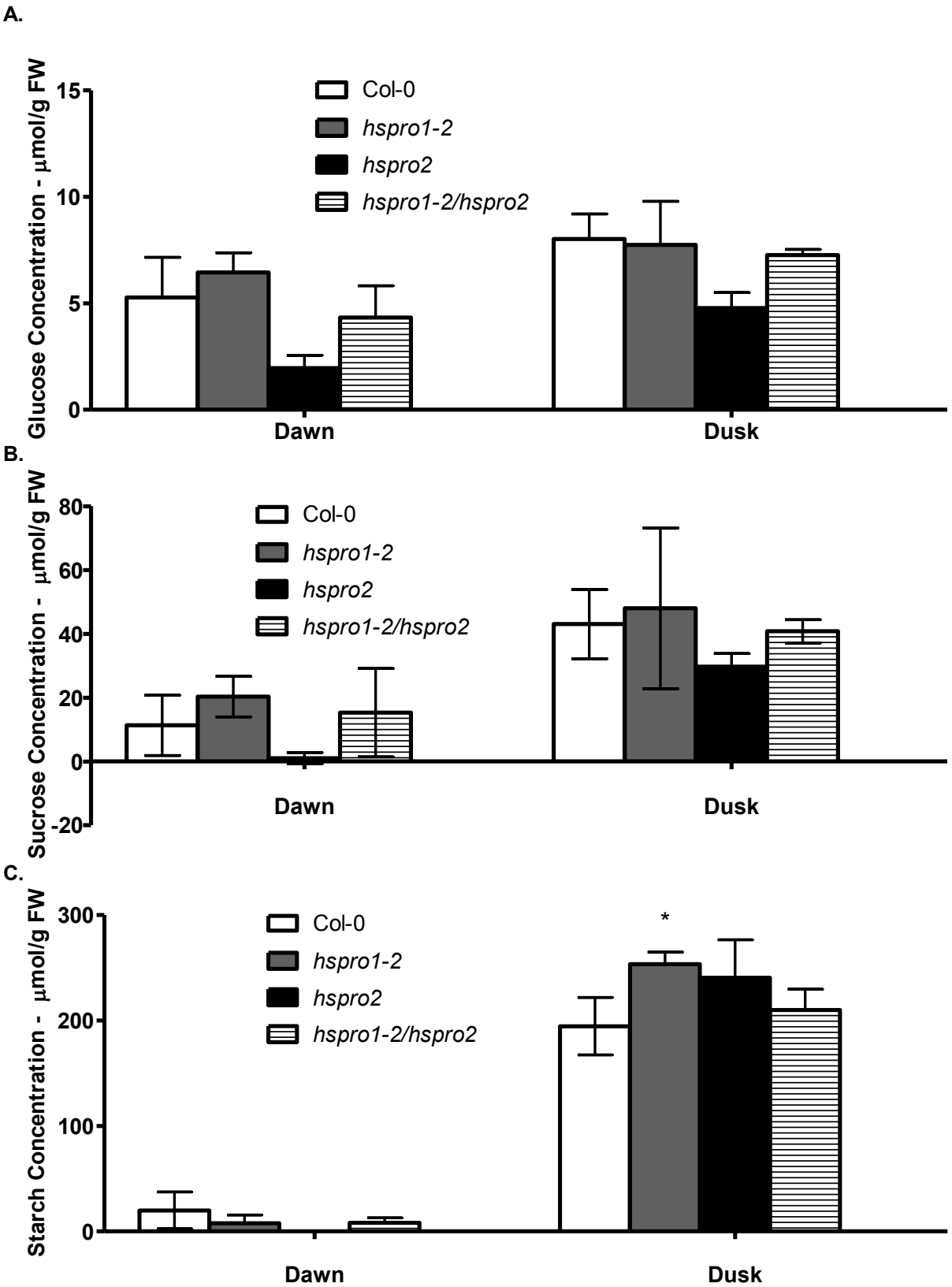


Figure 4.15: Carbohydrate content of *Arabidopsis* wild type and the mutants at the beginning and the end of the subjective day.

Seeds were germinated on soil and grown for 4 weeks under 16 h light/12 h dark cycles. Single leaves from a minimum of 6 plants were harvested and pooled at dawn and dusk on the same day. The 3 carbohydrates under investigation were extracted from the plant tissue and quantified. N = 3 separate pools and the error bars represent the standard deviation. This data is representative of only one experiment.

4.2.3 HSPRO function and the circadian clock

As previously discussed in chapter 1, numerous sugar-responsive genes display a diurnal pattern of expression that mimics the internal carbohydrate concentrations across the photoperiod (Bläsing *et al.*, 2005) and it is thought that these fluctuations in sugar levels drive the diurnal gene expression. The circadian clock is also an input into the regulation of diurnal gene expression and sugar levels can enhance this regulation. Furthermore, sugars have also been shown to alter the expression of circadian regulated genes (Bläsing *et al.*, 2005). Circadian rhythm and photoperiodism terms are enriched for in the GO analysis of the *hspro* array data, while circadian and light regulated promoter motifs are enriched in the *hspro* microarray data. This combined knowledge directed inquiry into the possible involvement of *HSPRO* genes in diurnal and circadian regulatory function in *Arabidopsis*.

4.2.3.1 HSPRO2 expression follows a diurnal pattern

Following on the idea that *HSPRO2* is dark inducible, repressed by sugars during carbon fixation – processes that coincide with the end of night and beginning of the day - it was decided to investigate the potential diurnal regulation of gene expression. The online resource DIURNAL was utilised in this investigation, whereby both *HSPRO1* and *HSPRO2* gene IDs were queried in the database and an output of expression profiles generated. Using the stringency recommended by the tool, only *HSPRO2* was determined to display a diurnal regulatory pattern as is illustrated in Figure 4.16. *HSPRO2* expression consistently peaks towards the end of the night period under short, long or half day conditions and in short or long day conditions expression gradually lowers during the day finally reaching the lowest level at dusk. However, in half day conditions the *HSPRO2* repression is more extreme and expression levels are at their lowest only 4 h after dawn and are maintained here until just before the end of the night period. The temperature across all 3 of these data profiles was constant eliminating the effects of temperature changes on these observed diurnal patterns. The Pearson‘s correlation coefficients comparing the observed expression profiles and the predicted expression models were strongly positive reinforcing the robustness of these diurnal rhythms.

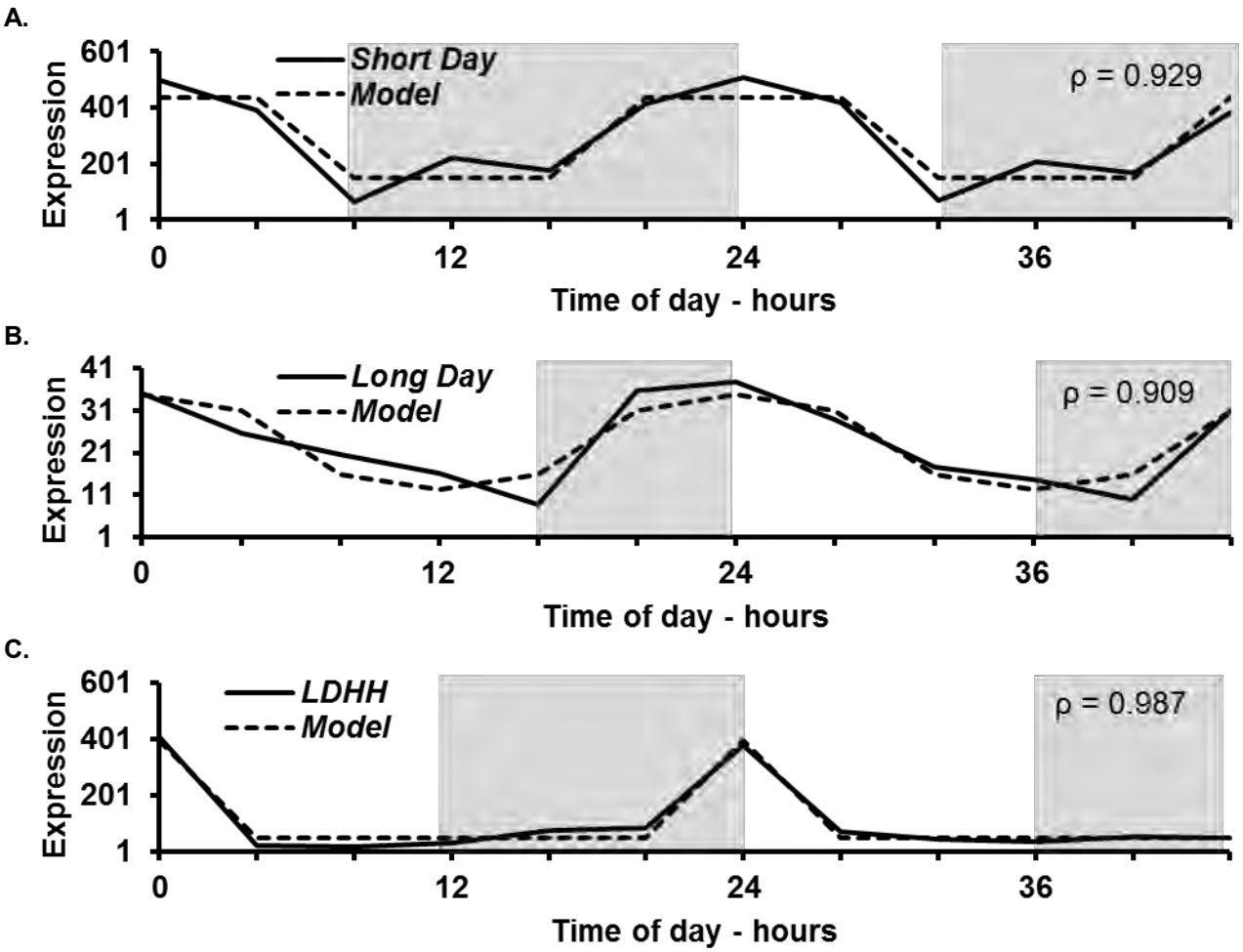


Figure 4.16: Diurnal *HSPRO2* gene expression under varying photoperiods.

Normalised data from the DIURNAL tool for short day plants (A), long day plants (B) and 12h light/12 h dark plants (C). — is actual gene expression and **----** the predicted gene expression model. ρ = Pearson's correlation coefficient between actual gene and model expression profiles. White bars are light and grey bars are dark conditions.

4.2.3.2 HSPRO2 expression appears to be circadian regulated

Luciferase reporter genes are a long established tool used in investigating temporal regulation of genes without having to perform intensive RT-qPCR expression profiling (Nakamichi *et al.*, 2004). To confirm the temporal regulation of the *HSPRO2* promoter the 1.5 kb region directly upstream of the ATG start codon was amplified via PCR and cloned into the pART27 binary vector via *SacI* and *EcoRI* restriction sites. The pART27 vector affords one the opportunity to investigate promoter regulation since whatever promoter sequence is cloned into the vector drives the expression of luciferase, whose activity can be visualised via the luciferin-luciferase reaction, *in vivo*. Transgenic lines were identified via kanamycin selection on 0.5 x MS agar and progressively self-fertilised until homozygous T_3 lines displaying luciferase activity and completely resistant to the antibiotic were isolated. Plants were entrained in 12 h light/ 12 h dark days and either kept in half

day conditions or transferred into constant light at the start of promoter activity measurements. Plants transferred to constant light still displayed a diurnal rhythm similar to those maintained in 12 h light/ 12 h dark light conditions, suggesting a circadian input into the diurnal regulation of *HSPRO2* promoter activity. Regardless of light conditions, the phase for *HSPRO2* promoter activity seems to be around 2 h and the promoter-driven luciferase expression is repressed during subjective day and induced at night. There was a discrepancy between the diurnal expression profile generated via luciferase activity and that generated from the DIURNAL tool under 12 h light/12 h dark conditions. *HSPRO2* promoter activity appeared to be more rapidly responsive to dark conditions (Figure 4.17) than DIURNAL gene expression which was only induced 4 h before dawn (Figure 4.16).

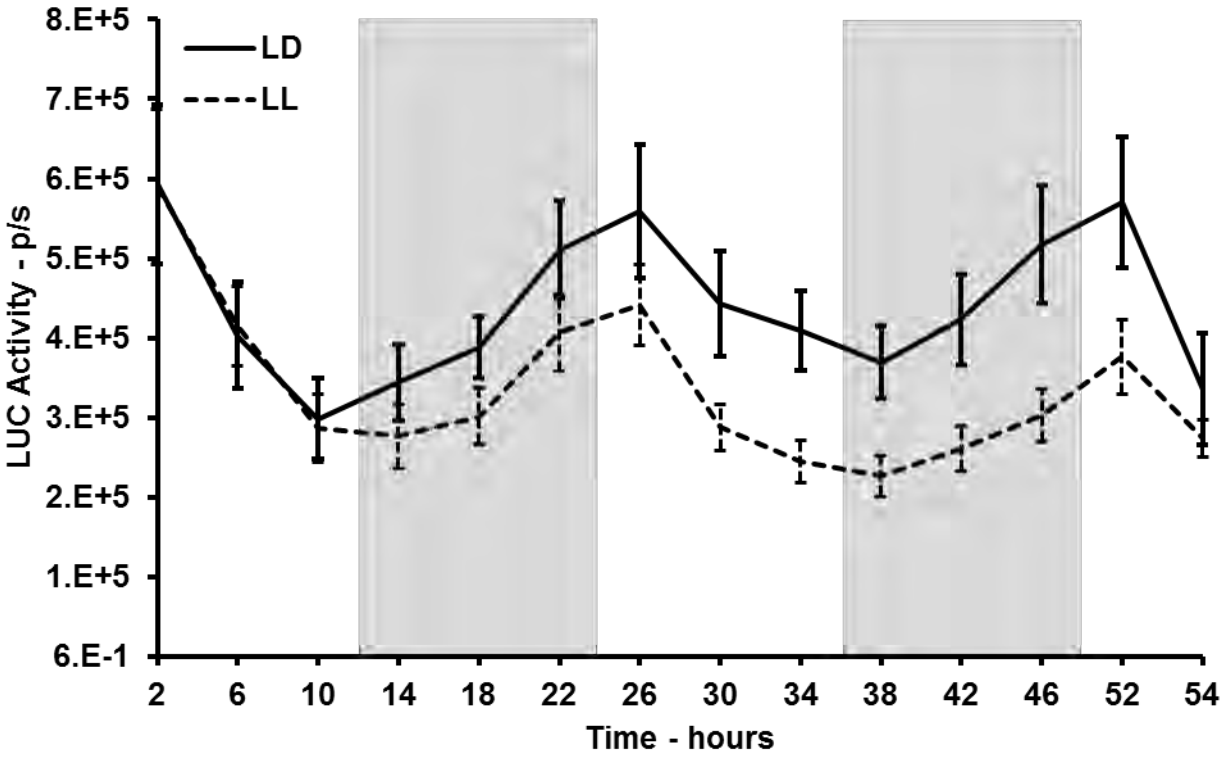


Figure 4.17: Circadian response of *HSPRO2::LUC* to constant light conditions

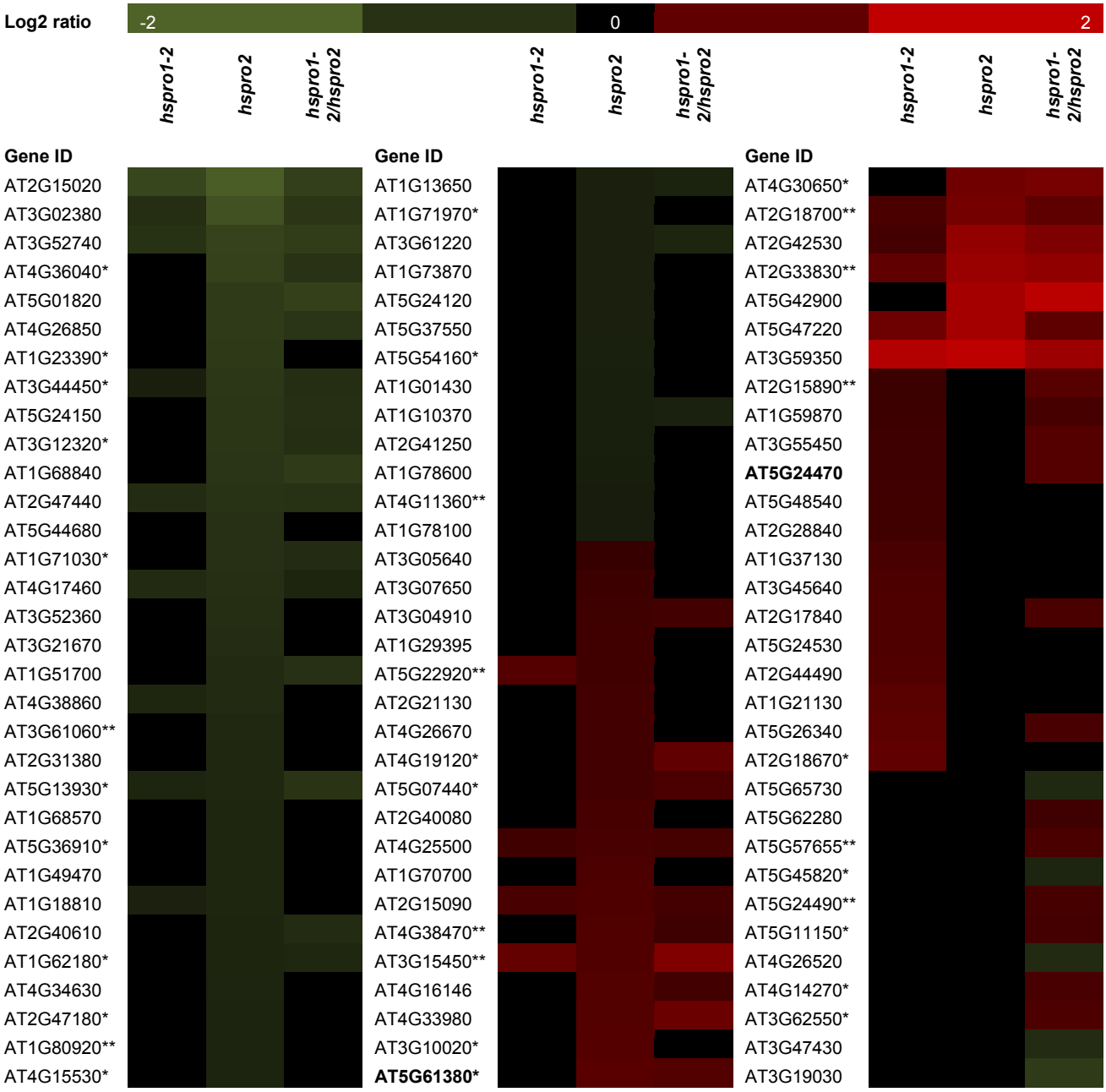
Two week old seedlings entrained in 12 h light/12 h dark light conditions were either kept at control conditions (LD —) or transferred to constant light conditions (LL ---) and promoter activity monitored via luciferase imaging every 4 h over a 56 h time period, starting 2 h after dawn. The white bars represent subjective day and grey bars subjective night. N = 30 and the error bars represent the S.E.M. This data is representative of two experiments.

4.2.3.3 Circadian clock regulated genes misregulated in the mutants

Having established that *HSPRO2* expression is diurnally and circadian clock regulated we decided to investigate whether disruptions in *HSPRO* gene functionality compromised the circadian clock. An initial look at the potential role of *HSPRO* genes in clock regulation

involved looking at the differential expression of known circadian clock genes (Covington *et al.*, 2008) in the *hspro* mutant arrays (Table 4.9). *hspro2* and *hspro1-2/hspro2* had the highest total number of circadian related genes (Table 4.10). Approximately 25% of the genes differentially expressed in *hspro2* and *hspro1-2/hspro2* and 20% in *hspro1-2* were known circadian clock regulated genes. In *hspro2* 60% of these genes were repressed while 56% of the *hspro1-2/hspro2* genes circadian genes were induced. Knocking out *HSPRO* gene expression appeared to cause disruption in the expression of a subset of known circadian clock regulated genes in *Arabidopsis* potentially implicating *HSPRO* in the regulation of circadian clock dependent gene expression.

Table 4.9: Clock regulated genes that are differentially expressed in *hspro* mutants



Genes with one asterisk were identified as energy responsive (Figure 4.10) and genes with two asterisks are also KIN10 targets (Figure 4.9) in addition to being energy responsive. *TOC1* and *PRR5* are highlighted in bold.

Table 4.10: Summary of circadian regulated genes

	hspro1-2		*hspro2*		*hspro1-2/hpro2*	
	total	**%**		**%**		**%**
mutant gene list	205		382		256	
down	169	82	178	47	148	58
up	36	18	204	53	108	42
Circadian clock and mutant overlap	36	18	91	24	71	28
down	11	31	55	60	31	44
up	25	69	36	40	40	56
Energy, clock and mutant overlap	10	5	34	9	26	10

4.2.3.4 Phenotypic analysis of circadian clock disruption

The circadian clock regulates numerous developmental events including leaf movement (McClung, 2006), flowering time (Ortiz-Marchena *et al.*, 2014) and hypocotyl elongation (Niwa *et al.*, 2009). Additionally, flowering time and hypocotyl elongation are dependent on the length of the photoperiod and mirror each other under similar conditions (Niwa *et al.*, 2009). Classical methods to determine disruption in clock mechanisms involve looking at altered leaf movement patterns, hypocotyl elongation and flowering time phenotypes in candidate clock mutants. Considering that *HSPRO2* expression shows a circadian pattern, there is enrichment of circadian regulation motifs and circadian GO terms in the *hspro1-2* and *hspro2* microarray data, and circadian clock regulated genes are misregulated in the *hspro* mutants we investigated the possibility that knocking out the two genes disrupts the circadian clock in *Arabidopsis*.

Leaf movement analysis

A simple approach to identifying clock mutants is to analyse movement patterns of actively growing and developing young plant leaves. *Arabidopsis* leaves are supported by the petiole and rhythmic oscillations in the abaxial and adaxial cells of these organs cause the leaf to move up and down in a predictable and consistent manner. Wild type plant leaves lowered during the day and moved up during the night, peaking just after dawn as expected (Figure 4.18). All three mutants behaved similarly to wild type plants as mutant period lengths were near identical to the wild type (Table 4.11). Relative amplitude error is a measure of the robustness of a rhythm with values ranging from 0 to 1. A RAE value of zero is indicative of a perfect and robust cosine wave while values tending towards 1 approach the limits of statistical significance (Michael & McClung, 2002). In this experiment, all the mutant lines had very strong rhythms with RAE values of approximately 0.11. These data indicate that there is no break in rhythmicity in the absence of either one or both of the genes. It is interesting though that the phase of leaf movement is different in the mutants when compared to Col-0 plants and this translates to possible altered regulation of upward leaf movement in the mutants. This upward leaf movement is called hyponastic growth and is responsible for leaf movement in response to challenging environmental cues and involves overlaps in ethylene, ABA, JA and SA signalling (Benschop *et al.*, 2007; Polko *et al.*, 2013). Future work could investigate disruptions in hyponastic growth in the mutants.

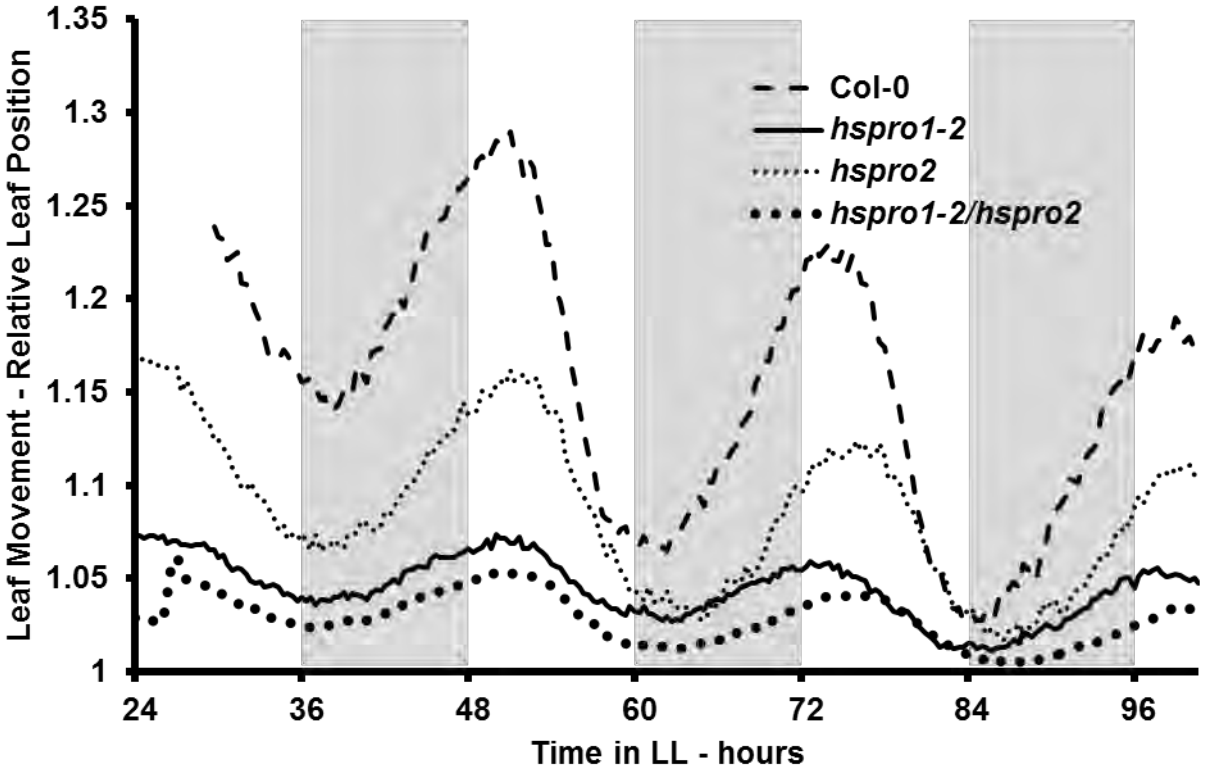


Figure 4.18: Leaf movement analysis of the mutant lines versus the wild type.

Seeds were stratified for three days, germinated and grown on standard 0.5 X MS agar media for four days under 12 h light/12 h dark conditions. On day five the light conditions were changed to constant light (LL) and leaf movements recorded for four days. The curves are average plots of 20, 12, 16 and 21 replicates for Col-0, *hspro1-2, hspro2* and *hspro1-2/hspro2* respectively. White and grey bars represent subjective day and night respectively. This data is representative of one experiment.

Table 4.11: Summary of rhythmicity

		Col-0	***hspro1-2***	***hspro2***	***hspro1-2/ hspro2***
Period	*Weighted Mean*	26.14	26.14	26.46	26.50
	Weighted S.E.M	0.11	0.15	0.22	0.16
Relative Amplitude Error (RAE)	*Weighted Mean*	0.11	0.12	0.11	0.11
	Weighted S.E.M	0.00	0.01	0.01	0.00

Hypocotyl elongation

Hypocotyl growth has been shown to have a diurnal rhythm that is largely dependent on photoperiodic and circadian clock cues and mutants with altered hypocotyl elongation rates and patterns have often been utilised to identify clock mutants and infer gene function in circadian clock regulation (Dowson-Day & Millar, 1999; Niwa *et al.*, 2009; Nozue *et al.*, 2007; Nusinow *et al.*, 2011). Along with circadian clock terms the *hspro* mutant transcriptomics study revealed enrichment of terms and promoter motifs

associated with light perception, photoperiodism and response to dark. GO terms associated with this theme are enriched for in the *hspro1-2*, *hspro2* and *hspro1-2/hspro2* microarray FATIGO analysis. Certain DNA promoter motifs – including but not limited to the G-box and I-box motifs - have been shown to be intrinsic to the induction of transcription of light responsive genes (Hudson & Quail, 2003). 2 copies of the I-box motif are present in the putative promoter of *HSPRO1* while *HSPRO2* has one copy in its promoter. Interestingly, the *HSPRO2* ECGG is enriched for I-box promoter motifs and the *hspro2* ECGGs and *hspro1-2/hspro2*-induced ECGG are G-box motif enriched. With this background knowledge, *HSPRO* genes were evaluated for potential involvement in the photoperiod and circadian clock dependent control of hypocotyl growth under differing light conditions. *Arabidopsis* Col-0 and *hspro* mutant seedlings were germinated under varying light conditions and grown for seven days. As expected, the wild type hypocotyls that grew in the absence of light were etiolated and significantly more elongated than both the short/long day plants (Figure 4.19). However, there was no discernible difference between the growths of the knockout mutant plants versus wild type regardless of treatment, signifying that HSPRO1 and HSPRO2 are not required for circadian clock and light dependent hypocotyl elongation.

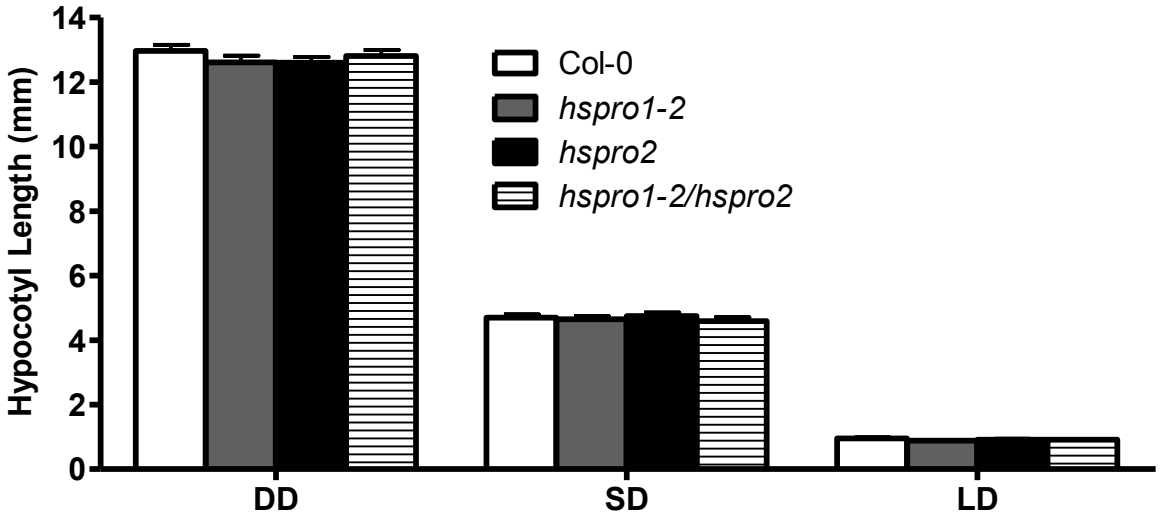


Figure 4.19: Photoperiod dependent hypocotyl growth.

Seeds were surface sterilised, sown on 0.5 x MS agar and stratified at 4^{o}C for 3 days before being transferred to the growth chambers. Constant dark (DD) plates were covered in aluminium foil, short day plants (SD) were grown in 8 h light/16 h dark conditions, long day (LD) plants were grown in 16 h light/8 h dark and all plants were grown vertically at 22^{o}C for 7 days. Hypocotyl measurement was conducted in ImageJ. The data represents the means and S.E.M from at least 25 biological replicates. Statistical significance was evaluated via the Student‘s t-test. This data is representative of two experiments.

Plant flowering time

Being able to detect seasonal changes in day length is important in regulating and inducing seasonally appropriate flowering time. *Arabidopsis thaliana* is a facultative long day plant whose flowering time is delayed in short day conditions. Clock and photoreceptor mutants have been shown to have altered flowering time and flowering time is also influenced by carbon availability (Ortiz-Marchena *et al.*, 2014). A final attempt at evaluating the involvement of *HSPRO* genes in circadian clock function involved assessing the flowering times of the mutants under different photoperiods. The *hspro* mutants and Col-0 plants were grown on soil under either long or short days and leaf counts monitored. Leaf counts at flowering time and the time to flowering itself in the wild type were both increased in short day conditions as expected. However, there was no significant difference between the flowering times of the wild type and the mutants. It would appear *HSPRO1* and *HSPRO2* are not essential for photoperiodic and circadian regulation of flowering time.

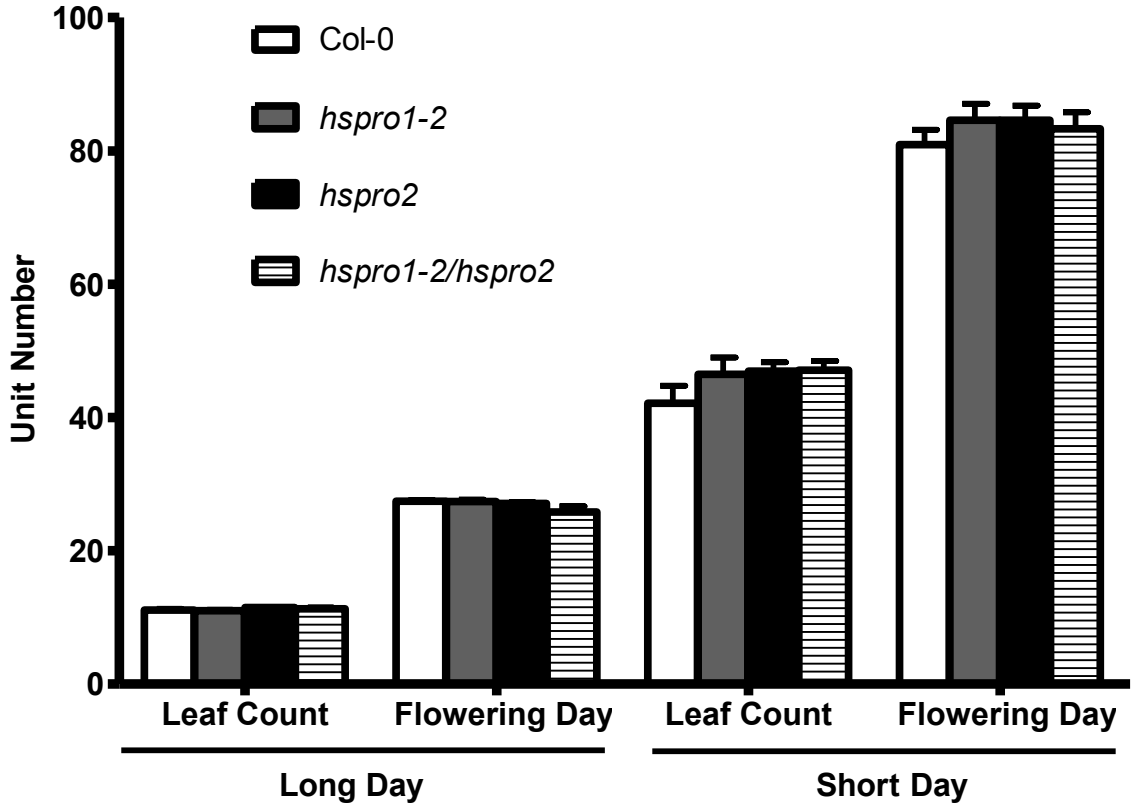


Figure 4.20: Day length dependent flowering time.

The flowering time of *Arabidopsis* plants was monitored under long day (16 h light/8 h dark) and short day (8 h light/16 h dark) conditions. Flowering time was measured as the number of days it took for the first flower to bloom per plant and also as the number of rosette leaves at flowering time. This data is representative of one experiment.

4.3 DISCUSSION

In this chapter transcriptome profiling was used to identify other biological processes *HSPRO* genes may be involved in *Arabidopsis*. GO analysis and promoter content analysis of differential expression gene lists in the *hspro* mutants revealed enrichment of genes and TF binding sites associated with various biological processes. These processes included responses to a/biotic stress and stress signalling hormones. Additionally, GO terms and TF sites associated with carbohydrate responses, signalling and transport, and circadian gene function were also enriched for providing insights into novel biological processes *HSPRO* genes may be involved in. Gene expression and mutant phenotype analysis supported some of the hypothesis generated from the transcriptome data and these results support roles for *HSPRO2* in energy perception and/or signalling.

4.3.1 HSPRO genes potentially have overlapping and unique effects on gene regulation in Arabidopsis

Microarray analysis of gene expression profiles in the *hspro* mutants growing under standard conditions revealed interesting insights into the functionality of the *HSPRO* genes. Knocking out the *HSPRO2* gene alone appeared to have a greater impact on gene misregulation than knocking out either *HSPRO1* alone or knocking out both *HSPRO* genes (Figure 4.3). There were observed overlaps in gene expression amongst the three mutant expression profiles and typically genes that were misregulated in more than one mutant behaved the same across all mutants and perhaps this is indicative of potential redundancy in the regulation of a subset of genes. However, there were also a number of genes that were misregulated in specific mutants only – with *hspro2* having more than double of either *hspro1-2* or *hspro1-2/hspro2* specific differentially expressed genes – suggesting that although there appear to be overlaps in *HSPRO*-dependent gene regulation, the *HSPRO* genes may also be involved in unique processes independent of each other.

4.3.2 A role for HSPRO2 in energy sensing and signalling

GO functional analysis of *hspro* mutant data revealed that *HSPRO* genes may be involved in carbohydrate perception or signalling. Furthermore, *HSPRO1* and *HSPRO2* are known to interact with the βγ sub-unit of the SnRK1 complex which plays a central role in energy and stress signalling (Gissot *et al.*, 2006). Plant mutants or ecotypes with alterations in sugar regulation can display sugar specific phenotypes such as altered germination

efficiency or aberrant growth morphology and since the *hspro2* mutation results in a hypersensitivity to glucose during germination, it was hypothesised that *HSPRO2* may be involved in sugar detection or signalling.

4.3.2.1 HSPRO2 may be involved in the regulation of extended dark responses

Transcriptome analysis revealed overlaps in *hspro2* gene misregulation and several sugar availability based analyses. Cluster analysis revealed two sets of expression patterns with one set of data showing energy responsive genes in *hspro2* displaying a pattern of expression mimicking energy starved plants (Figure 4.11 A and B) and the other set showed *hspro2* behaving as plants responding to high sugar levels would (Figure 4.11 C and D). Since *HSPRO2* promoter activity was up-regulated at night (Figure 4.17) and microarray analysis suggested the gene was starvation inducible it is tempting to conclude that *HSPRO2* expression is required for extended dark period starvation responses. *DIN6*, a marker for extended dark responses appears to be misregulated in the *hspro2* and *hspro1-2/hspro2* mutants following extended dark treatment (in the presence or absence of exogenous glucose) implicating *HSPRO2* in the extended dark-dependent induction of *DIN6* expression. Looking at *PR1* (Figure 4.13) and *DIN6* (Figure 4.14) expression, it appears that HXK1-dependent signalling is unperturbed in the absence of *HSPRO2* but the SnRK1-dependent extended dark response potentially requires HSPRO2 for signal transduction. It is tempting to speculate that perhaps *HSPRO2* is important only during starvation as *hspro2* and *hspro1-2/hspro2* fail to perceive the low energy status experienced during extended dark but can perceive exogenous glucose. However, looking at the gene expression profiles in (Figure 4.11 C and D) whereby the *hspro* mutants behaved as if they were growing under high energy conditions it appears that HSPRO2 may also be required for high energy signalling. The role of HSPRO2 in KIN10-dependent energy signalling is not yet clear and further experimentation is required before more solid conclusions can be made. This could include investigating the expression patterns of the other KIN10 targets described in chapter 4 (Figure 4.9) in the *hspro* mutants.

The SnRK1 complex in plants is known as a central regulator of energy and stress signalling. Nutrient deprivation and stress affect energy levels and SnRK1 is utilised to regulate response mechanisms to stress conditions (Baena-González & Sheen, 2008). There is evidence for the repression of SnRK1 by sugars and induction during starvation across several species including *Arabidopsis,* spinach and sugar cane (Crozet *et al.*,

2014). *HSPRO2* expression across sugar limiting or abundant conditions is very typical of KIN10 target genes. KIN10 is a catalytic α sub-unit of the SnRK1 complex and since HSPRO2 interacts with the regulatory sub-unit AKINβγ it is possible that the SnRK1 complex regulates starvation responses (such as *DIN6* induction) through *HSPRO2* expression and function and perhaps there is a feedback loop whereby KIN10 induces *HSPRO2* and HSPRO2 interacts with the AKINβγ subunit to regulate KIN10 activity. Assessment of KIN10 protein kinase activity and the expression of the KIN10 gene targets misregulated in the *hspro* mutants versus Col-0 could possibly provide more insights into the biological functions of HSPRO in energy signalling.

Many of the energy responsive genes - including *DIN6* - that are misregulated in *hspro2* plants during normal growth have conserved G-box and I-box motifs in their promoters. Since HSPRO2 does not appear to contain any nuclear localisation sequences or DNA binding domains it is unlikely that it directly regulates gene expression and future work could investigate if HSPRO2 indirectly regulates gene expression via interaction with other proteins such as TFs.

4.3.2.2 Diurnal expression of HSPRO2 appears to be circadian clock driven

It is well established that plant gene expression can be regulated in response to light/dark cycles and many life-driving processes are important in specific phases of day or night period. Plant sugars are known to fluctuate according to the time of day with maximal levels attained at the end of the light period, followed by a linear, near-complete depletion of sugars during the night (Graf *et al.*, 2010). Regulation of sugar levels has huge implications on the fitness of the plant since limited sugar availability can have a negative effect of vegetative growth and it is thought that these diurnal sugar fluctuations drive the expression of a subset of diurnally expressed genes (Bläsing *et al.*, 2005). Alterations in carbon availability induce and repress global changes in plant transcriptomes and metabolism. Low sugar levels result in the repression of polysaccharide biosynthetic processes and other anabolic processes that utilise sugars such as starch and protein synthesis. Starvation also induces catabolic processes that salvage soluble sugars in an attempt to regulate the carbon balance (Thimm *et al.*, 2004; Thum *et al.*, 2004; Usadel *et al.*, 2008). Exogenous sugar supply or an abundance of endogenous sugar is typified by a reversal of these responses and so energy responsive genes typically display opposing responses under opposing conditions. Short periods of sugar starvation – extended dark

periods for instance – can cause rapid repression of plant growth and so many sugar regulated genes are activated before the end of the night period to pre-empt the effects of starvation (Bläsing *et al.*, 2005).

HSPRO2 expression appears to be diurnally regulated and is induced under dark conditions and expression peaks around dawn. Shortly after daybreak gene expression is repressed and this repression is inversely correlated with an increase in sugar levels. Microarray data showed that *HSPRO2* expression is specifically energy responsive as gene expression is repressed during CO_2 fixation during the light period – which leads to an increase in internal sugar levels - but is not repressed in plants growing in light and less than 50 ppm CO_2 (no increase in photosynthesis derived carbohydrate levels) (Bläsing *et al.*, 2005). Furthermore, qPCR analysis revealed that *HSPRO2* expression is repressed following treatment with 6% glucose supporting this hypothesis that sugars levels input directly into the regulation of *HSPRO2* (Figure 4.10 B).

There appears to be a circadian regulation component of *HSPRO2* expression, as *HSPRO2* promoter activity maintains the diurnal pattern even under constant light and this is typical of many diurnally regulated genes (McClung, 2006; Schaffer *et al.*, 2001). To test whether the rhythmic expression of *HSPRO2* is driven by changes in endogenous sugars during the day, it would be interesting to measure luciferase activity of plants grown on exogenous sugar media or under energy limiting conditions (e.g. growth under the photosynthesis limiting conditions of <50 ppm CO_2) since misregulation of sugars in mutants such as *pgm* overrides circadian behaviour (Usadel *et al.*, 2008).

Even though *HSPRO2* expression appeared to be circadian regulated, the core clock genes *TIMING OF CAB EXPRESSION1* (*TOC1*) and *PSEUDO-RESPONSE REGULATOR5* (*PRR5*) were differentially expressed in the *hspro* array data and there was enrichment for GO terms associated with clock function in the *hspro2* array, *HSPRO2* itself does not appear to be essential for normal clock function as indicated by assessment of clock regulated phenotypes such as leaf movement, flowering time and hypocotyl elongation. Interestingly though, during leaf movement analysis the three mutants displayed phase changes in their leaf movement patterns implying altered hyponastic growth functions. Hyponastic growth is a response to adverse environmental conditions and simply put is upward leaf movement and since the *hspro* mutants have smaller phases

they also have shorter ranges of motion (Polko *et al.*, 2013). Hyponasty is under the regulation of the phytohormones ethylene, ABA, JA and SA and microarray GO analysis revealed that *hspro* mutants may have misregulated phytohormone signalling pathways. Future work could perhaps look into the involvement of the *HSPRO* genes in hyponastic growth (Benschop *et al.*, 2007; Polko *et al.*, 2013).

4.3.3 Summary

Microarray analysis showed that subsets of the energy signalling and circadian clock regulated genes are misregulated in the *hspro* mutants under standard conditions. Seed germination revealed that mutants lacking *HSPRO2* were increasingly sensitive to exogenous glucose while *DIN6* gene expression analysis in the *hspro2* and *hspro1-2/hspro2* mutants suggested a potential disruption in SnRK1-dependent signalling under extended dark conditions. Although inconclusive and unconfirmed, there was evidence for potential altered carbohydrate levels in the *hspro2* mutant and the combined energy signalling results suggest a previously undescribed role for *HSPRO2* in energy sensing and signalling. Although *HSPRO2* expression was also seen to be diurnally regulated through integration with the circadian clock, and some circadian genes are misregulated in *hspro2*, *HSPRO2* did not appear to be essential for circadian clock function.

CHAPTER 5

General discussion

CHAPTER 5: GENERAL DISCUSSION

The results presented here describe the first phenotypic characterisation of the *hspro1* mutant and the discovery of additional phenotypes for the *hspro2* mutant. Although several phenotypes have been revealed for *hspro* mutants in this thesis, a major limitation of this work is the fact that only one allele per mutant was characterised and therefore the conclusions made here are preliminary pending analysis of a second null allele for each gene.

The phenotypes of *hspro* mutants support roles for both *HSPRO* genes in abiotic and biotic stress responses in *Arabidopsis* and interestingly enough these roles appear to be antagonistic under certain conditions. HSPRO2 appears to be a positive regulator of PTI while HSPRO1 negatively regulates the defence response to virulent *P. syringae*. However, there is a reversal of roles during osmotic stress tolerance responses in the roots of *Arabidopsis* seedlings, with HSPRO1 and HSPRO2 behaving as positive and negative regulators of osmotic tolerance respectively. This potential antagonistic relationship has not been described before and interestingly during the defence response to *Pst*, knocking out both genes does not result in a disease phenotype at all while during osmotic stress responses, the *hspro2* mutation appears to be epistatic to *hspro1-2*.

Computational analysis also provided evidence for the involvement of the *HSPRO* genes in a/biotic stress responses as both *HSPRO1* and *HSPRO2* are co-regulated with genes involved in both types of stresses. Microarray analysis of *hspro* mutants also revealed additional evidence for possible regulatory functions of *HSPRO* in stress as genes involved in a/biotic stress responses are misregulated and enriched for in *hspro1-2* and *hspro2* mutants. The *hspro* microarray data and ECGG analysis also revealed a potential biological function of *HSPRO* genes in carbohydrate signalling and/or transport, as a subset of genes differentially expressed in the *hspro* mutants were also shown to be energy responsive. Phenotypic analysis revealed that *hspro2* and *hspro1-2/hspro2* seeds were increasingly sensitive to exogenous glucose while gene expression analysis showed that only *HSPRO2* expression was glucose responsive and that loss-of-function of *HSPRO2* may alter energy perception signalling during the dark period. *HSPRO2* expression may be of importance in carbohydrate metabolism and gene expression appears to be regulated not only by energy levels but also via the plant circadian clock. In summary, this study has identified several and novel phenotypes of *hspro* mutants that

can easily be tested and this has helped to establish the functions of *HSPRO* genes in *Arabidopsis*.

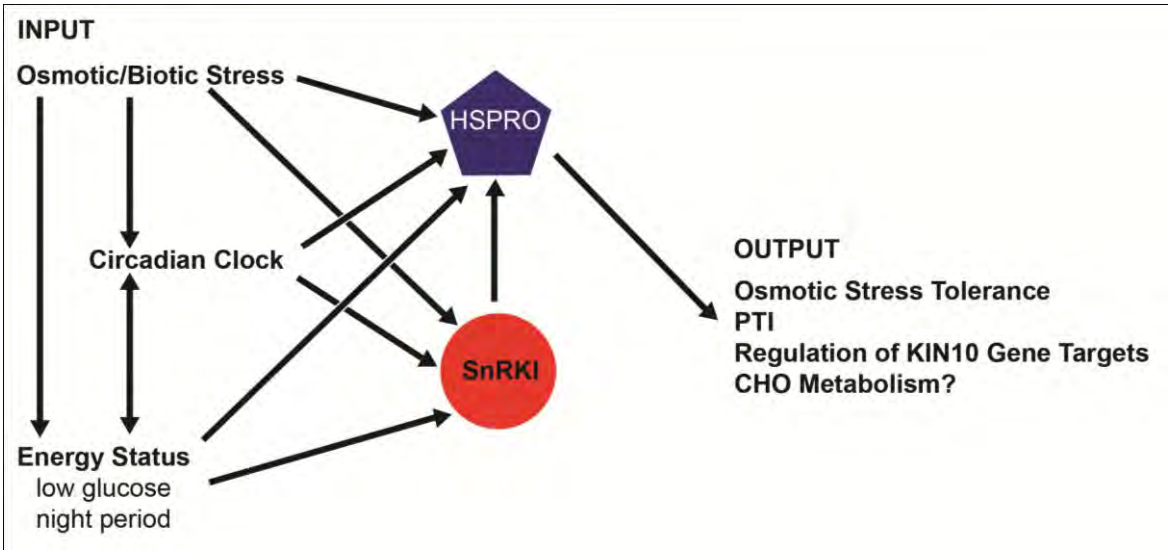


Figure 5.1: Preliminary model for *HSPRO* regulation

As illustrated in Figure 5.1 there are multiple possible stimuli for *HSPRO* expression and of additional importance is the potential for all these stimuli to interact with each other. Stress and the circadian clock can have dramatic effects on the plant energy status and regulate *HSPRO* function possibly through the interaction with the SnRK1 complex. The SnRK1 complex is likely not the only intermediary complex between stimuli and *HSPRO* downstream effects and there are potentially alternative regulatory mechanisms that drive gene expression and function. However, regardless on input and signal transduction pathways it appears the *HSPRO* genes have varying extents of involvement in abiotic and biotic stress responses and this is possibly through known KIN10 targets.

Once again it is important to note the results presented here are preliminary and experimental analysis of additional *hspro1* and *hspro2* alleles will be required before more substantial conclusions can be made. Insights established from this work can serve as a launching platform for future research into *HSPRO* functionality *in planta*.

5.1 *HSPRO* GENES ARE INVOLVED IN MULTIPLE STRESS RESPONSES

Due to their sessile nature, plants typically encounter multiple environmental stresses on a daily basis and have evolved complex and interactive networks that perceive and respond to internal and environmental cues. Generally speaking, most stress-related studies have

only focused on the biological responses to individual stresses in isolation and this is useful in determining important regulators and pathways in particular stresses but it is not necessarily a true reflection of the combinatorial effects of multiple stresses that plants are often and repeatedly exposed to. The effects of a particular stress can be additive or antagonistic to the effects of another stress during combinatorial stress events and plants are also capable of activating unique and specific responses to combinatorial stresses. Comparative analysis of multiple stress related microarray profiles has classified *HSPRO* genes and 22 other SnRK1 targets as ‗multi-stress' responsive genes involved in general stress signalling (Baena-González & Sheen, 2008). This is reflected in the results presented here as both *HSPRO* genes appear to be involved in both biotic (*Pst* infection) and abiotic stress (mannitol treatment) responses. It is interesting that both genes appear to have antagonistic functions under both types of stresses and an additional curiosity is the fact that both genes can positively or negatively regulate biological responses depending on the type of stress. This is not unique to HSPRO proteins as another SnRK1 target, the NAC transcription factor ATAF1, also displays opposing regulatory roles depending on the type of perceived stress (Baena-González, 2010; Sheen, 2010; Wu *et al.*, 2009). It is unclear how the two *HSPRO* genes evolved in *Arabidopsis* but perhaps their differences in function could be a result of gene duplication events as gene duplication may result in the evolution of novel functions (Hanzawa *et al.*, 2005; Lynch & Conery, 2000; Lynch & Conery, 2000; Taylor & Raes, 2004). It would be interesting to investigate the effects of combinatorial stresses on *HSPRO* expression and functionality in an effort to evaluate their regulatory effects under conditions more likely to occur in uncontrolled and natural environments.

Consistent with the proposed generalist stress functions of the *HSPRO* genes, knocking out either or both of the *HSPRO* genes resulted in the misregulation of genes involved in several stress-associated phytohormone signalling networks and in responses to numerous stresses (Appendix Table 11). SA and JA/ET have been well characterised as the main hormones involved in response to infection with biotrophic and necrotrophic pathogens respectively (Glazebrook, 2005; Spoel *et al.*, 2007). Additionally signalling cross-talk occurs between the hormone signalling networks and these can result in synergistic or antagonistic signal transduction (Derksen *et al.*, 2013). Signalling overlaps between the disease response phytohormones and the main abiotic stress hormone ABA

have also been characterised and these network interactions have been shown to be important in the regulation of genes responsive to both biotic and abiotic stresses

Gibberellins and auxin have established signal transduction and regulatory roles in numerous developmental processes such as seed germination, stem elongation, leaf expansion and induction of flowering (Achard & Genschik, 2009; Davies, 1995; Davière & Achard, 2013). Gibberellins (Navarro *et al.*, 2006) and auxin (Park *et al.*, 2007a; Robert-Seilaniantz *et al.*, 2011) have been linked to plant-pathogen interactions and typically regulate disease responses through their interactions with SA, JA and/or ethylene signalling pathways (Derksen *et al.*, 2013). However there is increasing evidence supporting the involvement of various gibberellins in abiotic stress responses and auxin is thought to be involved in the regulation of developmental growth in plants during cold stress responses (Rahman, 2013). Suppression of GA and GA-dependent signalling has been shown to result in stunted plant growth upon treatment with cold, salt and osmotic stress and GA signalling has also been linked to stress tolerance (Colebrook *et al.*, 2014). If *HSPRO* genes are in fact general stress response genes perhaps they mediate their functions through interactions with hormone signalling networks involved in multiple stress signalling networks. In order to test this, one approach would be to generate *hspro*/signalling double mutants and determine whether the *hspro* phenotypes described here still occur in these plants. Conversely, to determine whether HSPRO1 and HSPRO2 are required for normal signalling, the *hspro* mutants could be tested for aberrant behaviour in response to hormone treatment. For example, can ET induce apical hook formation in *hspro1-2* and *hspro2* and is this inhibited by JA-treatment as in wild-type plants.

5.2 HSPRO2 MAY PLAY A ROLE IN ENERGY PERCEPTION AND/OR SIGNALLING

Microarray data (Figure 4.9 and gene expression analysis Figure 4.10) revealed that *HSPRO2* expression is responsive to both high and low energy conditions, with gene induction occurring under low energy conditions. Although transcriptome analysis suggests that both *HSPRO* genes are KIN10 targets (Baena-González *et al.*, 2007), *HSPRO1* is only responsive to low energy conditions and the gene is not differentially expressed in response to high energy conditions (Figure 4.9). The *hspro2* and *hspro1-2/hspro2* mutants are more sensitive to exogenous glucose and germinate at a lower rate than the wild type (Figure 4.12) while high sugar does not appear to cause a change in the germination rate of the *hspro1-2* mutant. Additionally, 26% of the genes misregulated in *hspro2* are energy responsive (Table 4.6) including a subset of the downstream targets of KIN10 (Figure 4.9) and (Figure 4.11) and SnRK1-dependent *DIN6* expression appears to be misregulated in *hspro2* and *hspro1-2/hspro2* during extended dark-induced starvation conditions (Figure 4.14). Finally the *hspro2* mutant is potentially compromised in its ability to regulate linear carbon depletion during the night period as the sugars glucose, sucrose and starch appear to be more depleted in *hspro2* when compared to the wild type at dawn (Figure 4.15). This particular result is yet to be confirmed in independent experiments and should be interpreted with a considerable amount of caution. Taken together this data suggests a role for HSPRO2 in the perception or regulation of energy signal transduction, particularly under low energy conditions. Since *HSPRO* genes are KIN10 targets and certain KIN10 targets are misregulated in the *hspro* mutants it is possible that the *HSPRO* genes are an intermediary step in KIN10 signal transduction that regulate downstream targets or alternatively function through a feedback mechanism to regulate the kinase activity of KIN10 through their interaction with AKINβγ. This mechanism can be investigated in the future through evaluation of KIN10 kinase activity in the *hspro* mutants compared to the wild type controls under various states of energy availability. Additionally, HSPRO-dependent expression of other KIN10 targets besides *DIN6* needs to be evaluated before involvement of the HSPRO proteins in SnRK1 dependent energy signalling is confirmed.

Research has shown connections and overlaps between sugar signalling and the ABA signalling network. Both glucose and ABA are known to inhibit seed germination and glucose mutants such as *gin5* and *gin6* are often allelic to ABA mutants (Arenas-Huertero *et al.*, 2000; Gibson, 2005; León & Sheen, 2003). Several glucose responsive genes such

as *CAB1* and *PC* have been shown to be non-responsive to glucose in ABA biosynthesis mutants such as *aba2* and glucose treatment is known to induce ABA biosynthesis and signalling genes (Cheng *et al.*, 2002), providing further evidence for the relationship between glucose and ABA signalling. FatiGO analysis of the *HSPRO2* ECGG (Figure 3.11) and genes induced in the *hspro2* (Figure 4.6) and *hspro1-2/hspro2* (Figure 4.8) microarray data showed enrichment of genes expressed in response to ABA stimulus. Additionally the FatiGO analysis of *hspro2* and *hspro1-2/*hspro2-induced gene lists revealed a partial overlap of the genes expressed in response to ABA and those responsive to carbohydrate stimulus. This information provides evidence for the potential involvement of ABA signalling in HSPRO2-dependent responses to altered plant energy status. Public array data suggests that *HSPRO* genes are induced in response to ABA (Figure 3.16) but experimental validation needs to be conducted before any bold conclusions can be made. Other methods such as germination rates in response to ABA and/or combinations of ABA and sugars, or evaluation of *HSPRO* gene expression in ABA biosynthesis/signalling mutants can also be utilised to further dissect the possible role of HSPRO2 in ABA-dependent energy responses.

5.3 THE BIOTIC STRESS PHENOTYPE IN *hspro2* MUTANTS MAY BE LINKED TO ALTERED SUGAR METABOLISM

Sugar regulation of gene expression is a well-established phenomenon *in planta* with numerous biological processes under regulation by sugars. Carbohydrate availability affects responses to both a/biotic stress and conversely sugar levels are altered during stress response. Biosynthesis of antimicrobials and defence proteins is taxing on energy levels (Bolton, 2009) and abiotic stress potentially affects sugar levels by altering photosynthetic sugar production (Block *et al.*, 2005). Cell wall invertases that convert sucrose to glucose and fructose are also crucial in a/biotic stress function as down-regulation of invertases results in decreased availability of soluble sugars and increased susceptibility to disease (Essmann *et al.*, 2008) and sensitivity to abiotic stress (Wingler & Roitsch, 2008).

Plants with soluble sugars levels lower than wild type have been shown to be more susceptible to pathogen infection (Essmann *et al.*, 2008) and the *hspro2* plants that are susceptible to pathogen infection may also have lower than normal endogenous sugar levels at the beginning of the day (the time at which *Pst* infections were carried out). It seems altered sugar levels have a profound effect on disease susceptibility and future work could look at the interactions between *P. syringae* and *hspro2* mutants pre-treated with sugars or comparing infections of normally grown *hspro2* versus carbon fixation limited wild type plants. The potential link between lower sugar levels and increased stress sensitivity is not reflected in the osmotic stress data set and perhaps other factors are masking this effect.

Looking at microarray data (Figure 4.9 and Figure 4.16) and monitoring *HSPRO2* promoter activity (Figure 4.17) it appears *HSPRO2* is consistently induced towards the end of the night period - presumably in response to declining sugar levels – and during low energy conditions. The fact that *HSPRO2* expression is induced after *Pst* infection is potentially important in the regulation of carbohydrate levels during the defence response. Since infection with pathogens including *Pst* can result in a transition from source to sink functionality with photosynthetic gene repression and induction of sugar catabolism genes such as invertases (Bonfig *et al.*, 2006), perhaps *HSPRO2* induction is important in the regulation of catabolic generation of soluble sugars from storage carbohydrates during the defence response. Since SnRK1 represses energy consuming anabolic processes and

activates catabolic genes in low energy environments (Baena-González *et al.*, 2007), the interaction between HSPRO2 and SnRK1 could be instrumental in the proposed role of HSPRO2 during plant defence.

5.4 A CIRCADIAN LINK TO SUGAR SIGNALLING

It has been reported that energy altering processes such as photosynthesis are under the regulation of the circadian clock in plants (Dodd *et al.*, 2005; Harmer, 2000) and conversely circadian rhythms can be entrained through fluctuations in endogenous energy levels (Haydon *et al.*, 2013). This link between circadian regulation and energy signalling is reflected within the overlap of genes differentially regulated in response to varying energy levels and in circadian signalling mutants or the differential expression of circadian clock genes in response to sugar (Bläsing *et al.*, 2005) or the fact that some clock mutants cannot regulate the linear depletion of carbohydrates during the night period (Graf *et al.*, 2010). With this in mind it was interesting to note that some of the energy responsive genes misregulated in the *hspro* mutants –albeit a small percentage – such as *TOC1* (AT5G61380) and *PRR5* (AT5G24470) are core clock genes (Table 4.9 and Table 4.10). Although *HSPRO2* expression does not appear to be critical for general clock function (Figure 4.18, Figure 4.19 and Figure 4.20) perhaps it is utilised within circadian sugar signalling. The morning-expressed gene *PRR7* is an example of how a gene can be important for the sugar-dependent entrainment of the circadian clock (Haydon *et al.*, 2013).

5.5 FUTURE WORK

Although multiple and diverse methodologies were utilised to characterise the *HSPRO* genes, most of the gene expression and phenotype analyses described here were conducted on only one allele of either *hspro* single knockout mutant. This was based on the assumption that the consistency between *hspro1* alleles (Figure 3.12), and *hspro2* and the complemented line, *hspro2(HSPRO2)* (Murray *et al.*, 2007), in response to *P. syringae* would hold true across all experimental treatments. To be more confident in the claims made throughout this thesis it would therefore be necessary to repeat the more significant experiments with either second alleles of both *hspro1* and *hspro2*, or consider analysis of complemented *hspro1-2(HSPRO1)* and *hspro2(HSPRO2)* mutants. The latter approach is less favourable as it may result in additional pleiotropic effects of non-targeted insertional mutagenesis and ultimately the observation of gene-independent but insertion site specific effects and phenotypes.

It is necessary to evaluate HSPRO protein expression and mechanisms of post-translational regulation as it is often the case that changes in gene mRNA expression do not necessarily translate into protein biosynthesis and/or activation. An attempt to generate an antibody to HSPRO2 was unsuccessful as the antibody was non-specific and interacted with multiple proteins *in vitro* (Dr Robert Ingle, pers. comm.). Future attempts at generating antibodies to HSPRO proteins could utilise peptide antigens to commercially generate protein specific antibodies.

Since HSPRO proteins are interacting partners of SnRK1 complex they are possibly regulated via the kinase activity of KIN10 during responses to environmental stimuli. It follows then that as part of a SnRK1-dependent signal transduction pathway they could have other unidentified interacting partners and targets downstream of KIN10. Protein co-immunoprecipitation assays can be utilised to identify these potential interacting partners of the HSPRO proteins but protein specific antibodies would need to be generated that can specifically bind and discriminate between the two proteins since they have high amino acid sequence similarities. Co-immunoprecipitation assays can be conducted following treatment with the particular stresses of interest and interacting partners identified via GC-MS. It would be interesting to see if interaction partners vary depending on the type of stress response. Protein interaction studies are particularly useful regarding identification of additional activators and not just the targets of HSPRO proteins.

Yeast-two-hybrid screens could also be utilised to further identify other potential HSPRO interaction partners and provide supporting evidence for the data obtained using co-immunoprecipitation analysis. The main advantage of this technique is that it is not dependent on either stimulus specific induction of HSPRO protein expression or on protein expression levels *in planta*. Both HSPRO proteins can be used independently as bait molecules to screen *Arabidopsis* cDNA yeast-two-hybrid libraries generated from root and/or shoot tissue treated with osmotic stress, exposed to altered energy conditions or infected with virulent *P. syringae* to assess putative interacting partners with HSPRO during a/biotic stress and energy signalling responses. This technique allows for the identification of both upstream and downstream HSPRO interacting partners and identification of these putative partners could highlight new SnRK1-independent signalling pathways the HSPRO proteins could be linked to.

Since both HSPRO proteins interact with a common complex is it possible that their antagonistic modes of function could be as a result of reciprocal binding. Proteins with known opposing effects on gene regulation have been shown to display this reciprocal binding, with the ratios of protein interacting with the central complex being an intrinsic factor in the overall outcome of gene regulation (Nalabothula *et al.*, 2014; Portolés & Más, 2010; Zhu *et al.*, 2008). Perhaps the HSPRO proteins utilise this mechanism in their interaction with the SnRK1 complex to direct downstream effects and future work could investigate this possibility.

5.6 CONCLUSION

The current results collectively provide evidence for the involvement of *HSPRO* genes in both biotic and abiotic stress responses in *Arabidopsis*. HSPRO1 and HSPRO2 both localise to the cytoplasm and behave antagonistically in response to infection with *Pst* and treatment with osmotic stress. Although both proteins interact with the SnRK1 complex and *HSPRO* gene expression is targeted by KIN10, it appears that HSPRO2 may be more important in energy status-dependent signalling than HSPRO1. Expression of *HSPRO2* can receive input from the circadian clock and displays a rhythmic pattern, and can additionally be driven by external stimuli such as bacteria and exogenous glucose. We propose that *HSPRO1* and *HSPRO2* are general stress response genes whose functionality may be linked to altered energy levels during various stress responses. This study sets the stage for further investigation of *HSPRO* gene and protein function and identification of their interacting partners and downstream targets will allow the placement of HSPRO into specific signal transduction pathways and networks.

CHAPTER 6

Appendices

CHAPTER 6: APPENDICES

Appendix Table 1: The *HSPRO1* ECGG

Gene	Description	R^2	ρ	p-value
At2g22880	VQ motif-containing protein	0.56	0.75	8.82E-307
At1g29690	constitutively activated cell death 1 (CAD1)	0.56	0.75	2.84E-304
At3g59080	aspartyl protease family protein	0.5	0.71	1.12E-262
At1g09940	HEMA2	0.5	0.71	8.17E-258
At3g02840	immediate-early fungal elicitor family protein	0.5	0.71	1.06E-257
At5g66070	zinc finger (C3HC4-type RING finger) family protein	0.5	0.71	2.27E-257
At5g52050	MATE efflux protein-related	0.49	0.70	9.49E-252
At1g76600	unknown protein	0.48	0.69	4.20E-246
At1g80840	WRKY40	0.48	0.69	2.33E-244
At5g17350	unknown protein	0.48	0.69	6.26E-243
At5g41100	unknown protein	0.47	0.69	8.91E-239
At1g25400	unknown protein	0.47	0.69	1.97E-238
At5g13190	unknown protein	0.44	0.66	2.33E-217
At4g36030	ARMADILLO REPEAT ONLY 3 (ARO3)	0.44	0.66	2.63E-216
At4g01250	WRKY22	0.43	0.66	1.19E-213
At2g41640	transferase transferring glycosyl groups	0.43	0.66	7.41E-211
At2g38470	WRKY33	0.43	0.66	3.43E-209
At4g24570	mitochondrial substrate carrier family protein	0.43	0.66	8.67E-209
At1g15010	unknown protein	0.42	0.65	3.69E-208
At4g29780	unknown protein	0.42	0.65	1.11E-207
At1g02400	GIBBERELLIN 2-OXIDASE 6 (GA2OX6)	0.42	0.65	5.43E-207
At3g53810	lectin protein kinase putative	0.42	0.65	5.43E-207
At1g61340	F-box family protein	0.42	0.65	5.89E-204
At1g28370	ERF DOMAIN PROTEIN 11 (ERF11)	0.42	0.65	9.53E-204
At5g49520	WRKY48	0.41	0.64	1.06E-201
At1g02660	lipase class 3 family protein	0.41	0.64	1.25E-201
At1g18570	MYB DOMAIN PROTEIN 51 (MYB51)	0.41	0.64	4.42E-201
At3g57760	protein kinase family protein	0.41	0.64	1.91E-200
At2g37430	zinc finger (C2H2 type) family protein (ZAT11)	0.41	0.64	7.28E-200
At3g23250	MYB DOMAIN PROTEIN 15 (MYB15)	0.41	0.64	2.92E-199
At5g10695	unknown protein	0.41	0.64	4.37E-198
At1g66160	U-box domain-containing protein	0.41	0.64	1.11E-197
At2g25735	unknown protein	0.41	0.64	3.92E-197
At4g17500	ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR 1 (ATERF-1)	0.4	0.63	6.94E-195
At2g01180	PHOSPHATIDIC ACID PHOSPHATASE 1 (ATPAP1)	0.4	0.63	1.94E-192
At1g05575	unknown protein	0.4	0.63	2.54E-192
At1g72520	lipoxygenase putative	0.4	0.63	2.82E-192
At2g32030	GCN5-related N-acetyltransferase (GNAT) family protein	0.4	0.63	5.50E-192
At1g76070	unknown protein	0.4	0.63	1.03E-191

At2g40000	**ARABIDOPSIS ORTHOLOG OF SUGAR BEET HS1 PRO-1 2 (HSPRO2)**	**0.4**	0.63	**1.27E-191**
At1g69890	unknown protein	0.4	0.63	7.56E-191
At2g27080	HIN1-related	0.39	0.62	1.67E-189
At4g17230	Scarecrow-like 13 (SCL13)	0.39	0.62	2.42E-187
At5g64660	U-box domain-containing protein	0.39	0.62	7.51E-187
At2g27500	glycosyl hydrolase family 17 protein	0.39	0.62	2.02E-186
At1g27730	salt tolerance zinc finger (STZ)	0.39	0.62	2.83E-186
At3g26910	hydroxyproline-rich glycoprotein family protein	0.39	0.62	5.42E-186
At2g27690	CYP94C1; FUNCTIONS IN: fatty acid (omega-1)-hydroxylase activity oxygen binding	0.39	0.62	1.76E-185
At2g35930	PLANT U-BOX 23 (PUB23); FUNCTIONS IN: ubiquitin-protein ligase activity	0.38	0.62	2.94E-183
At5g27420	zinc finger (C3HC4-type RING finger) family protein; FUNCTIONS IN: protein binding zinc ion binding	0.38	0.62	5.96E-183
At2g39650	unknown protein	0.38	0.62	7.47E-182
At4g39890	Arabidopsis Rab GTPase homolog H1c (AtRABH1c)	0.38	0.62	3.28E-181
At3g18690	MAP kinase substrate 1 (MKS1)	0.38	0.62	5.93E-181
At4g17490	ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR 6 (ATERF6)	0.38	0.62	1.86E-180
At4g25810	XYLOGLUCAN ENDOTRANSGLYCOSYLASE 6 (XTR6)	0.38	0.62	2.43E-180
At4g28350	lectin protein kinase family protein	0.38	0.62	4.08E-180
At4g23190	CYSTEINE-RICH RLK11 (CRK11)	0.38	0.62	5.03E-180
At2g26530	AR781	0.38	0.62	1.03E-179
At5g22250	CCR4-NOT transcription complex protein putative	0.38	0.62	7.12E-178
At1g65390	ARABIDOPSIS THALIANA PHLOEM PROTEIN 2 A5 (ATPP2-A5); FUNCTIONS IN: carbohydrate binding;	0.37	0.61	5.33E-177
At1g76650	CALMODULIN-LIKE 38 (CML38); FUNCTIONS IN: calcium ion binding	0.37	0.61	1.25E-176
At5g66210	CPK28; FUNCTIONS IN: protein serine/threonine kinase activity calmodulin-dependent protein kinase activity	0.37	0.61	1.30E-176
At3g55980	SALT-INDUCIBLE ZINC FINGER 1 (SZF1); FUNCTIONS IN: transcription factor activity	0.37	0.61	1.80E-176
At1g23710	unknown protein	0.37	0.61	6.48E-176
At3g15210	ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR 4 (ERF4)	0.37	0.61	1.21E-175
At4g33920	protein phosphatase 2C family protein / PP2C family protein	0.37	0.61	1.97E-175
At2g23320	WRKY15; FUNCTIONS IN: transcription factor activity calmodulin binding	0.37	0.61	2.88E-175
At1g19380	unknown protein	0.37	0.61	6.30E-175
At5g59550	zinc finger (C3HC4-type RING finger) family protein; FUNCTIONS IN: protein binding zinc ion binding	0.37	0.61	6.87E-175
At5g42380	CALMODULIN LIKE 37 (CML37); FUNCTIONS IN: calcium ion binding; INVOLVED IN: response to ozone	0.37	0.61	7.05E-175
At4g35480	RHA3B; FUNCTIONS IN: protein binding zinc ion binding; INVOLVED IN: response to chitin	0.37	0.61	1.30E-174
At3g57450	unknown protein	0.37	0.61	2.51E-172
At1g59910	formin homology 2 domain-containing protein / FH2 domain-containing protein	0.37	0.61	4.38E-172
At2g30040	MAPKKK14; FUNCTIONS IN: protein serine/threonine kinase activity protein kinase activity	0.37	0.61	1.77E-171
At4g34150	C2 domain-containing protein; INVOLVED IN: response to cold	0.36	0.60	5.67E-171
At1g76700	DNAJ heat shock N-terminal domain-containing protein	0.36	0.60	5.84E-171
At4g34390	extra-large GTP-binding protein 2 (XLG2)	0.36	0.60	2.62E-170
At5g22690	disease resistance protein (TIR-NBS-LRR class) putative	0.36	0.60	5.41E-170
At3g04640	glycine-rich protein	0.36	0.60	5.59E-170
At3g13430	zinc finger (C3HC4-type RING finger) family protein	0.36	0.60	5.80E-170
At1g35140	PHOSPHATE-INDUCED 1 (PHI-1)	0.36	0.60	6.09E-170

At3g05200	ATL6; FUNCTIONS IN: protein binding zinc ion binding; INVOLVED IN: response to chitin	0.36	0.60	2.25E-168
At1g15430	unknown protein	0.36	0.60	2.38E-168
At2g34600	JASMONATE-ZIM-DOMAIN PROTEIN 7 (JAZ7)	0.36	0.60	2.41E-168
At5g54490	PINOID-BINDING PROTEIN 1 (PBP1); FUNCTIONS IN: protein binding calcium ion binding	0.36	0.60	4.37E-167
At2g36220	unknown protein	0.36	0.60	6.51E-167
At2g35710	glycogenin glucosyltransferase (glycogenin)-related	0.36	0.60	1.03E-166
At5g59820	RESPONSIVE TO HIGH LIGHT 41 (RHL41)	0.36	0.60	1.32E-166
At3g09830	protein kinase putative	0.36	0.60	3.03E-166
At3g61190	BON ASSOCIATION PROTEIN 1 (BAP1); FUNCTIONS IN: protein binding phospholipid binding	0.36	0.60	8.75E-166

Appendix Table 2: The *HSPRO2* ECGG

Gene	Description	R^2	ρ	p-value
At1g23710	unknown protein	0.67	0.82	0
At5g04760	myb family transcription factor; FUNCTIONS IN: transcription factor activity DNA binding	0.64	0.80	0
At3g46600	scarecrow transcription factor family protein; FUNCTIONS IN: transcription factor activity	0.63	0.79	0
At2g41640	transferase transferring glycosyl groups	0.61	0.78	0
At3g55980	SALT-INDUCIBLE ZINC FINGER 1 (SZF1); FUNCTIONS IN: transcription factor activity	0.59	0.77	0
At4g27280	calcium-binding EF hand family protein; FUNCTIONS IN: calcium ion binding	0.58	0.76	0
At3g57530	CALCIUM-DEPENDENT PROTEIN KINASE 32 (CPK32)	0.58	0.76	0
At1g25400	unknown protein	0.57	0.75	2.68E-315
At4g37610	BTB AND TAZ DOMAIN PROTEIN 5 (BT5); FUNCTIONS IN: protein binding transcription regulator activity	0.56	0.75	2.08E-306
At2g18700	ATTPS11; FUNCTIONS IN: transferase activity transferring glycosyl groups	0.56	0.75	9.58E-306
At4g33940	zinc finger (C3HC4-type RING finger) family protein; FUNCTIONS IN: protein binding zinc ion binding	0.56	0.75	1.11E-305
At1g32920	unknown protein; FUNCTIONS IN: molecular_function unknown; INVOLVED IN: response to wounding	0.56	0.75	3.70E-305
At4g17500	ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR 1 (ATERF-1); FUNCTIONS IN: transcription factor activity	0.55	0.74	1.68E-302
At2g23810	TETRASPANIN8 (TET8); FUNCTIONS IN: molecular_function unknown; INVOLVED IN: aging	0.55	0.74	1.85E-298
At5g49450	Arabidopsis thaliana basic leucine-zipper 1 (AtbZIP1)	0.54	0.73	1.89E-295
At5g54730	AtATG18f INVOLVED IN: response to starvation;	0.54	0.73	3.87E-295
At3g46620	zinc finger (C3HC4-type RING finger) family protein; FUNCTIONS IN: protein binding zinc ion binding	0.54	0.73	3.94E-295
At1g27100	unknown protein	0.54	0.73	6.42E-295
At5g10695	unknown protein	0.54	0.73	1.04E-294
At1g70290	ATTPS8;	0.54	0.73	5.53E-294
At3g15760	unknown protein	0.54	0.73	2.72E-293
At2g32150	haloacid dehalogenase-like hydrolase family protein; FUNCTIONS IN: hydrolase activity catalytic activity	0.54	0.73	3.17E-290
At2g38470	WRKY33; FUNCTIONS IN: transcription factor activity; INVOLVED IN: in 10 processes	0.54	0.73	2.32E-288
At1g22190	AP2 domain-containing transcription factor putative	0.54	0.73	1.00E-287
At1g19770	ATPUP14; FUNCTIONS IN: purine transmembrane transporter activity; INVOLVED IN: purine transport	0.53	0.73	3.70E-285
At1g76600	unknown protein	0.53	0.73	1.83E-281
At2g30040	MAPKKK14; FUNCTIONS IN: protein serine/threonine kinase activity protein kinase activity kinase activity	0.53	0.73	7.95E-281
At3g15770	unknown protein	0.53	0.73	4.14E-280
At2g02710	PAS/LOV PROTEIN B (PLPB); FUNCTIONS IN: two-component sensor activity signal transducer activity	0.52	0.72	8.10E-279

At1g27730	salt tolerance zinc finger (STZ);	0.52	0.72	1.40E-278
At2g27830	unknown protein	0.52	0.72	2.45E-272
At1g05575	unknown protein	0.51	0.71	6.88E-268
At4g29780	unknown protein	0.51	0.71	1.26E-266
At2g27500	glycosyl hydrolase family 17 protein	0.51	0.71	3.13E-266
At5g62570	calmodulin-binding protein; FUNCTIONS IN: calmodulin binding	0.51	0.71	9.26E-266
At2g06530	VPS2.1; INVOLVED IN: vesicle-mediated transport; LOCATED IN: ESCRT III complex	0.5	0.71	1.85E-263
At3g05200	ATL6; FUNCTIONS IN: protein binding zinc ion binding; INVOLVED IN: response to chitin	0.5	0.71	1.08E-262
At5g59820	RESPONSIVE TO HIGH LIGHT 41 (RHL41)	0.5	0.71	1.33E-257
At1g29340	PLANT U-BOX 17 (PUB17); FUNCTIONS IN: ubiquitin-protein ligase activity; INVOLVED IN: defense response	0.5	0.71	1.83E-257
At1g80920	J8; FUNCTIONS IN: unfolded protein binding heat shock protein binding	0.49	0.70	1.42E-256
At5g04720	ADR1-like 2 (ADR1-L2)	0.49	0.70	3.52E-256
At5g27420	zinc finger (C3HC4-type RING finger) family protein; FUNCTIONS IN: protein binding zinc ion binding	0.49	0.70	1.76E-255
At5g45110	NPR1-LIKE PROTEIN 3 (NPR3); FUNCTIONS IN: protein binding; INVOLVED IN: defense response to fungus	0.49	0.70	5.04E-254
At5g61600	ethylene-responsive element-binding family protein	0.49	0.70	4.13E-253
At1g50600	SCL5; FUNCTIONS IN: transcription factor activity; INVOLVED IN: regulation of transcription;	0.49	0.70	1.21E-251
At4g33920	protein phosphatase 2C family protein / PP2C family protein	0.48	0.69	2.60E-248
At4g25390	protein kinase family protein	0.48	0.69	3.42E-248
At2g40140	CZF1; FUNCTIONS IN: transcription factor activity; INVOLVED IN: defense response to fungus response	0.48	0.69	1.96E-245
At5g57560	Touch 4 (TCH4); FUNCTIONS IN: hydrolase activity acting on glycosyl bonds xyloglucan	0.48	0.69	2.57E-245
At4g17490	ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR 6 (ATERF6)	0.48	0.69	1.98E-244
At3g12740	ALA-INTERACTING SUBUNIT 1 (ALIS1); FUNCTIONS IN: phospholipid transporter activity	0.48	0.69	2.80E-244
At3g12400	ELC; FUNCTIONS IN: ubiquitin binding; INVOLVED IN: trichome branching cell division	0.47	0.69	8.84E-242
At5g62020	AT-HSFB2A; FUNCTIONS IN: transcription factor activity DNA binding; INVOLVED IN: response to chitin	0.47	0.69	7.06E-241
At3g04640	glycine-rich protein	0.47	0.69	1.17E-239
At1g09070	SOYBEAN GENE REGULATED BY COLD-2 (SRC2); FUNCTIONS IN: protein binding; INVOLVED IN: protein targeting	0.47	0.69	1.10E-238
At3g14090	exocyst subunit EXO70 family protein D3 (ATEXO70D3); FUNCTIONS IN: protein binding	0.47	0.69	7.19E-238
At4g11280	1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID (ACC) SYNTHASE 6 (ACS6)	0.47	0.69	1.37E-237
At5g58430	exocyst subunit EXO70 family protein B1 (ATEXO70B1); FUNCTIONS IN: protein binding	0.47	0.69	1.58E-237
At1g58180	BETA CARBONIC ANHYDRASE 6 (BCA6); FUNCTIONS IN: carbonate dehydratase activity zinc ion binding	0.47	0.69	2.03E-237
At4g24570	mitochondrial substrate carrier family protein; FUNCTIONS IN: binding	0.47	0.69	2.98E-237
At5g66210	CPK28	0.47	0.69	4.66E-237
At1g35140	PHOSPHATE-INDUCED 1 (PHI-1)	0.47	0.69	1.94E-236
At1g73500	MAP KINASE KINASE 9 (MKK9); FUNCTIONS IN: protein kinase activator activity MAP kinase kinase activity	0.46	0.68	5.38E-234
At1g32700	zinc-binding family protein; FUNCTIONS IN: binding	0.46	0.68	1.52E-233
At4g37260	MYB DOMAIN PROTEIN 73 (MYB73); FUNCTIONS IN: transcription factor activity DNA binding	0.46	0.68	1.23E-232
At3g52800	zinc finger (AN1-like) family protein; FUNCTIONS IN: DNA binding zinc ion binding	0.46	0.68	1.57E-231
At2g24550	unknown protein	0.46	0.68	3.09E-231
At2g26530	AR781	0.46	0.68	6.68E-231
At3g10930	unknown protein	0.46	0.68	3.02E-229
At1g02660	lipase class 3 family protein; FUNCTIONS IN: triacylglycerol lipase activity; INVOLVED IN: lipid metabolism	0.46	0.68	8.06E-229

At3g07780	OBERON1 (OBE1); FUNCTIONS IN: protein binding zinc ion binding; INVOLVED IN: embryonic development	0.45	0.67	2.19E-227
At5g16830	SYNTAXIN OF PLANTS 21 (SYP21); FUNCTIONS IN: SNAP receptor activity	0.45	0.67	8.80E-226
At2g23450	protein kinase family protein; FUNCTIONS IN: kinase activity	0.45	0.67	1.15E-225
At3g61190	BON ASSOCIATION PROTEIN 1 (BAP1); FUNCTIONS IN: protein binding phospholipid binding	0.45	0.67	1.24E-225
At1g11050	protein kinase family protein; FUNCTIONS IN: kinase activity;	0.45	0.67	3.83E-225
At5g54080	HOMOGENTISATE 12-DIOXYGENASE (HGO); FUNCTIONS IN: homogentisate 12-dioxygenase activity	0.45	0.67	4.51E-225
At1g59910	formin homology 2 domain-containing protein / FH2 domain-containing protein	0.45	0.67	2.63E-224
At5g05140	transcription elongation factor-related	0.45	0.67	1.69E-223
At1g80440	kelch repeat-containing F-box family protein	0.45	0.67	4.38E-223
At5g04040	SUGAR-DEPENDENT1 (SDP1); FUNCTIONS IN: triacylglycerol lipase activity	0.45	0.67	4.47E-223
At3g10020	unknown protein; INVOLVED IN: response to oxidative stress	0.45	0.67	1.24E-222
At2g36220	unknown protein	0.45	0.67	1.05E-221
At1g21450	SCARECROW-LIKE 1 (SCL1); FUNCTIONS IN: transcription factor activity	0.44	0.66	1.40E-221
At2g32030	GCN5-related N-acetyltransferase (GNAT) family protein	0.44	0.66	3.93E-221
At1g79380	copine-related; FUNCTIONS IN: protein binding zinc ion binding	0.44	0.66	9.11E-220
At4g24160	hydrolase alpha/beta fold family protein; FUNCTIONS IN: hydrolase activity	0.44	0.66	4.18E-219
At3g19580	ARABIDOPSIS ZINC-FINGER PROTEIN 2 (AZF2)	0.44	0.66	4.44E-218
At3g13430	zinc finger (C3HC4-type RING finger) family protein; FUNCTIONS IN: protein binding zinc ion binding	0.44	0.66	1.31E-217
At1g69490	NAC-like activated by AP3/PI (NAP); FUNCTIONS IN: transcription factor activity	0.44	0.66	2.59E-217
At3g45970	ARABIDOPSIS THALIANA EXPANSIN-LIKE A1 (ATEXLA1)	0.44	0.66	2.32E-216
At5g59550	zinc finger (C3HC4-type RING finger) family protein; FUNCTIONS IN: protein binding zinc ion binding	0.44	0.66	5.49E-216
At1g73540	Arabidopsis thaliana Nudix hydrolase homolog 21 (atnudt21); FUNCTIONS IN: hydrolase activity	0.44	0.66	8.19E-216
At1g66400	calmodulin-related protein putative; FUNCTIONS IN: calcium ion binding	0.44	0.66	2.45E-215
At1g75020	LYSOPHOSPHATIDYL ACYLTRANSFERASE 4 (LPAT4); FUNCTIONS IN: acyltransferase activity	0.43	0.66	6.93E-215
At3g14050	RELA-SPOT HOMOLOG 2 (RSH2); FUNCTIONS IN: GTP diphosphokinase activity	0.43	0.66	1.20E-214
At3g02340	zinc finger (C3HC4-type RING finger) family protein; FUNCTIONS IN: protein binding zinc ion binding	0.43	0.66	1.21E-214
At4g09460	ATMYB6; FUNCTIONS IN: transcription factor activity DNA binding; INVOLVED IN: in 9 processe	0.43	0.66	1.82E-214
At5g51390	unknown protein	0.43	0.66	6.69E-214
At3g10640	VPS60.1; INVOLVED IN: vesicle-mediated transport;	0.43	0.66	1.20E-213
At3g10985	SENESCENCE ASSOCIATED GENE 20 (SAG20);	0.43	0.66	9.75E-213
At4g20380	LESION SIMULATING DISEASE (LSD1); FUNCTIONS IN: transcription factor activity; INVOLVED IN: in 11 pr...//	0.43	0.66	3.63E-212
At5g66650	unknown protein;	0.43	0.66	6.25E-211
At1g10150	carbohydrate binding; FUNCTIONS IN: carbohydrate binding;	0.43	0.66	6.68E-211
At4g01090	extra-large G-protein-related; EXPRESSED IN: 23 plant structures;	0.43	0.66	6.79E-211
At2g01180	PHOSPHATIDIC ACID PHOSPHATASE 1 (ATPAP1); FUNCTIONS IN: phosphatidate phosphatase activity;	0.43	0.66	1.58E-210
At5g58650	plant peptide containing sulfated tyrosine 1 (PSY1)	0.43	0.66	3.16E-210
At1g18570	MYB DOMAIN PROTEIN 51 (MYB51);	0.42	0.65	3.03E-208
At5g05410	DREB2A; FUNCTIONS IN: transcription factor activity transcription activator activity DNA binding	0.42	0.65	2.30E-207
At1g80840	WRKY40; FUNCTIONS IN: transcription factor activity;	0.42	0.65	2.46E-206
At5g13190	unknown protein	0.42	0.65	9.06E-206
At3g52400	SYNTAXIN OF PLANTS 122 (SYP122); FUNCTIONS IN: SNAP receptor activity;	0.42	0.65	1.23E-205
At4g17230	Scarecrow-like 13 (SCL13); FUNCTIONS IN: transcription factor activity;	0.42	0.65	1.61E-205
At3g62260	protein phosphatase 2C putative / PP2C putative;	0.42	0.65	1.37E-204

At3g49530	Arabidopsis NAC domain containing protein 62 (anac062);	0.42	0.65	3.43E-204
At4g36040	DNAJ heat shock N-terminal domain-containing protein (J11);	0.42	0.65	3.45E-203
At3g27560	ATN1; FUNCTIONS IN: protein serine/threonine/tyrosine kinase activity protein serine/threonine kinas..//	0.42	0.65	4.02E-203
At4g05070	unknown protein;	0.41	0.64	6.38E-202
At4g29160	SNF7.1; INVOLVED IN: vesicle-mediated transport; LOCATED IN: ESCRT III complex;	0.41	0.64	8.56E-202
At1g76650	CALMODULIN-LIKE 38 (CML38); FUNCTIONS IN: calcium ion binding;	0.41	0.64	1.08E-201
At1g04960	FUNCTIONS IN: molecular_function unknown;	0.41	0.64	4.56E-201
At1g09940	HEMA2; FUNCTIONS IN: glutamyl-tRNA reductase activity;	0.41	0.64	4.88E-201
At4g34390	extra-large GTP-binding protein 2 (XLG2);	0.41	0.64	1.17E-200
At5g61210	SOLUBLE N-ETHYLMALEIMIDE-SENSITIVE FACTOR ADAPTOR PROTEIN 33 (SNAP33);	0.41	0.64	1.30E-200
At3g59080	aspartyl protease family protein; FUNCTIONS IN: DNA binding aspartic-type endopeptidase activity;	0.41	0.64	2.90E-200
At1g22930	T-complex protein 11;	0.41	0.64	6.05E-200
At1g03610	unknown protein;	0.41	0.64	1.83E-199
At5g11680	unknown protein;	0.41	0.64	4.74E-199
At1g21000	zinc-binding family protein; FUNCTIONS IN: binding;	0.41	0.64	5.22E-199
At3g43230	zinc finger (FYVE type) family protein; FUNCTIONS IN: phosphoinositide binding zinc ion binding;	0.41	0.64	1.15E-198
At4g36030	ARMADILLO REPEAT ONLY 3 (ARO3); FUNCTIONS IN: binding;	0.41	0.64	7.10E-198
At4g29950	microtubule-associated protein; FUNCTIONS IN: RAB GTPase activator activity	0.41	0.64	1.17E-197
At2g22300	SIGNAL RESPONSIVE 1 (SR1); FUNCTIONS IN: transcription regulator activity calmodulin binding	0.41	0.64	1.36E-197
At2g45170	AtATG8e; FUNCTIONS IN: microtubule binding;	0.41	0.64	1.36E-197
At4g18880	AT-HSFA4A; FUNCTIONS IN: transcription factor activity DNA binding; INVOLVED IN: response to chitin	0.41	0.64	3.85E-197
At1g19020	unknown protein;	0.41	0.64	4.87E-197
At5g42050	FUNCTIONS IN: molecular_function unknown;	0.41	0.64	6.88E-196
At3g07870	F-box family protein;	0.4	0.63	1.34E-195
At2g27310	F-box family protein;	0.4	0.63	2.02E-195
At3g08760	ATSIK;	0.4	0.63	2.51E-195
At5g11650	hydrolase alpha/beta fold family protein; FUNCTIONS IN: hydrolase activity; LOCATED IN: chloroplast;	0.4	0.63	2.55E-195
At5g04340	ZINC FINGER OF ARABIDOPSIS THALIANA 6 (ZAT6)	0.4	0.63	3.18E-195
At1g50740	unknown protein;	0.4	0.63	2.74E-194
At3g08720	ARABIDOPSIS THALIANA SERINE/THREONINE PROTEIN KINASE 2 (S6K2);	0.4	0.63	5.73E-193
At3g10800	BZIP28; FUNCTIONS IN: transcription factor activity DNA binding	0.4	0.63	1.23E-192
At3g45300	ISOVALERYL-COA-DEHYDROGENASE (IVD)	0.4	0.63	1.46E-192
At5g48655	zinc finger (C3HC4-type RING finger) family protein; FUNCTIONS IN: protein binding zinc ion binding	0.4	0.63	1.62E-192
At3g15210	ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR 4 (ERF4)	0.4	0.63	1.72E-192
At5g59420	OSBP(OXYSTEROL BINDING PROTEIN)-RELATED PROTEIN 3C (ORP3C); FUNCTIONS IN: oxysterol binding	0.4	0.63	2.13E-192
At3g61060	Arabidopsis thaliana phloem protein 2-A13 (AtPP2-A13); FUNCTIONS IN: carbohydrate binding	0.4	0.63	6.70E-192
At3g55840	**HSPRO1**	**0.4**	**0.63**	**1.27E-191**
At1g70740	protein kinase family protein; FUNCTIONS IN: protein serine/threonine kinase activity	0.4	0.63	1.43E-191
At1g63090	Phloem protein 2-A11 (AtPP2-A11); FUNCTIONS IN: carbohydrate binding	0.4	0.63	1.81E-191
At1g70590	F-box family protein	0.4	0.63	5.03E-191
At3g44260	CCR4-NOT transcription complex protein putative; FUNCTIONS IN: ribonuclease activity nucleic acid binding	0.4	0.63	1.21E-190
At3g16720	ATL2; FUNCTIONS IN: protein binding zinc ion binding; INVOLVED IN: response to chitin defense response	0.4	0.63	1.39E-190

At1g29690	constitutively activated cell death 1 (CAD1); INVOLVED IN: immune response cell death	0.4	0.63	1.93E-190
At2g44500	unknown protein;	0.4	0.63	1.12E-189
At1g44770	unknown protein	0.39	0.62	1.64E-189
At2g02220	PHYTOSULFOKIN RECEPTOR 1 (PSKR1); FUNCTIONS IN: peptide receptor activity	0.39	0.62	8.95E-189
At5g54490	PINOID-BINDING PROTEIN 1 (PBP1); FUNCTIONS IN: protein binding calcium ion binding; INVOLVED IN: res..//	0.39	0.62	2.83E-188
At1g22280	protein phosphatase 2C putative / PP2C putative;	0.39	0.62	3.44E-188
At4g21560	VACUOLAR PROTEIN SORTING-ASSOCIATED PROTEIN 28 HOMOLOG 1 (VPS28-1);	0.39	0.62	4.94E-188
At1g34300	lectin protein kinase family protein;	0.39	0.62	2.08E-187
At5g54940	eukaryotic translation initiation factor SUI1 putative; FUNCTIONS IN: translation initiation factor	0.39	0.62	6.52E-187
At3g52240	unknown protein	0.39	0.62	8.00E-187
At4g19640	ARA7; FUNCTIONS IN: GTP binding;	0.39	0.62	6.41E-186
At3g49780	PHYTOSULFOKINE 4 PRECURSOR (ATPSK4); FUNCTIONS IN: growth factor activity;	0.39	0.62	7.50E-186
At1g70520	protein kinase family protein; FUNCTIONS IN: kinase activity	0.39	0.62	1.09E-185
At5g63790	ARABIDOPSIS NAC DOMAIN CONTAINING PROTEIN 102 (ANAC102); FUNCTIONS IN: transcription factor activity	0.39	0.62	1.16E-185
At2g28400	unknown protein	0.39	0.62	1.17E-185
At1g75440	ubiquitin-conjugating enzyme 16 (UBC16)	0.39	0.62	1.44E-185
At2g23320	WRKY15; FUNCTIONS IN: transcription factor activity calmodulin binding; INVOLVED IN: response to chitin	0.39	0.62	2.34E-185
At3g18690	MAP kinase substrate 1 (MKS1); FUNCTIONS IN: protein binding; INVOLVED IN: defense response signalling	0.39	0.62	2.56E-185
At1g62422	unknown protein; EXPRESSED IN: 22 plant structures; EXPRESSED DURING: 13 growth stages	0.39	0.62	3.35E-185
At5g66070	zinc finger (C3HC4-type RING finger) family protein; FUNCTIONS IN: protein binding zinc ion binding	0.39	0.62	1.33E-184
At3g46930	protein kinase family protein; FUNCTIONS IN: protein serine/threonine/tyrosine kinase activity	0.39	0.62	2.52E-184
At1g11260	SUGAR TRANSPORTER 1 (STP1);	0.39	0.62	3.19E-184
At4g04960	lectin protein kinase putative; FUNCTIONS IN: kinase activity	0.38	0.62	2.99E-183
At1g13260	RAV1; FUNCTIONS IN: transcription repressor activity transcription factor activity DNA binding	0.38	0.62	7.20E-183
At2g17520	IRE1A; FUNCTIONS IN: endoribonuclease activity kinase activity	0.38	0.62	1.03E-182
At1g74450	unknown protein;	0.38	0.62	1.38E-182
At3g56880	VQ motif-containing protein	0.38	0.62	2.81E-182
At4g12040	zinc finger (AN1-like) family protein; FUNCTIONS IN: DNA binding zinc ion binding;	0.38	0.62	3.61E-182
At5g04850	VPS60.2; INVOLVED IN: vesicle-mediated transport	0.38	0.62	2.34E-181
At3g57450	unknown protein	0.38	0.62	4.22E-180
At1g28380	necrotic spotted lesions 1 (NSL1)	0.38	0.62	6.17E-180
At5g45710	ROOT HANDEDNESS 1 (RHA1); FUNCTIONS IN: transcription factor activity DNA binding	0.38	0.62	6.70E-180
At2g22880	VQ motif-containing protein	0.38	0.62	8.58E-180
At2g41430	EARLY RESPONSIVE TO DEHYDRATION 15 (ERD15); FUNCTIONS IN: protein binding;	0.38	0.62	1.41E-179
At1g26800	zinc finger (C3HC4-type RING finger) family protein; FUNCTIONS IN: protein binding zinc ion binding	0.38	0.62	3.67E-179
At2g32800	AP4.3A; FUNCTIONS IN: protein serine/threonine kinase activity protein tyrosine kinase activity	0.38	0.62	5.75E-179
At5g45340	CYP707A3; FUNCTIONS IN: oxygen binding (+)-abscisic acid 8'-hydroxylase activity;	0.38	0.62	6.02E-179
At3g01430		0.38	0.62	2.12E-178
At4g36730	GBF1; FUNCTIONS IN: transcription factor activity sequence-specific DNA binding;	0.38	0.62	8.23E-178
At1g04440	CASEIN KINASE LIKE 13 (CKL13); FUNCTIONS IN: protein serine/threonine kinase activity protein kinase	0.38	0.62	1.95E-177
At4g28085	unknown protein	0.37	0.61	3.41E-177

At5g18150	unknown protein	0.37	0.61	7.79E-177
At1g69890	unknown protein	0.37	0.61	1.87E-176
At4g33300	ADR1-like 1 (ADR1-L1); FUNCTIONS IN: protein binding ATP binding; INVOLVED IN: defense response	0.37	0.61	2.27E-176
At4g19140	unknown protein	0.37	0.61	4.14E-176
At4g20830	FAD-binding domain-containing protein; FUNCTIONS IN: electron carrier activity oxidoreductase activity	0.37	0.61	7.41E-176
At1g63720	EXPRESSED IN: 21 plant structures; EXPRESSED DURING: 13 growth stages	0.37	0.61	8.76E-176
At4g18140	phosphatase; FUNCTIONS IN: phosphatase activity	0.37	0.61	2.60E-175
At1g13450	DNA binding protein GT-1; FUNCTIONS IN: transcription factor activity;	0.37	0.61	7.33E-175
At5g46780	VQ motif-containing protein	0.37	0.61	8.56E-175
At2g21120	unknown protein	0.37	0.61	1.01E-174
At5g02020	unknown protein;	0.37	0.61	1.84E-174
At1g08720	ENHANCED DISEASE RESISTANCE 1 (EDR1)	0.37	0.61	3.49E-174
At4g27652	unknown protein	0.37	0.61	5.47E-174
At1g78080	related to AP2 4 (RAP2.4); FUNCTIONS IN: transcription factor activity DNA binding	0.37	0.61	8.53E-174
At4g31550	WRKY11; FUNCTIONS IN: transcription factor activity calmodulin binding; INVOLVED IN: defense response	0.37	0.61	1.05E-173
At2g31945	unknown protein	0.37	0.61	1.14E-173
At3g15500	ARABIDOPSIS NAC DOMAIN CONTAINING PROTEIN 55 (ANAC055)	0.37	0.61	1.91E-173
At5g08350	GRAM domain-containing protein / ABA-responsive protein-related;	0.37	0.61	2.10E-173
At5g64660	U-box domain-containing protein; FUNCTIONS IN: ubiquitin-protein ligase activity binding	0.37	0.61	2.13E-173
At3g25600	calcium ion binding; FUNCTIONS IN: calcium ion binding	0.37	0.61	2.42E-173
At5g44290	protein kinase family protein	0.37	0.61	2.54E-173
At3g02070	OTU-like cysteine protease family protein; FUNCTIONS IN: cysteine-type peptidase activity	0.37	0.61	2.63E-173
At5g49520	WRKY48; FUNCTIONS IN: transcription factor activity; INVOLVED IN: response to chitin	0.37	0.61	6.15E-173
At3g57760	protein kinase family protein	0.37	0.61	9.72E-173
At5g38210	serine/threonine protein kinase family protein	0.37	0.61	1.52E-172
At3g48760	zinc finger (DHHC type) family protein; FUNCTIONS IN: zinc ion binding	0.37	0.61	1.96E-172
At1g70530	protein kinase family protein; FUNCTIONS IN: kinase activity	0.37	0.61	5.88E-172
At1g14370	PROTEIN KINASE 2A (APK2A); FUNCTIONS IN: protein serine/threonine kinase activity	0.37	0.61	7.08E-172
At5g63620	oxidoreductase zinc-binding dehydrogenase family protein	0.37	0.61	1.05E-171
At1g66160	U-box domain-containing protein; FUNCTIONS IN: ubiquitin-protein ligase activity binding	0.37	0.61	1.61E-171
At1g27290	unknown protein	0.37	0.61	1.68E-171
At4g13530	unknown protein	0.36	0.60	5.22E-171
At5g58350	WITH NO K (=LYSINE) 4 (WNK4); FUNCTIONS IN: protein kinase activity kinase activity	0.36	0.60	6.94E-171
At1g69840	band 7 family protein	0.36	0.60	1.01E-170
At3g62720	XYLOSYLTRANSFERASE 1 (XT1)	0.36	0.60	1.26E-170
At3g02140	TWO OR MORE ABRES-CONTAINING GENE 2 (TMAC2)	0.36	0.60	1.67E-169
At3g23030	INDOLE-3-ACETIC ACID INDUCIBLE 2 (IAA2); FUNCTIONS IN: transcription factor activity	0.36	0.60	2.59E-169
At5g51070	EARLY RESPONSIVE TO DEHYDRATION 1 (ERD1)	0.36	0.60	2.68E-169
At5g18630	lipase class 3 family protein; FUNCTIONS IN: triacylglycerol lipase activity	0.36	0.60	3.81E-169
At2g39650	unknown protein	0.36	0.60	4.10E-169
At1g10140	FUNCTIONS IN: molecular_function unknown	0.36	0.60	5.96E-169
At5g06320	NHL3; FUNCTIONS IN: molecular_function unknown; INVOLVED IN: defense response to virus defense response	0.36	0.60	6.90E-169
At5g17350	unknown protein	0.36	0.60	1.25E-168

At1g79670	RESISTANCE TO FUSARIUM OXYSPORUM 1 (RFO1); FUNCTIONS IN: kinase activity	0.36	0.60	1.39E-168
At1g80180	unknown protein	0.36	0.60	2.57E-168
At1g18740	unknown protein	0.36	0.60	3.65E-168
At1g72940	disease resistance protein (TIR-NBS class) putative; FUNCTIONS IN: transmembrane receptor activity	0.36	0.60	4.81E-168
At1g71697	CHOLINE KINASE 1 (ATCK1); FUNCTIONS IN: choline kinase activity; INVOLVED IN: response to salt stress	0.36	0.60	6.49E-168
At2g46500	phosphatidylinositol 3- and 4-kinase family protein / ubiquitin family protein;	0.36	0.60	1.43E-167
At5g61900	BONZAI 1 (BON1); FUNCTIONS IN: calcium-dependent phospholipid binding	0.36	0.60	2.76E-167
At3g51130	unknown protein; INVOLVED IN: response to salt stress	0.36	0.60	3.32E-167
At1g33050	unknown protein	0.36	0.60	5.47E-167
At2g41010	ARABIDOPSIS THALIANA CALMODULIN (CAM)-BINDING PROTEIN OF 25 KDA (ATCAMBP25)	0.36	0.60	7.37E-167
At5g66250	kinectin-related	0.36	0.60	8.37E-167
At1g02860	nitrogen limitation adaptation (NLA); FUNCTIONS IN: ubiquitin-protein ligase activity;	0.36	0.60	2.00E-166
At5g04080	unknown protein	0.36	0.60	3.08E-166
At4g30390	unknown protein	0.36	0.60	3.22E-166
At4g29900	AUTOINHIBITED CA(2+)-ATPASE 10 (ACA10); FUNCTIONS IN: calmodulin binding calcium-transporting ATPase..//	0.36	0.60	9.05E-166

Appendix Table 3: FatiGO analysis of the *HSPRO1* ECGG

Term	ECGG vs genome	Genes in ECGG containing term	p value	Adjusted p value
GO Biological Process - Level 3 to 9				
response to chitin (GO:0010200)	25.56% 0.42%	At5g66070, at1g80840, at4g01250, at2g38470, at5g49520,at2g37430, at3g23250, at1g66160, at4g17500, at4g17230,at5g64660, at1g27730, at2g35930, at5g27420, at4g17490,at3g55980, at3g15210, at2g23320, at5g59550, at4g35480,at3g05200, at2g34600, at5g59820,	2.35E-33	2.03E-30
response to carbohydrate stimulus (GO:0009743)	26.67% 0.65%	At5g66070, at1g80840, at4g01250, at2g38470, at5g49520,at2g37430, at3g23250, at1g66160, at4g17500, at4g17230,at5g64660, at1g27730, at2g35930, at5g27420, at4g17490,at3g55980, at3g15210, at2g23320, at5g59550, at4g35480,at4g34390, at3g05200, at2g34600, at5g59820,	4.01E-31	1.729E-28
defense response (GO:0006952)	21.11% 2.67%	At1g80840, at4g01250, at2g38470, at1g18570, at4g17500,at2g01180, at1g72520, at2g40000, at2g27500, at2g35930,at3g18690, at4g17490, at4g23190, at1g65390, at3g15210,at5g22690, at3g05200, at2g34600, at3g61190,	3.55E-12	1.021E-09
immune response (GO:0006955)	11.11% 1.11%	At1g29690, at1g80840, at2g01180, at2g40000, at2g35930,at3g18690, at4g23190, at1g65390, at3g15210, at5g22690,	8.15E-08	0.00001758
innate immune response (GO:0045087)	8.89% 0.96%	At2g01180, at2g40000, at2g35930, at3g18690, at4g23190,at1g65390, at3g15210, at5g22690,	2.97E-06	0.0005128
response to bacterium (GO:0009617)	7.78% 0.78%	At1g80840, at2g38470, at1g18570, at1g72520, at2g40000,at4g23190, at3g15210,	8.18E-06	0.001176
response to wounding (GO:0009611)	6.67% 0.55%	At1g80840, at1g72520, at1g27730, at1g76650, at5g59820,at3g61190,	1.28E-05	0.001578
defense response to bacterium (GO:0042742)	6.67% 0.58%	At1g80840, at2g38470, at1g18570, at2g40000, at4g23190,at3g15210,	1.78E-05	0.001916
regulation of defense response (GO:0031347)	4.44% 0.18%	At1g80840, at3g18690, at3g15210, at3g61190,	2.99E-05	0.002862
response to ozone (GO:0010193)	3.33%	At3g02840, at1g72520, at5g42380,	8.42E-05	0.007263

	0.08%			
response to other organism (GO:0051707)	8.89% 1.62%	At3g02840, at1g80840, at2g38470, at1g18570, at1g72520,at2g40000, at4g23190, at3g15210,	0.000119	0.009312
response to ethylene (GO:0009723)	6.67% 0.84%	At1g28370, at1g18570, at3g23250, at4g17500, at4g17490,at3g15210,	0.000131	0.009403
response to biotic stimulus (GO:0009607)	8.89% 1.82%	At3g02840, at1g80840, at2g38470, at1g18570, at1g72520,at2g40000, at4g23190, at3g15210,	0.000255	0.01694
response to hormone stimulus (GO:0009725)	11.11% 2.97%	At1g28370, at1g18570, at3g23250, at4g17500, at1g27730,at5g27420, at4g17490, at3g15210, at4g34390, at5g54490,	0.00036	0.0222
response to external stimulus (GO:0009605)	6.67% 1.23%	At1g80840, at1g72520, at1g27730, at1g76650, at5g59820,at3g61190,	0.000916	0.04942
response to salicylic acid (GO:0009751)	4.44% 0.46%	At1g80840, at1g18570, at2g40000, at3g61190,	0.000916	0.04942

Appendix Table 4: FatiGO analysis of the *HSPRO2* ECGG

Term	**ECGG vs genome**	**Genes in ECGG containing term**	**p value**	**Adjusted p value**
GO Biological Process - Level 3 to 9				
response to chitin (GO:0010200)	11.92% 0.42%	At3g46600, at3g55980, at4g37610, at4g33940, at4g17500,at3g46620, at2g38470, at1g27730, at3g05200, at5g59820,at5g27420, at2g40140, at4g17490, at5g62020, at4g37260,at3g52800, at3g19580, at5g59550, at1g80840, at4g17230,at3g49530, at4g18880, at5g48655, at3g15210, at3g16720,at2g23320, at5g66070, at4g31550, at5g64660, at5g49520,at1g66160,	4.417E-33	3.812E-30
response to carbohydrate stimulus (GO:0009743)	12.31%0.65%	At3g46600, at3g55980, at4g37610, at4g33940, at4g17500,at3g46620, at2g38470, at1g27730, at3g05200, at5g59820,at5g27420, at2g40140, at4g17490, at5g62020, at4g37260,at3g52800, at3g19580, at5g59550, at1g80840, at4g17230,at3g49530, at4g34390, at4g18880, at5g48655, at3g15210,at3g16720, at2g23320, at5g66070, at4g31550, at5g64660,at5g49520, at1g66160,	2.564E-29	1.106E-26
defense response (GO:0006952)	11.15% 2.67%	At2g40000, at4g17500, at2g38470, at2g27500, at3g05200,at1g29340, at5g04720, at5g45110, at5g61600, at2g40140,at4g17490, at4g11280, at3g61190, at4g20380, at2g01180,at1g18570, at1g80840, at3g52400, at3g15210, at3g16720,at3g18690, at1g28380, at4g33300, at4g31550, at5g51070,at5g06320, at1g72940, at5g61900, at1g02860,	1.898E-10	5.461E-08
response to biotic stimulus (GO:0009607)	8.85% 1.82%	At2g40000, at2g38470, at1g29340, at5g45110, at5g61600,at2g40140, at3g10985, at4g20380, at1g18570, at1g80840,at3g52400, at5g61210, at2g22300, at3g10800, at3g15210,at3g44260, at2g17520, at2g41430, at1g08720, at4g31550,at5g06320, at1g79670, at1g02860,	9.961E-10	2.149E-07
response to wounding (GO:0009611)	5% 0.55%	At1g32920, at1g27730, at5g59820, at4g11280, at1g73500,at3g61190, at3g14050, at1g80840, at1g76650, at3g61060,at3g44260, at2g02220, at1g71697,	5.306E-09	9.159E-07
response to external stimulus (GO:0009605)	6.92% 1.23%	At1g32920, at5g54730, at1g27730, at5g59820, at5g57560,at4g11280, at1g73500, at3g61190, at3g14050, at1g80840,at1g76650, at5g61210, at2g45170, at3g61060, at3g44260,at2g02220, at5g45710, at1g71697,	8.191E-09	0.000001178
response to other organism	7.31% 1.62%	At2g40000, at2g38470, at1g29340, at5g45110	9.468E-08	0.000011

(GO:0051707)		, at5g61600,at2g40140, at3g10985, at4g2038 0, at1g18570, at1g80840,at3g52400, at5g612 10, at3g15210, at2g41430, at1g08720,at4g31 550, at5g06320, at1g79670, at1g02860,		67
response to fungus (GO:0009620)	3.85% 0.40%	At2g38470, at1g29340, at5g45110, at5g61600 , at2g40140,at3g10985, at1g80840, at3g5240 0, at1g08720, at1g79670,	2.138E-07	0.000023 07
immune response (GO:0006955)	5.77% 1.11%	At2g40000, at1g29340, at5g45110, at4g20380 , at2g01180,at1g80840, at3g52400, at3g1521 0, at1g29690, at3g18690,at1g28380, at5g063 20, at1g72940, at5g61900, at1g02860,	3.951E-07	0.000035 93
response to hormone stimulus (GO:0009725)	9.62% 2.97%	At3g57530, at4g37610, at4g17500, at1g22190 , at1g27730,at5g27420, at5g61600, at5g5756 0, at4g17490, at4g11280,at4g37260, at3g195 80, at3g14050, at4g09460, at4g20380,at1g18 570, at4g34390, at3g15210, at5g54490, at1g1 3260,at5g45710, at1g08720, at1g78080, at3g 02140, at3g23030,	4.163E-07	0.000035 93
response to ethylene (GO:0009723)	5% 0.84%	At4g17500, at1g22190, at5g61600, at4g17490 , at4g11280,at4g37260, at4g09460, at4g2038 0, at1g18570, at3g15210,at1g13260, at1g087 20, at1g78080,	6.008E-07	0.000047 14
plant-type hypersensitive response (GO:0009626)	2.31% 0.15%	At4g20380, at2g01180, at3g52400, at1g28380 , at5g61900,at1g02860,	0.00000481 1	0.000311 4
host programmed cell death induced by symbiont (GO:0034050)	2.31% 0.15%	At4g20380, at2g01180, at3g52400, at1g28380 , at5g61900,at1g02860,	0.00000541 3	0.000311 4
response to water (GO:0009415)	3.85% 0.59%	At2g38470, at1g27730, at3g19580, at5g05410 , at2g41430,at5g45340, at1g08720, at3g1550 0, at5g61900, at2g41010,	0.00000520 8	0.000311 4
cell death (GO:0008219)	4.62% 0.87%	At1g29340, at5g04720, at4g11280, at4g20380 , at2g01180,at3g52400, at1g29690, at1g2838 0, at4g33300, at1g08720,at5g61900, at1g028 60,	0.00000508 8	0.000311 4
response to salicylic acid (GO:0009751)	3.46% 0.46%	At2g40000, at4g37260, at3g61190, at4g09460 , at4g20380,at1g18570, at1g80840, at3g5240 0, at1g02860,	0.00000585 2	0.000315 6
response to bacterium (GO:0009617)	4.23% 0.78%	At2g40000, at2g38470, at5g45110, at1g18570 , at1g80840,at3g15210, at2g41430, at1g0872 0, at4g31550, at5g06320,at1g02860,	0.00000983 5	0.000499 3
innate immune response (GO:0045087)	4.62% 0.96%	At2g40000, at1g29340, at5g45110, at4g20380 , at2g01180,at3g52400, at3g15210, at3g1869 0, at1g28380, at1g72940,at5g61900, at1g028 60,	0.000012	0.000562 7
defense response to fungus (GO:0050832)	2.69% 0.27%	At2g38470, at1g29340, at5g45110, at5g61600 , at2g40140,at1g80840, at3g52400,	0.00001239	0.000562 7
response to heat (GO:0009408)	3.08% 0.40%	At2g38470, at5g59820, at5g57560, at3g61190 , at5g05410,at3g08720, at3g10800, at5g4571 0,	0.00001477	0.000607 1
regulation of defense response (GO:0031347)	2.31% 0.18%	At3g61190, at1g80840, at3g52400, at3g15210 , at3g18690,at5g61900,	0.00001409	0.000607 1
response to water deprivation (GO:0009414)	3.46% 0.56%	At2g38470, at1g27730, at3g19580, at5g05410 , at2g41430,at5g45340, at1g08720, at3g1550 0, at2g41010,	0.00002394	0.000939
response to salt stress (GO:0009651)	5% 1.21%	At3g57530, at2g38470, at1g27730, at5g59820 , at1g73500,at4g37260, at3g19580, at4g0946 0, at1g18570, at3g08720,at1g28380, at3g021 40, at2g41010,	0.00002542	0.000953 9
defense response to bacterium (GO:0042742)	3.46% 0.58%	At2g40000, at2g38470, at5g45110, at1g18570 , at1g80840,at3g15210, at4g31550, at5g0632 0, at1g02860,	0.00003265	0.001174
regulation of salicylic acid metabolic process (GO:0010337)	1.15% 0.02%	At1g28380, at2g41010, at1g02860,	0.00003565	0.001231

programmed cell death (GO:0012501)	3.85% 0.77%	At1g29340, at5g04720, at4g11280, at4g20380, at2g01180,at3g52400, at1g28380, at4g33300, at5g61900, at1g02860,	0.00005034	0.001671
response to osmotic stress (GO:0006970)	5% 1.31%	At3g57530, at2g38470, at1g27730, at5g59820, at1g73500,at4g37260, at3g19580, at4g09460, at1g18570, at3g08720,at1g28380, at3g02140, at2g41010,	0.00005512	0.001762
response to abscisic acid (GO:0009737)	4.23% 0.97%	At3g57530, at1g27730, at5g27420, at4g37260, at3g19580,at3g14050, at4g09460, at1g18570, at4g34390, at3g15210,at3g02140,	0.00006477	0.001996
two-component signal transduction system (phosphorelay) (GO:0000160)	3.46% 0.72%	At4g17500, at1g22190, at2g02710, at5g61600, at4g17490,at4g20380, at3g15210, at1g13260, at1g78080,	0.0001564	0.004499
ethylene-activated signaling pathway (GO:0009873)	3.08% 0.56%	At4g17500, at1g22190, at5g61600, at4g17490, at4g20380,at3g15210, at1g13260, at1g78080,	0.0001541	0.004499
salicylic acid metabolic process (GO:0009696)	1.15% 0.04%	At1g28380, at2g41010, at1g02860,	0.0003385	0.009424
response to jasmonic acid (GO:0009753)	2.69% 0.51%	At4g11280, at4g37260, at4g09460, at1g18570, at3g52400,at3g15210, at3g15500,	0.0005154	0.0139
response to mechanical stimulus (GO:0009612)	1.15% 0.05%	At5g57560, at4g11280, at5g61210,	0.0005746	0.01503
response to oxidative stress (GO:0006979)	3.46% 0.94%	At2g40000, at1g27730, at5g59820, at3g10020, at4g20380,at5g05410, at1g09940, at1g19020, at4g20830,	0.001006	0.02553
response to temperature stimulus (GO:0009266)	4.23% 1.38%	At2g38470, at1g27730, at5g59820, at2g40140, at5g57560,at3g61190, at5g05410, at3g08720, at3g10800, at5g45710,at5g61900,	0.00123	0.02947
intracellular signaling cascade (GO:0007242)	7.31% 3.25%	At3g57530, at4g17500, at1g22190, at5g61600, at4g17490,at4g20380, at3g52400, at4g29950, at3g10800, at3g15210,at4g19640, at3g18690, at1g13260, at2g17520, at1g08720,at1g78080, at3g15500, at3g02140, at3g23030,	0.001227	0.02947
protein transport (GO:0015031)	5% 1.87%	At2g06530, at3g12400, at1g09070, at5g16830, at3g10640,at3g52400, at4g29160, at5g61210, at2g45170, at5g11650,at4g21560, at4g19640, at5g04850,	0.001532	0.03549
defense response, incompatible interaction (GO:0009814)	1.92% 0.31%	At2g40000, at1g29340, at5g45110, at4g20380, at1g02860,	0.001563	0.03549
endoplasmic reticulum unfolded protein response (GO:0030968)	0.77% 0.02%	At3g10800, at2g17520,	0.002053	0.04392
protein localization (GO:0008104)	5% 1.95%	At2g06530, at3g12400, at1g09070, at5g16830, at3g10640,at3g52400, at4g29160, at5g61210, at2g45170, at5g11650,at4g21560, at4g19640, at5g04850,	0.002137	0.04392
hormone-mediated signaling pathway (GO:0009755)	4.23% 1.48%	At3g57530, at4g17500, at1g22190, at5g61600, at4g17490,at4g20380, at3g15210, at1g13260, at1g78080, at3g02140,at3g23030,	0.002115	0.04392
response to auxin (GO:0009733)	3.46% 1.05%	At4g37610, at5g57560, at4g11280, at4g37260, at4g09460,at1g18570, at5g54490, at5g45710, at3g23030,	0.002121	0.04392
vesicle-mediated transport (GO:0016192)	3.46% 1.06%	At2g06530, at3g14090, at5g58430, at5g16830, at3g10640,at3g52400, at4g29160, at5g61210, at5g04850,	0.002212	0.04439
regulation of programmed cell death (GO:0043067)	1.15% 0.09%	At3g10800, at2g17520,	0.002467	0.04819
response to unfolded protein (GO:0006986)	0.77% 0.02%	At3g10800, at2g17520,	0.002625	0.04819
cellular response to unfolded protein (GO:0034620)	0.77% 0.02%	At3g10800, at2g17520,	0.002625	0.04819
camalexin biosynthetic process (GO:0010120)	0.77% 0.02%	At2g38470, at1g73500,	0.002625	0.04819

Appendix Table 5: GO analysis of genes induced by *hspro1-2* knockout mutation

Term	hspro1-2 vs genome	p value	Adjusted p value
GO BIOLOGICAL PROCESS - level 3 to 9			
response to other organism (GO:0051707)	19.53% 1.48%	2.14E-26	1.87E-23
response to biotic stimulus (GO:0009607)	20.12% 1.67%	5.94E-26	2.60E-23
defense response (GO:0006952)	19.53% 2.57%	2.47E-19	7.20E-17
innate immune response (GO:0045087)	12.43% 0.87%	1.01E-17	1.77E-15
response to fungus (GO:0009620)	9.47% 0.35%	9.59E-18	1.77E-15
immune response (GO:0006955)	13.02% 1.02%	1.65E-17	2.40E-15
response to bacterium (GO:0009617)	10.06% 0.71%	1.90E-14	2.38E-12
defense response, incompatible interaction (GO:0009814)	7.10% 0.26%	1.36E-13	1.48E-11
response to carbohydrate stimulus (GO:0009743)	8.88% 0.58%	2.21E-13	2.15E-11
defense response to fungus (GO:0050832)	6.51% 0.24%	1.62E-12	1.42E-10
response to chitin (GO:0010200)	7.10% 0.37%	5.24E-12	4.17E-10
defense response to bacterium (GO:0042742)	7.10% 0.53%	2.78E-10	2.03E-08
oxylipin metabolic process (GO:0031407)	3.55% 0.08%	1.84E-08	1.24E-06
defense response by callose deposition (GO:0052542)	2.96% 0.04%	3.51E-08	2.2E-06
plant-type hypersensitive response (GO:0009626)	3.55% 0.13%	1.56E-07	9.08E-06
host programmed cell death induced by symbiont (GO:0034050)	3.55% 0.13%	1.78E-07	9.75E-06
regulation of defense response (GO:0031347)	3.55% 0.16%	5.30E-07	2.73E-05
systemic acquired resistance (GO:0009627)	2.96% 0.08%	6.58E-07	3.2E-05
programmed cell death (GO:0012501)	5.92% 0.74%	8.38E-07	3.86E-05
response to external stimulus (GO:0009605)	7.10% 1.17%	1.07E-06	4.68E-05
defense response by callose deposition in cell wall (GO:0052544)	2.37% 0.04%	1.42E-06	5.93E-05
GO:0007047	6.51% 1.04%	2.25E-06	8.96E-05
callose deposition in cell wall (GO:0052543)	2.37% 0.05%	2.37E-06	9.03E-05
cell death (GO:0008219)	5.92% 0.85%	2.57E-06	9.38E-05
cell wall thickening (GO:0052386)	2.37% 0.05%	2.99E-06	0.000105
response to oxidative stress (GO:0006979)	5.92% 0.88%	3.77E-06	0.000127
response to wounding (GO:0009611)	4.73% 0.52%	4.29E-06	0.000139
defense response to fungus, incompatible interaction (GO:0009817)	2.37% 0.07%	9.52E-06	0.000287
response to ozone (GO:0010193)	2.37% 0.07%	9.52E-06	0.000287
response to osmotic stress (GO:0006970)	6.51% 1.22%	1E-05	0.000293
defense response to bacterium, incompatible interaction (GO:0009816)	2.37% 0.07%	1.12E-05	0.000316
response to fructose stimulus (GO:0009750)	1.78% 0.02%	1.27E-05	0.000348
fatty acid biosynthetic process (GO:0006633)	4.14% 0.50%	3.12E-05	0.000826
response to light intensity (GO:0009642)	2.96% 0.21%	4.24E-05	0.001065

response to absence of light (GO:0009646)	1.78% 0.03%	4.26E-05	0.001065
response to insect (GO:0009625)	1.78% 0.04%	5.4E-05	0.001312
cell wall modification (GO:0042545)	3.55% 0.41%	9.62E-05	0.002275
response to temperature stimulus (GO:0009266)	5.92% 1.32%	0.000105	0.002416
glucosinolate metabolic process (GO:0019760)	2.37% 0.14%	0.000112	0.002523
response to salicylic acid (GO:0009751)	3.55% 0.44%	0.000137	0.002999
response to glucose stimulus (GO:0009749)	1.78% 0.05%	0.000141	0.003007
response to salt stress (GO:0009651)	5.33% 1.13%	0.000166	0.003461
cellular aromatic compound metabolic process (GO:0006725)	5.92% 1.42%	0.00019	0.003863
fatty acid metabolic process (GO:0006631)	4.14% 0.68%	0.000198	0.003937
response to light stimulus (GO:0009416)	5.92% 1.45%	0.000222	0.00432
response to cold (GO:0009409)	4.73% 0.95%	0.000262	0.004991
response to radiation (GO:0009314)	5.92% 1.49%	0.000277	0.005156
regulation of hydrogen peroxide metabolic process (GO:0010310)	1.18% 0.01%	0.000287	0.005234
response to water deprivation (GO:0009414)	3.55% 0.52%	0.000316	0.005637
response to water (GO:0009415)	3.55% 0.55%	0.000404	0.007069
salicylic acid mediated signaling pathway (GO:0009863)	1.78% 0.08%	0.000412	0.007069
secondary metabolic process (GO:0019748)	5.33% 1.38%	0.000675	0.01136
defense response to insect (GO:0002213)	1.18% 0.02%	0.000795	0.01313
response to sucrose stimulus (GO:0009744)	1.78% 0.11%	0.000971	0.01573
xenobiotic metabolic process (GO:0006805)	1.18% 0.03%	0.001269	0.02019
lipid transport (GO:0006869)	2.96% 0.47%	0.001449	0.02225
response to jasmonic acid (GO:0009753)	2.96% 0.47%	0.001449	0.02225
carbohydrate transport (GO:0008643)	2.37% 0.29%	0.001719	0.02593
systemic acquired resistance, salicylic acid mediated signaling pathway (GO:0009862)	1.18% 0.03%	0.001848	0.02741
cell surface receptor linked signal transduction (GO:0007166)	4.73% 1.32%	0.002115	0.03033
lipid metabolic process (GO:0006629)	7.10% 2.66%	0.002108	0.03033
phospholipid transport (GO:0015914)	1.18% 0.04%	0.002531	0.0346
regulation of oxygen and reactive oxygen species metabolic process (GO:0080010)	1.18% 0.04%	0.002531	0.0346
response to xenobiotic stimulus (GO:0009410)	1.18% 0.04%	0.002531	0.0346
transmembrane transport (GO:0055085)	3.55% 0.80%	0.002661	0.03552
sulfur metabolic process (GO:0006790)	2.96% 0.55%	0.002679	0.03552
negative regulation of defense response (GO:0031348)	1.18% 0.04%	0.00291	0.03637
response to hypoxia (GO:0001666)	1.18% 0.04%	0.00291	0.03637
nitrate assimilation (GO:0042128)	1.18% 0.04%	0.00291	0.03637
salicylic acid metabolic process (GO:0009696)	1.18% 0.04%	0.00291	0.03637
lipid biosynthetic process (GO:0008610)	4.73% 1.45%	0.003678	0.04532

Appendix Table 6: GO analysis of genes repressed by *hspro2* knockout mutation

Term	hspro2 vs genome	p value	Adjusted p value
GO BIOLOGICAL PROCESS - level 3 to 9			
response to ethylene (GO:0009723)	7.35% 0.77%	1.40E-10	1.23E-07
response to hormone stimulus (GO:0009725)	11.76% 2.85%	7.68E-09	3.36E-06
response to jasmonic acid (GO:0009753)	4.90% 0.46%	6.70E-08	1.96E-05
glucosinolate metabolic process (GO:0019760)	2.94% 0.13%	5.39E-07	0.000118
response to metal ion (GO:0010038)	6.37% 1.22%	2.06E-06	0.000353
sulfur metabolic process (GO:0006790)	4.41% 0.53%	2.42E-06	0.000353
response to cadmium ion (GO:0046686)	5.39% 1.02%	1.15E-05	0.001436
ethylene-activated signaling pathway (GO:0009873)	3.92% 0.52%	1.62E-05	0.001776
response to oxidative stress (GO:0006979)	4.90% 0.88%	1.96E-05	0.001828
response to chitin (GO:0010200)	3.43% 0.39%	2.09E-05	0.001828
response to temperature stimulus (GO:0009266)	5.88% 1.32%	2.32E-05	0.001842
secondary metabolic process (GO:0019748)	5.88% 1.37%	3.48E-05	0.002537
response to external stimulus (GO:0009605)	5.39% 1.17%	4.02E-05	0.002708
response to auxin (GO:0009733)	4.90% 1.05%	7.84E-05	0.0049
two-component signal transduction system (phosphorelay) (GO:0000160)	3.92% 0.68%	9.86E-05	0.005751
defense response (GO:0006952)	7.84% 2.63%	0.000123	0.006751
response to heat (GO:0009408)	2.94% 0.37%	0.000141	0.007252
anthocyanin-containing compound biosynthetic process (GO:0009718)	1.47% 0.05%	0.000245	0.01192
response to biotic stimulus (GO:0009607)	5.88% 1.74%	0.000306	0.01345
response to carbohydrate stimulus (GO:0009743)	3.43% 0.61%	0.000308	0.01345
response to salicylic acid (GO:0009751)	2.94% 0.44%	0.000378	0.01574
response to salt stress (GO:0009651)	4.41% 1.14%	0.000661	0.02513
anthocyanin-containing compound metabolic process (GO:0046283)	1.47% 0.07%	0.000636	0.02513
flavonoid biosynthetic process (GO:0009813)	1.96% 0.19%	0.000775	0.02824
response to wounding (GO:0009611)	2.94% 0.53%	0.000904	0.03064
response to cold (GO:0009409)	3.92% 0.95%	0.000911	0.03064
response to osmotic stress (GO:0006970)	4.41% 1.23%	0.001134	0.03648
response to gibberellin (GO:0009739)	2.45% 0.37%	0.001167	0.03648

Appendix Table 7: GO analysis of genes induced by *hspro2* knockout mutation

Term	hspro2 vs genome	p value	Adjusted p value
GO Biological process - level 3 to 9			
defense response (GO:0006952)	13.48% 2.60%	7.73E-11	6.77E-08
response to external stimulus (GO:0009605)	8.99% 1.16%	5.73E-10	2.51E-07
response to water deprivation (GO:0009414)	6.18%	3.70E-09	9.16E-07

	0.50%		
response to wounding (GO:0009611)	6.18% 0.51%	4.19E-09	9.16E-07
response to water (GO:0009415)	6.18% 0.53%	6.03E-09	1.02E-06
circadian rhythm (GO:0007623)	3.93% 0.13%	7.62E-09	1.02E-06
response to osmotic stress (GO:0006970)	8.43% 1.21%	8.16E-09	1.02E-06
response to other organism (GO:0051707)	8.99% 1.53%	2.66E-08	2.91E-06
response to biotic stimulus (GO:0009607)	8.99% 1.73%	1.31E-07	1.27E-05
response to cold (GO:0009409)	6.74% 0.94%	1.97E-07	1.72E-05
response to jasmonic acid (GO:0009753)	5.06% 0.46%	2.38E-07	1.9E-05
two-component signal transduction system (phosphorelay) (GO:0000160)	5.62% 0.67%	5.40E-07	3.94E-05
response to temperature stimulus (GO:0009266)	7.30% 1.31%	9.83E-07	5.85E-05
jasmonic acid mediated signaling pathway (GO:0009867)	2.81% 0.09%	1E-06	5.85E-05
oxylipin metabolic process (GO:0031407)	2.81% 0.09%	1E-06	5.85E-05
response to hormone stimulus (GO:0009725)	10.67% 2.87%	1.23E-06	6.42E-05
response to salt stress (GO:0009651)	6.74% 1.13%	1.25E-06	6.42E-05
response to carbohydrate stimulus (GO:0009743)	5.06% 0.60%	1.92E-06	9.34E-05
response to desiccation (GO:0009269)	2.25% 0.05%	2.92E-06	0.000134
intracellular signaling cascade (GO:0007242)	10.67% 3.13%	4.25E-06	0.000186
cold acclimation (GO:0009631)	2.25% 0.05%	4.57E-06	0.000191
ethylene-activated signaling pathway (GO:0009873)	4.49% 0.52%	6.01E-06	0.000239
response to abscisic acid (GO:0009737)	5.62% 0.90%	6.96E-06	0.000265
response to chitin (GO:0010200)	3.93% 0.39%	8.64E-06	0.000315
response to ozone (GO:0010193)	2.25% 0.07%	1.17E-05	0.000409
response to oxidative stress (GO:0006979)	5.06% 0.89%	4.01E-05	0.001351
response to absence of light (GO:0009646)	1.69% 0.03%	4.97E-05	0.00161
hormone-mediated signaling pathway (GO:0009755)	6.18% 1.41%	5.63E-05	0.001758
response to bacterium (GO:0009617)	4.49% 0.74%	7.22E-05	0.002179
defense response to bacterium (GO:0042742)	3.93% 0.55%	7.56E-05	0.002205
response to fungus (GO:0009620)	3.37% 0.38%	8.36E-05	0.00236
hyperosmotic salinity response (GO:0042538)	2.25% 0.12%	9.58E-05	0.00262
response to ethylene (GO:0009723)	4.49% 0.79%	0.000107	0.002842
defense response by callose deposition (GO:0052542)	1.69% 0.05%	0.000139	0.003573
hyperosmotic response (GO:0006972)	2.25% 0.14%	0.000176	0.004397
regulation of defense response (GO:0031347)	2.25% 0.16%	0.000276	0.006519
fatty acid metabolic process (GO:0006631)	3.93% 0.68%	0.000272	0.006519
regulation of long-day photoperiodism, flowering (GO:0048586)	1.12% 0.01%	0.000318	0.007332
response to light stimulus (GO:0009416)	5.62% 1.45%	0.000337	0.00755

fatty acid biosynthetic process (GO:0006633)	3.37% 0.50%	0.000354	0.007748
response to radiation (GO:0009314)	5.62% 1.50%	0.000418	0.008924
cellular response to water deprivation (GO:0042631)	1.12% 0.01%	0.000476	0.009913
response to light intensity (GO:0009642)	2.25% 0.22%	0.000732	0.01489
immune response (GO:0006955)	4.49% 1.07%	0.000774	0.01539
vegetative to reproductive phase transition of meristem (GO:0010228)	2.25% 0.22%	0.000813	0.0158
carboxylic acid metabolic process (GO:0019752)	7.30% 2.59%	0.000883	0.0168
response to fructose stimulus (GO:0009750)	1.12% 0.02%	0.001129	0.02102
long-day photoperiodism, flowering (GO:0048574)	1.12% 0.03%	0.001406	0.02563
innate immune response (GO:0045087)	3.93% 0.91%	0.001448	0.02586
defense response to fungus (GO:0050832)	2.25% 0.27%	0.001562	0.02734
photoperiodism, flowering (GO:0048573)	1.69% 0.12%	0.001648	0.02827
photoperiodism (GO:0009648)	1.69% 0.13%	0.002022	0.03402
carbohydrate transport (GO:0008643)	2.25% 0.29%	0.002076	0.03427
plant-type hypersensitive response (GO:0009626)	1.69% 0.14%	0.002157	0.03495
host programmed cell death induced by symbiont (GO:0034050)	1.69% 0.14%	0.002298	0.03656
cell surface receptor linked signal transduction (GO:0007166)	4.49% 1.32%	0.002912	0.04549

Appendix Table 8: GO analysis of genes repressed by *hspro1-2*/*hspro2* knockout mutation

Term	hspro1-2/hspro2 vs genome	p value	Adjusted p value
GO BIOLOGICAL PROCESS (levels from 3 to 9)			
response to chitin (GO:0010200)	9.26% 0.38%	2.35E-11	2.06E-08
response to carbohydrate stimulus (GO:0009743)	9.26% 0.59%	1.60E-09	7.02E-07
response to ethylene (GO:0009723)	9.26% 0.78%	1.94E-08	5.66E-06
ethylene-activated signaling pathway (GO:0009873)	6.48% 0.52%	2.09E-06	0.000458
two-component signal transduction system (phosphorelay) (GO:0000160)	6.48% 0.68%	1.11E-05	0.00195
response to hormone stimulus (GO:0009725)	12.04% 2.88%	0.000016	0.002334
defense response (GO:0006952)	10.19% 2.63%	0.000146	0.01607
response to salicylic acid (GO:0009751)	4.63% 0.45%	0.000147	0.01607
immune response (GO:0006955)	6.48% 1.07%	0.000184	0.01654
response to jasmonic acid (GO:0009753)	4.63% 0.47%	0.000189	0.01654
response to wounding (GO:0009611)	4.63% 0.53%	0.000317	0.02523
innate immune response (GO:0045087)	5.56% 0.91%	0.000533	0.03884

Appendix Table 9: GO analysis of genes induced by *hspro1-2/hspro2* knockout mutation

Term	hspro1-2/hspro2 vs genome	p value	Adjusted p value

GO Biological process – levels from 3 to 9			
response to cold (GO:0009409)	10.81% 0.93%	1.47E-12	1.29E-09
response to temperature stimulus (GO:0009266)	11.49% 1.30%	1.92E-11	8.40E-09
response to osmotic stress (GO:0006970)	10.81% 1.20%	6.39E-11	1.86E-08
two-component signal transduction system (phosphorelay) (GO:0000160)	8.11% 0.66%	6.15E-10	1.08E-07
response to water deprivation (GO:0009414)	7.43% 0.50%	5.22E-10	1.08E-07
response to water (GO:0009415)	7.43% 0.53%	8.57E-10	1.25E-07
response to hormone stimulus (GO:0009725)	14.19% 2.86%	2.27E-09	2.83E-07
ethylene-activated signaling pathway (GO:0009873)	6.76% 0.51%	8.70E-09	9.51E-07
cold acclimation (GO:0009631)	3.38% 0.05%	3.24E-08	3.154E-06
response to other organism (GO:0051707)	9.46% 1.54%	1.04E-07	9.127E-06
response to external stimulus (GO:0009605)	8.11% 1.17%	2.57E-07	2.047E-05
response to ethylene (GO:0009723)	6.76% 0.78%	3.85E-07	2.665E-05
response to carbohydrate stimulus (GO:0009743)	6.08% 0.60%	4.11E-07	2.665E-05
response to biotic stimulus (GO:0009607)	9.46% 1.73%	4.27E-07	2.665E-05
defense response (GO:0006952)	11.49% 2.62%	4.66E-07	0.0000272
response to light intensity (GO:0009642)	4.05% 0.21%	1.126E-06	0.0000616
response to salt stress (GO:0009651)	7.43% 1.13%	1.334E-06	6.864E-05
hormone-mediated signaling pathway (GO:0009755)	8.11% 1.40%	1.622E-06	7.885E-05
response to glucose stimulus (GO:0009749)	2.70% 0.05%	1.765E-06	8.127E-05
response to ozone (GO:0010193)	2.70% 0.07%	5.634E-06	0.0002465
response to abscisic acid (GO:0009737)	6.08% 0.90%	1.064E-05	0.0004433
oxylipin metabolic process (GO:0031407)	2.70% 0.09%	1.561E-05	0.0006208
response to wounding (GO:0009611)	4.73% 0.52%	1.738E-05	0.0006613
response to absence of light (GO:0009646)	2.03% 0.03%	2.867E-05	0.001045
intracellular signaling cascade (GO:0007242)	10.14% 3.14%	0.0000772	0.002598
response to light stimulus (GO:0009416)	6.76% 1.45%	7.446E-05	0.002598
response to desiccation (GO:0009269)	2.03% 0.05%	8.039E-05	0.002605
fatty acid metabolic process (GO:0006631)	4.73% 0.68%	8.687E-05	0.002715
response to radiation (GO:0009314)	6.76% 1.49%	9.355E-05	0.002823
fatty acid biosynthetic process (GO:0006633)	4.05% 0.50%	0.0001307	0.003812
response to fungus (GO:0009620)	3.38% 0.39%	0.0003294	0.009298
response to chitin (GO:0010200)	3.38% 0.39%	0.0003544	0.00969
systemic acquired resistance (GO:0009627)	2.03% 0.09%	0.0004233	0.01122
innate immune response (GO:0045087)	4.73% 0.91%	0.0004911	0.01264
carboxylic acid metabolic process (GO:0019752)	8.11% 2.60%	0.0005494	0.01373
response to sucrose stimulus (GO:0009744)	2.03% 0.11%	0.0006609	0.01606

response to fructose stimulus (GO:0009750)	1.35% 0.02%	0.0007832	0.01852
response to bacterium (GO:0009617)	4.05% 0.75%	0.0009984	0.02299
hyperosmotic salinity response (GO:0042538)	2.03% 0.13%	0.001041	0.02335
response to high light intensity (GO:0009644)	2.03% 0.13%	0.001115	0.02439
immune response (GO:0006955)	4.73% 1.07%	0.001218	0.026
circadian rhythm (GO:0007623)	2.03% 0.14%	0.001357	0.02828
lipid biosynthetic process (GO:0008610)	5.41% 1.45%	0.001618	0.0317
defense response to bacterium (GO:0042742)	3.38% 0.56%	0.001619	0.0317
hyperosmotic response (GO:0006972)	2.03% 0.15%	0.00163	0.0317
regulation of ethylene-activated signaling pathway (GO:0010104)	1.35% 0.04%	0.001676	0.03188
regulation of defense response (GO:0031347)	2.03% 0.17%	0.002274	0.04234

Appendix Table 10: Genes significantly differentially expressed in at least one mutant line in the *hspro* microarray

Gene ID	*hspro1-2* log$_2$	*hspro2* log$_2$	*hspro1-2/hspro2* log$_2$
AT2G15020	-1.413	-1.881	-1.285
AT1G73540		-1.756	-1.355
AT3G02380	-0.911	-1.653	-1.094
AT1G22190		-1.537	-1.035
AT1G72430	-1.223	-1.465	-1.328
AT1G74930		-1.460	-1.174
AT3G46080		-1.373	
AT3G56360		-1.359	
AT3G10113	-0.650	-1.358	-1.215
AT1G32920		-1.355	-1.767
AT5G35480		-1.355	
AT3G52740	-0.982	-1.342	-1.221
AT5G54585		-1.340	-0.813
AT1G13245		-1.324	
AT4G36040		-1.317	-1.019
AT4G29905		-1.299	-0.848
AT4G08300		-1.185	
AT5G01820		-1.178	-1.294
AT4G26850		-1.177	-1.054
AT5G56550	-0.641	-1.145	
AT5G61600		-1.145	-1.473
AT1G23390		-1.144	
AT5G05250		-1.136	
AT3G44450	-0.612	-1.112	-0.918
AT3G19710		-1.097	
AT2G04795	-0.641	-1.086	-0.617
AT5G53450		-1.086	
AT5G24150		-1.084	-0.957
AT3G12320		-1.083	-0.922
AT3G30180	-0.766	-1.080	
AT1G68840		-1.060	-1.183
AT3G06145		-1.044	-1.066
AT2G14610	2.965	-1.038	2.056
AT1G10020	-0.813	-1.032	-1.191
AT3G16420		-1.028	
AT2G47440	-0.880	-1.010	-0.983
AT1G76960	0.980	-1.006	
AT5G67420		-0.990	-0.785
AT4G38620	-0.768	-0.987	
AT5G57560		-0.983	
AT3G18773		-0.983	
AT2G30650	-0.737	-0.977	-1.007
AT5G51390		-0.974	
AT5G44680		-0.971	
AT1G71030		-0.968	-0.863
AT2G38160	-0.875	-0.967	
AT1G32928		-0.967	-0.907
AT1G66230		-0.954	
AT1G56430		-0.954	
AT4G17460	-0.838	-0.946	-0.738
AT5G24660		-0.942	-1.095
AT2G38310	-1.120	-0.940	
AT4G13395		-0.939	
AT2G38210		-0.923	-1.365
AT1G76600		-0.921	-0.989
AT3G52360		-0.920	
AT1G04770		-0.917	-0.897
AT4G15248	-0.698	-0.915	
AT5G60400		-0.913	
AT2G17300		-0.912	
AT5G44420	0.946	-0.912	
AT3G21670		-0.910	
AT5G64060		-0.906	-1.004
AT2G21185		-0.900	-0.971
AT1G78080		-0.885	-0.875
AT4G16880		-0.872	-0.929

Gene ID	*hspro1-2* log$_2$	*hspro2* log$_2$	*hspro1-2/hspro2* log$_2$
AT1G22770		0.763	
AT5G48380	1.283	0.763	1.200
AT5G45750	0.762	0.776	
AT1G20840		0.778	
AT2G47060	0.900	0.781	
AT3G50960	0.909	0.783	
AT2G31810		0.788	0.835
AT3G23550		0.791	
AT5G65310		0.799	
AT1G22510	0.691	0.799	
AT1G70700		0.800	
AT1G28480	0.762	0.815	
AT4G23180	1.143	0.817	0.673
AT2G15090	0.756	0.820	0.715
AT4G02380	1.390	0.825	1.257
AT2G13800	0.955	0.836	0.854
AT3G24420		0.837	0.691
AT1G13210	1.068	0.839	0.765
AT3G51060		0.846	
AT4G38470		0.846	0.653
AT3G15450	1.035	0.850	1.337
AT4G36670	0.950	0.855	1.185
AT4G16146		0.860	0.690
AT3G03341	0.627	0.863	1.487
AT4G01026	0.671	0.867	1.033
AT4G33980		0.873	1.104
AT3G10020		0.886	
AT5G02020		0.887	1.405
AT5G06860		0.888	
AT5G44210		0.890	1.170
AT1G07000	1.631	0.894	
AT5G57220	0.963	0.894	
AT5G61380		0.921	0.859
AT5G04480		0.922	
AT3G21070	0.999	0.922	
AT3G53330		0.932	0.620
AT1G52720		0.933	
AT2G24600	1.156	0.933	
AT2G21660		0.937	
AT1G74950		0.937	
AT2G34930		0.953	0.916
AT1G77680		0.958	1.104
AT1G30250		0.958	1.011
AT5G47240		0.964	0.936
AT1G21680		0.966	
AT1G20030		0.976	0.885
AT5G20230	1.537	0.977	1.251
AT5G11330	1.014	0.978	1.021
AT1G73080		0.983	
AT5G52310		0.991	1.088
AT5G25350		1.010	1.136
AT1G02710		1.038	
AT3G50950	1.532	1.038	1.187
AT1G20510	0.783	1.039	
AT1G60140	0.923	1.045	
AT3G43850		1.046	
AT5G54170		1.049	
AT4G28490	0.683	1.052	
AT2G41100	1.411	1.056	0.733
AT1G07050		1.070	1.152
AT2G02100		1.074	1.103
AT5G15970		1.079	1.260
AT2G42540		1.083	0.932
AT5G24470	0.758	1.093	0.814
AT2G39800		1.093	
AT4G21570	0.908	1.094	1.214

AT3G13310		-0.871	
AT2G40330		-0.866	
AT1G13260		-0.865	
AT1G47400		-0.865	
AT3G47640		-0.861	
AT5G19190		-0.861	
AT1G51700		-0.855	-0.972
AT3G02910		-0.854	-0.965
AT5G24780	-0.840	-0.851	
AT1G06830		-0.851	
AT3G23170	-0.715	-0.847	-0.786
AT4G38860	-0.757	-0.843	
AT1G55330		-0.840	-1.078
AT1G34060	-0.685	-0.836	
AT3G04140	-0.839	-0.827	-1.228
AT2G18160		-0.824	-0.609
AT4G04830		-0.820	
AT5G20150	-0.701	-0.817	
AT5G53500		-0.816	
AT3G10930		-0.813	
AT3G61060		-0.811	
AT4G28290		-0.809	-0.731
AT1G53870		-0.802	
AT1G18590		-0.802	
AT2G07671		-0.801	
AT2G39030	0.625	-0.794	
AT1G76240		-0.786	-0.744
AT2G31380		-0.780	
AT3G46620		-0.779	-0.807
AT5G13930	-0.717	-0.779	-1.044
AT1G45201	-0.945	-0.778	
AT2G37760		-0.778	
AT5G49740		-0.776	-0.651
AT3G19550		-0.776	
AT1G25400		-0.775	
AT1G16410		-0.774	
AT1G68570		-0.773	
AT1G75830	0.803	-0.772	
AT5G36910		-0.769	
AT3G50060		-0.769	-0.768
AT1G18300		-0.761	
AT1G49470		-0.760	
AT1G18810	-0.661	-0.759	
AT1G72416		-0.758	
AT2G09970		-0.756	
AT1G23710		-0.753	-0.794
AT3G22840	-0.668	-0.752	-0.915
AT2G16280		-0.752	
AT1G07610		-0.751	
AT1G70810		-0.747	
AT1G76990		-0.747	
AT2G40610		-0.746	-0.869
AT1G02816		-0.743	
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AT5G01790	-0.698	-0.740	-0.807
AT4G21870		-0.740	
AT4G27280		-0.738	-1.070
AT1G62180		-0.737	-0.784
AT2G14247		-0.731	
AT4G34630		-0.728	
AT4G26350		-0.726	
AT1G72910		-0.724	-1.037
AT2G05050		-0.723	-0.804
AT5G67390		-0.723	-0.670
AT5G54490		-0.717	
AT3G03190	-0.731	-0.713	
AT4G38850		-0.712	
AT3G61270		-0.712	
AT2G47180		-0.706	
AT1G80920		-0.706	
AT4G15530		-0.703	
AT3G16470		-0.703	
AT1G62560		-0.703	
AT2G26530		-0.701	-0.821
AT5G35490		-0.699	
AT2G32880		-0.699	

AT4G31800	0.690	1.124	
AT1G20450	0.946	1.129	1.563
AT1G64380	0.583	1.132	0.889
AT5G41600		1.137	1.250
AT1G28330	0.811	1.141	1.354
AT3G57520		1.149	0.914
AT3G24520		1.152	
AT1G73480	1.001	1.162	1.031
AT5G52320		1.167	
AT4G30650		1.169	1.249
AT1G09970	0.875	1.173	0.764
AT4G36850		1.176	
AT1G05680	1.343	1.189	0.750
AT2G29720	1.113	1.211	
AT2G18700	0.782	1.225	0.988
AT1G32640		1.298	
AT5G15650	0.848	1.298	1.311
AT2G40230		1.308	0.934
AT4G24960		1.310	1.467
AT5G27520		1.317	0.848
AT4G17230	1.199	1.318	
AT3G50260	1.397	1.338	1.297
AT2G06050	0.896	1.470	1.599
AT2G42530	0.711	1.540	1.323
AT5G66210	1.563	1.544	1.296
AT4G36010		1.576	1.339
AT4G28040		1.586	
AT2G33830	1.012	1.600	1.509
AT1G61890	0.965	1.679	
AT5G42900		1.721	1.955
AT5G47220	1.139	1.729	0.980
AT3G25760	0.794	1.738	1.616
AT1G72520	1.419	1.761	0.846
AT3G47340	0.653	1.939	1.585
AT3G59350	1.872	1.970	1.630
AT1G17420	1.419	2.125	1.303
AT5G56870			0.980
AT2G15890	0.616		0.913
AT3G62550			0.807
AT5G65730			-0.802
AT2G04790			-0.710
AT3G01290	1.010		1.006
AT1G02660	0.754		
AT2G28630			0.725
AT1G72070	0.658		0.965
AT2G15960			1.288
AT3G47430			-0.875
AT1G03850	0.892		
AT1G64500			-0.729
AT1G03870			-0.840
AT2G39400			0.807
AT5G24490			0.747
AT5G01015			-0.786
AT1G10340	1.147		
AT1G76590			0.757
AT5G57655			0.788
AT2G26560	1.655		0.956
AT4G25810			-0.755
AT4G19530			-0.730
AT1G08930			0.750
AT1G14345	-0.672		-0.880
AT5G45820			-0.719
AT4G26520			-0.859
AT4G12730			-0.917
AT1G56510	1.056		
AT5G62280			0.647
AT2G18670	1.005		
AT1G65390	0.784		0.925
AT5G17640			-0.719
AT3G55980	0.618		-1.152
AT3G10720	0.632		
AT4G14270			0.767
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AT4G12490	0.874	-0.684	
AT5G59080		-0.682	
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AT3G19680	-0.998	-0.678	
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AT4G27657		-0.671	
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AT3G11090	-0.619	-0.657	-0.791
AT2G45660		-0.657	
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AT1G23020		-0.646	
AT3G03780		-0.645	
AT1G13650		-0.641	-0.675
AT1G71970		-0.639	
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AT1G73870		-0.637	
AT5G24120		-0.637	
AT3G19580		-0.636	
AT5G37550		-0.634	
AT4G13770		-0.634	
AT2G23430		-0.633	
AT5G54160		-0.633	
AT3G63200		-0.628	
AT5G15310		-0.624	
AT2G27420		-0.624	-0.774
AT1G01430		-0.624	
AT3G02020		-0.623	
AT1G10370		-0.621	-0.654
AT3G14200		-0.621	
AT2G41250		-0.619	
AT4G17870		-0.615	-0.595
AT4G37800		-0.613	
AT2G05440		-0.605	
AT4G01340		-0.603	
AT3G56240		-0.601	
AT1G78600		-0.599	
AT5G57350		-0.596	
AT5G27350		-0.596	
AT1G26210		-0.595	-0.986
AT1G48480		-0.593	-0.835
AT5G26200		-0.582	
AT4G28240		-0.582	
AT3G58990		-0.570	
AT5G04080		-0.567	
AT4G11360		-0.560	
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AT3G05640		0.568	
AT5G17860		0.575	
AT1G02405		0.576	
AT5G11990		0.584	
AT1G72450		0.586	
AT4G29900		0.592	
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AT4G08410		0.604	
AT4G15450		0.609	
AT2G22470		0.613	
AT1G61360		0.622	
AT4G36900		0.630	
AT4G03420		0.630	0.963
AT3G07650		0.632	

AT4G15610			0.684
AT1G37130	0.753		
AT2G40270	0.580		
AT2G30250	0.655		
AT5G24530	0.822		
AT2G23810	0.831		
AT4G16860	0.726		
AT2G05530			0.737
AT5G42830	1.077		
AT1G21310	1.241		0.931
AT4G25030	0.743		
AT1G21130	0.928		
AT5G05410	0.823		
AT3G50480	0.802		
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AT1G76930	0.949		
AT2G25490			0.690
AT2G18690	1.150		0.722
AT5G47230			-0.723
AT1G72940			-0.804
AT3G06770			-0.662
AT5G22690	0.759		
AT5G26920	1.071		
AT3G19030			-1.170
AT4G33050	1.194		
AT3G56400			-1.088
AT1G50740	0.888		
AT4G13800			-0.665
AT2G39210	0.618		
AT5G02290	0.790		
AT3G55450	0.650		0.876
AT2G21210			-0.824
AT1G80840			-1.010
AT2G38790	0.637		
AT3G52430	0.828		
AT1G17665			0.767
AT2G05380			1.066
AT4G37990	0.763		
AT5G47070	0.769		
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AT2G15390	0.665		
AT4G34390	0.693		
AT3G45640	0.797		
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AT4G22780			0.745
AT1G19020	0.749		
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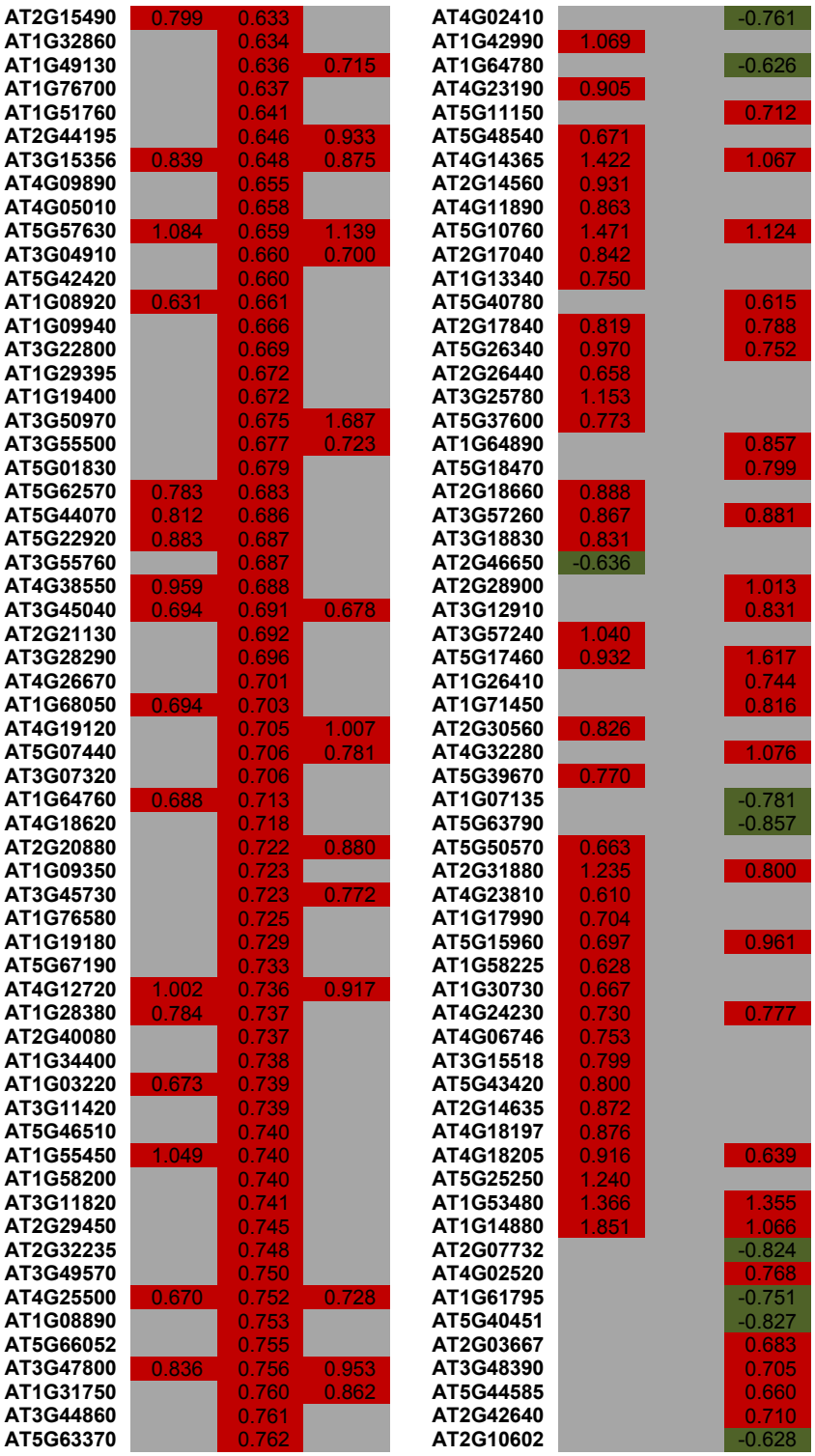
AT5G15730	0.743		
AT1G10960			-0.677
AT2G21320			-0.704
AT1G26380	0.749		
AT2G44490	0.847		
AT5G62360			1.029
AT3G25840			0.737
AT2G32240			0.780
AT4G12480	0.957		0.681
AT1G72920			-0.824
AT5G51570			0.634
AT1G01470			0.983
AT5G57800			-0.651
AT1G35720			0.724
AT5G66740			-0.765
AT3G26210			0.775
AT3G48090	0.741		
AT3G61630			0.609
AT4G34120	0.800		0.775
AT1G20440			1.338
AT2G37940	1.308		
AT3G25610	0.697		



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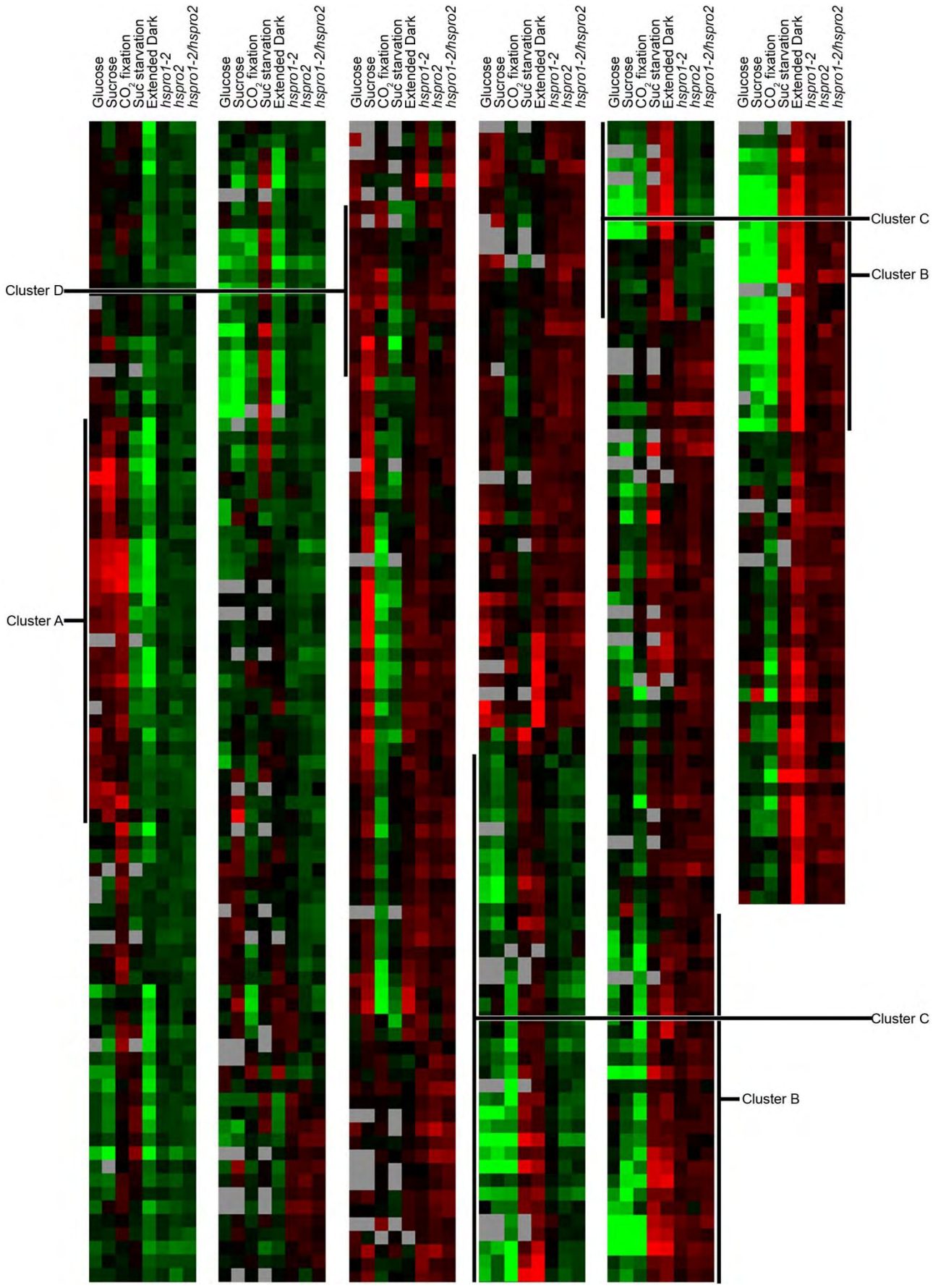
AT1G49130		0.636	0.715	AT1G64780			-0.626
AT1G76700		0.637		AT4G23190	0.905		
AT1G51760		0.641		AT5G11150			0.712
AT2G44195		0.646	0.933	AT5G48540	0.671		
AT3G15356	0.839	0.648	0.875	AT4G14365	1.422		1.067
AT4G09890		0.655		AT2G14560	0.931		
AT4G05010		0.658		AT4G11890	0.863		
AT5G57630	1.084	0.659	1.139	AT5G10760	1.471		1.124
AT3G04910		0.660	0.700	AT2G17040	0.842		
AT5G42420		0.660		AT1G13340	0.750		
AT1G08920	0.631	0.661		AT5G40780			0.615
AT1G09940		0.666		AT2G17840	0.819		0.788
AT3G22800		0.669		AT5G26340	0.970		0.752
AT1G29395		0.672		AT2G26440	0.658		
AT1G19400		0.672		AT3G25780	1.153		
AT3G50970		0.675	1.687	AT5G37600	0.773		
AT3G55500		0.677	0.723	AT1G64890			0.857
AT5G01830		0.679		AT5G18470			0.799
AT5G62570	0.783	0.683		AT2G18660	0.888		
AT5G44070	0.812	0.686		AT3G57260	0.867		0.881
AT5G22920	0.883	0.687		AT3G18830	0.831		
AT3G55760		0.687		AT2G46650	-0.636		
AT4G38550	0.959	0.688		AT2G28900			1.013
AT3G45040	0.694	0.691	0.678	AT3G12910			0.831
AT2G21130		0.692		AT3G57240	1.040		
AT3G28290		0.696		AT5G17460	0.932		1.617
AT4G26670		0.701		AT1G26410			0.744
AT1G68050	0.694	0.703		AT1G71450			0.816
AT4G19120		0.705	1.007	AT2G30560	0.826		
AT5G07440		0.706	0.781	AT4G32280			1.076
AT3G07320		0.706		AT5G39670	0.770		
AT1G64760	0.688	0.713		AT1G07135			-0.781
AT4G18620		0.718		AT5G63790			-0.857
AT2G20880		0.722	0.880	AT5G50570	0.663		
AT1G09350		0.723		AT2G31880	1.235		0.800
AT3G45730		0.723	0.772	AT4G23810	0.610		
AT1G76580		0.725		AT1G17990	0.704		
AT1G19180		0.729		AT5G15960	0.697		0.961
AT5G67190		0.733		AT1G58225	0.628		
AT4G12720	1.002	0.736	0.917	AT1G30730	0.667		
AT1G28380	0.784	0.737		AT4G24230	0.730		0.777
AT2G40080		0.737		AT4G06746	0.753		
AT1G34400		0.738		AT3G15518	0.799		
AT1G03220	0.673	0.739		AT5G43420	0.800		
AT3G11420		0.739		AT2G14635	0.872		
AT5G46510		0.740		AT4G18197	0.876		
AT1G55450	1.049	0.740		AT4G18205	0.916		0.639
AT1G58200		0.740		AT5G25250	1.240		
AT3G11820		0.741		AT1G53480	1.366		1.355
AT2G29450		0.745		AT1G14880	1.851		1.066
AT2G32235		0.748		AT2G07732			-0.824
AT3G49570		0.750		AT4G02520			0.768
AT4G25500	0.670	0.752	0.728	AT1G61795			-0.751
AT1G08890		0.753		AT5G40451			-0.827
AT5G66052		0.755		AT2G03667			0.683
AT3G47800	0.836	0.756	0.953	AT3G48390			0.705
AT1G31750		0.760	0.862	AT5G44585			0.660
AT3G44860		0.761		AT2G42640			0.710
AT5G63370		0.762		AT2G10602			-0.628

Appendix Figure 1: Global gene expression of energy responsive genes in the *hspro* mutants



A subset of genes differentially expressed in at least one of the three *hspro* mutants (but not necessarily all of them) was compared to genes differentially expressed under varying energy status. Cluster A genes are repressed in *hspro* mutants and sugar induced/starvation repressed, cluster B genes are induced in *hspro*

mutants and sugar repressed/starvation induced, while cluster C genes are repressed in the mutants but sugar repressed/starvation induced and cluster D genes are induced in the mutants and sugar induced/starvation repressed.

Appendix Table 11: Broad range of stress associated GO categories enriched for in *hspro* mutants microarray

	***hspro1-2* up**	***hspro2* down**	***hspro2* up**	***hspro1-2/hspro2* down**	***hspro1-2/hspro2* up**
RESPONSE TO HORMONE STIMULUS					
JA	✓	✓	✓	✓	
SA	✓	✓		✓	
Auxin		✓			
ET		✓	✓	✓	✓
GA		✓			
ABA			✓		✓
HORMONE SIGNAL TRANSDUCTION					
ET mediated signalling		✓	✓	✓	
JA mediated signalling			✓		
STRESS RESPONSES					
Water deprivation	✓		✓		✓
Water stimulus	✓		✓		✓
Osmotic stress	✓	✓	✓		✓
Salt stress	✓	✓	✓		✓
Cold stress			✓		✓
Cold acclimation			✓		
Temperature stimulus	✓	✓	✓		✓
Heat stress		✓			
Light intensity/stimulus	✓		✓		✓
Hypoxia	✓				
Ozone	✓		✓		✓
Oxidative stress	✓	✓	✓		
Radiation	✓		✓		✓
Wounding	✓	✓	✓	✓	✓

JA – jasmonic acid, SA – salicylic acid, ET – ethylene, GA – gibberellin, ABA – abscisic acid

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