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Understanding the role of ubiquitin signalling in barley to improve crop health

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Abstract

Barley (*Hordeum vulgare*) is the second most important crop in the UK and ranks fourth among cereal crops globally. Yet, it is highly vulnerable to diseases, including those caused by fungal pathogens, such as *Puccinia hordei*, which can lead to yield losses of up to 40%, posing a serious threat to food security. Understanding and enhancing plant immune responses against pests is therefore essential to safeguarding crop productivity, and there is a pressing need for innovative, mechanistically informed strategies.

Ubiquitination is a complex protein posttranslational modification, which serves various functions from modulating protein activity, localisation, and stability, to promoting protein degradation. Previous studies showed that it plays a pivotal role in regulating plant immune responses. Most of this work, however, is undertaken in model plant species, and little is known about immune-related ubiquitination in crops. This knowledge gap has hindered efforts to utilise the ubiquitin pathway as a strategy for enhancing pest resistance in crops. In this study, I provide a comprehensive profiling of the barley immune ubiquitome, to uncover molecular mechanisms that could be harnessed to enhance resistance to fungal pathogens.

This work has been divided into three chapters. Given the strict regulations on genetically modified or edited crops and the urgent need to reduce pesticide use, alternative strategies to enhance crop resilience are essential. In Chapter 3, I explore the use of arbuscular mycorrhizal fungi (AMF) as tools to prime barley immune responses. These results suggest, that AMF colonisation alters the barley transcriptome and modulates expression of ubiquitin-related genes during *P. hordei* infection. This suggests that AMF-mediated priming involves the ubiquitin system as part of a broader immune regulatory network.

Chapter 4 addresses the dynamic nature of ubiquitin signalling in barley during the immune response. Here, I perform an immune ubiquitome profiling by identifying ubiquitin-regulated proteome during (i) hormone-induced immune responses, (ii) pathogen infection in the lab, and (iii) in field conditions. The data reveal that the barley immune ubiquitome is signal-specific and dynamic, with a clear modulation of protein abundance and turnover. Common pathways and novel ubiquitinated targets have been identified, providing a comprehensive view of how ubiquitin signalling

contributes to barley immune responses and offering new candidate proteins for the improvement of crop resistance.

E3 ubiquitin ligases are the central specificity determinants of the ubiquitination cascade. Identifying immune-related E3 ligases and their substrates is key to understanding the regulatory nodes within the ubiquitin system. In Chapter 5 I employed a substrate-trapping approach to identify substrates of HvRGLG2, a putative immune-related E3 ligase in barley. The captured targets were implicated in core immune processes, and I demonstrated that their ubiquitination directly modulates immune function at the molecular level.

Taken together, these findings highlight two complementary routes to strengthen barley immunity: (i) exploiting beneficial associations with arbuscular mycorrhizal fungi (AMF) as sustainable strategies to enhance crop health, and (ii) targeting ubiquitin-mediated immune regulation to uncover novel resistance mechanisms. The immune ubiquitome generated in this study provides a valuable resource for understanding stress-responsive pathways and identifying new targets for resistance breeding. Furthermore, the substrate-trapping method developed here offers a scalable approach for mapping E3 ligase–substrate interactions in crops. Collectively, this research lays the groundwork for innovative tools to improve crop health and resilience.

Lay summary

Plant diseases pose a serious threat to food security, causing up to 40% of total crop yield losses. As climate change is predicted to provide more favourable conditions for many crop pathogens, it is important to develop strategies to protect our most valuable crops. Barley is the second most important crop in the UK and the most important in Scotland; however, it is vulnerable to various diseases, including brown rust caused by the fungal pathogen *Puccinia hordei*. There are many ways in which we can improve plant health. Some of them use symbiotic microorganisms to boost plant immune responses. Others involve breeding techniques to improve plant's ability to fight diseases. By understanding how plants protect themselves against pathogens, we can develop effective ways to improve crop health.

As sessile organisms, plants need to adjust quickly to changes in their environment. Proteins are the "workhorses" of the cell, responsible for all plant responses at the molecular level. Their activity, localisation and stability can be controlled by a small modifier – ubiquitin. Ubiquitin has previously been shown to regulate plant immunity; however, little is known about immune-related ubiquitination in crops. In this thesis, I explore how we can improve barley health and demonstrate how ubiquitin regulates barley immune responses.

Our results show that root-associated symbiotic fungi can prepare barley for pathogen attack, by "priming" the plant immune responses and modulating the ubiquitin system. I further generated a comprehensive map of ubiquitin-regulated proteins in barley during brown rust infection, identifying key pathways and proteins under ubiquitin control. Finally, I confirmed the role of a specific ubiquitination enzyme in targeting two immune proteins for ubiquitination. Together, these findings establish ubiquitination as a central regulator of barley immunity, highlighting practical avenues for improving crop health and resilience.

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Declaration

I declare that the thesis has been composed by myself and that the work has not be submitted for any other degree or professional qualification. I confirm that the work submitted is my own, except where work which has formed part of jointly-authored publications has been included. My contribution and those of the other authors to this work have been explicitly indicated below. I confirm that appropriate credit has been given within this thesis where reference has been made to the work of others.

The work presented in Chapter 3 was previously published in FEMS Microbiology Letters as “AMF primes immune genes against *Puccinia hordei* (Brown rust) in *Hordeum vulgare* but does not reduce pathogen burden.” by Moulton-Brown, C., Brzezinska, K., Orosa-Puente, B. & Helgason, T. This study was conceived by all of the authors. I carried out the experimental setup, root staining, data collection and processing for the leaf tissue, transcriptomic sample preparation and processing, data analysis and visualisation.

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List of Abbreviations

ACX	Acyl-CoA oxidase
AIM1	Abnormal inflorescence meristem
AMF	Arbuscular mycorrhizal fungi
AOC	Allene oxide cyclase
AOS	Allene oxide synthase
ATL	Arabidopsis toxicos en levadura
BA2H	Benzoic acid 2-hydroxylase
BD1	Bromodomain-containing protein 1
BIK1	Botrytis-induced kinase 1
BP	Biological process
CAM5	Calmodulin 5
CAT2	Catalase 2
cDNA	complementary DNA
CDPK	Calcium-dependent protein kinases
CEBiP	Chitin elicitor binding protein
CERK1	Chitin elicitor receptor kinase 1
CM	Chorismate mutase
COI1	Coronatine insensitive 1
COs	Chitooligosaccharides
CRL	Cullin-RING ligase
CTR1	Constitutive triple response 1
CUL1	CULLIN
DEG	Differentially expressed gene
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin ligase
EDS5	Enhanced disease susceptibility 5
EDTA	Ethylenediaminetetraacetic acid
EIN	Ethylene insensitive
EF1a	Elongation factor 1a

EPS1	Enhanced pseudomonas susceptibility 1
ERF	Ethylene responsive transcription factor
ET	Ethylene
ETI	Effector triggered immunity
GO	Gene Ontology
HECT	Homologous to E6-associated protein 7 C-Terminus
HSP70	Heat shock protein 70
ICS	Isochorismate synthase
ISR	Induced systemic resistance
JA	Jasmonic acid
JAR1	Jasmonate Resistant 1
JAZ	Jasmonate-zim domain
KAT	Ketoacyl-CoA thiolase
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC-MS/MS	Liquid chromatography coupled with tandem mass spectrometry
LOX	Lipoxygenase
MAMP	Microbial-associated molecular patterns
MAPK	Mitogen-activated protein kinase
MCL	Markov Cluster Algorithm
MIR	Mycorrhiza-induced resistance
MLA	Mildew Locus a
NB-LRR	Nucleotide binding and leucine rich repeat
NILs	Near-isogenic lines
NLR	Nucleotide-binding domain and C-terminal leucine-rich repeats
NPR1	Non-expressor of pathogenesis related gene 1
OPDA	Cis-12-oxophytodienoic acid
OPR3	Oxophytodienoic acid reductase 3
PAL	Phenylalanine ammonia lyase
PBS3	avrPphB SUSCEPTIBLE3
Psm	*Pseudomonas syringae pv. maculicola*
PTI	Pattern triggered immunity
PR	Pathogenesis related

PRR	Pattern recognition receptor
PUB	Plant U-box
RBR	RING between RING
RBX1	RING-box 1
RGLG2	Ring domain ligase 2
RING	Really Interesting New Gene
RLK1	Receptor-like serine/threonine-protein kinase 1
ROS	Reactive Oxygen Species
Rph	Resistance to *Puccinia hordei* (gene)
SA	Salicylic acid
SAR	Systemic acquired resistance
SCF	Skp1/Cul1/F-box
SynRGLG2	Synthetic RGLG2
tCA	Trans-cinnamic acid
TLP5	Thaumatin-Like Protein
TUBE	Tandem-repeated ubiquitin-binding entities
UBA1	Ubiquitin activating enzyme
UBC26	Ubiquitin conjugating-enzyme 26
UBD	Ubiquitin-binding domain
UPL1	Ubiquitin protein ligase 1
UPS	Ubiquitin proteasome system
USP2	Ubiquitin carboxyl-terminal hydrolase 2

Chapter 1

Introduction

1.1 Pathogens and food security

According to the Food and Agriculture Organisation of the United Nations, 1.28 billion ton increase in annual crop yield is required to meet the growing demand for cereal products in the next 10 years (OECD-FAO, 2024). Achieving this target will depend not only on improving productivity but also on mitigating crop losses caused by diseases, the leading cause of both pre- and postharvest yield reduction. It is estimated that up to 40% of global crop yield is lost annually due to pests and pathogens (FAO, 2021). These losses pose a significant threat to global food security and place considerable strain on the agricultural economy by increasing input requirements, labour costs, and revenue losses throughout the supply chain.

1.1.1 Barley as an important crop

Around 45% of calories consumed globally come from cereals (OECD-FAO, 2024). They are an important food staple, providing source of protein and fibre to both people and animals. Barley is one of the global primary crops, as defined by the United Nations Statistics Division (UNSD) Central Product Classification. It is the second most important crop in the UK and fourth most important cereal crop worldwide, providing human and animal food source, and used for malt production (National Statistics, 2021).

Even though barley is not the major cereal crop worldwide, it has a potential to become a more important food source in the near future. Due to its ability to withstand harsh conditions such as low soil nutrient content, drought and high salinity, it can be grown in regions unsuitable for wheat cultivation (Harlan, 1995). This could be important for expanding cultivation into the regions that experience drastic changes in precipitation and temperature, due to the progressing climate change, as well as land experiencing high soil degradation, due to unsustainable agricultural practices. Additionally, high fibre content and nutritional value contribute to barley's health benefits, such as decreasing the risk of cardiovascular disease by lowering blood cholesterol (Tosh & Bordenave, 2020).

Barley (*Hordeum vulgare ssp.* vulgare) use and cultivation started 10 000 years ago and some of the modern varieties are very similar to its wild progenitor *Hordeum vulgare ssp. spontaneum* (Badr et al., 2000). Nowadays, there are two major types of barley grown for agricultural purposes 2-row barley, most popular and similar to its

ancestor, and 6-row barley, which provides a higher yield with smaller grain. Additionally, existence of winter and spring varieties allows for cultivation throughout the year. Winter barley usually results in higher yield, but lower grain quality and is therefore used for animal feed, while spring barley, due to the better grain quality, is primarily used for human consumption and malting. Although spring barley is of greater commercial importance, it typically yields approximately 20% less than winter barley and is more prone to diseases and lodging (Spunar et al., 2002).

1.1.2 Emerging pathogens

Global agriculture faces numerous challenges, among which the emergence of new pests and pathogens is particularly critical (Bebber et al., 2014). New pathogens emerge by different means, for example, by the introduction of a new host or, a new host species, or environmental changes that create favourable ecological niches for pathogen establishment. Climate change influences both of these factors and is increasingly recognised as a key driver in the changing distribution, severity, and emergence of plant diseases. As a result, previously minor or geographically restricted pathogens are becoming significant threats to modern crop systems (Bebber, 2015).

Rising temperatures have led to a noticeable shift in crop cultivation patterns in the UK, with an increasing number of novel crops being introduced. For example, the grapevine growing area has doubled between 2010 and 2020 and is predicted to increase further (Nesbitt et al., 2022). Unfortunately, the introduction of new crops also increases the risk of introducing new pathogens. Changes to the climate can create more favourable conditions for already widespread but previously insignificant pathogens, enabling them to expand into new regions and become economically damaging. For example, in 2013, Germany experienced a first in decades outbreak of *Puccinia graminis f. sp. Tritici*, the causal agent of wheat stem rust, re-emerged after decades of absence. This outbreak was attributed to unusually favourable weather conditions, including a cold spring followed by an early, hot summer (Oliveira Firpo et al., 2017). Just a few years later, the first UK occurrence of wheat stem rust in over 60 years was reported, and it has been speculated that ongoing climate change may facilitate the further spread of this pathogen (Lewis et al., 2018).

Emerging plant pathogens are predominantly viral, followed closely by fungal species (Anderson et al., 2004). In the case of fungal diseases, both climatic conditions and

introduction events are equally important drivers of emergence (Anderson et al., 2004). Given that the most damaging barley pathogens are fungal in nature, climate change represents a significant threat to barley production, as it may facilitate the spread, severity, and geographic expansion of these pathogens.

1.2 *Puccinia hordei*

Fungal pathogens pose a major threat to agriculture, causing 10-23% of yield loss preharvest and a further 10-20% postharvest (Fisher et al., 2012). These losses occur despite the widespread use of fungicides, highlighting the complexity of the problem. Contributing factors to the success of fungal pathogens include high soil moisture, favourable environmental conditions, and unsustainable agricultural practices that may inadvertently promote pathogen survival and spread (AHDB, 2024). There are eight major foliar diseases affecting barley in the UK (*Septoria tritici*, Yellow rust, Brown rust, Powdery mildew, Rhynchosporium, Net blotch, Ramularia, Tan spot), all of which are caused by fungal pathogens. *Puccinia hordei* is one of these, and an emerging pathogen in the UK.

1.2.1 Disease progression

P. hordei is a biotrophic fungal pathogen causing brown leaf rust on barley. The disease symptoms appear as rusty coloured pustules containing uredospores up to 0.5 mm in size, visible on the upper or lower side of the leaf blade. The pustules are scattered around the leaf and can be surrounded by yellow chlorotic rings. When the leaf starts to senesce, green islands develop around the pustules (Clifford, 1985). Later in the season, teliospore containing dark pustules can form on the leaf and stem surface.

Puccinia hordei is a macrocyclic, heteroecious pathogen, meaning its life cycle comprises of five stages and requires two distinct hosts. Barley hosts the uredinial and telial stages of the life cycle, while aecia have been reported on members of the Liliaceae family (Clifford, 1985). The importance of the alternate host varies however, with lower importance in the north-western Europe (Clifford, 1985). As *P. hordei* uredia can overwinter on winter barley, it can create a detrimental reservoir leading to increased infection in the spring barley crop (AHDB, 2024).

The effect of *P. hordei* on barley crop is dependent on the degree of infection. As a biotrophic pathogen, it establishes a long-term association with its host to sequester nutrients and water. This results in resource depletion from the host, reduction in the rate of photosynthesis and leads to the reduction in growth, both of the shoot and root tissue (Swarbrick et al. 2006). This can be specifically important when the disease occurs earlier in the season, which can have a negative impact on grain development, resulting in lower quality grain and smaller yield.

1.2.2 Economic importance

In the past, *Puccinia hordei* has not been considered an important pathogen of barley in the UK as, although widespread, was not contributing to significant yield losses. This is due to the variation in geographical and seasonal severity. Milder winters, intensive barley cultivation and proximity of winter and spring barley cultivation can be beneficial for the success of the pathogen. Local epidemics have been reported in the UK in the past (Clifford, 1985) and it was estimated, that between years 2001 and 2005 *P. hordei* caused yield losses of £12 million (Edwards & Dodgson, 2009). In favourable conditions it can cause up to 62% yield loss in susceptible cultivars (Park et al., 2015).

It is predicted that due to the climate change, *P. hordei* can become a more threatening pathogen. Milder winter temperatures and warmer and wetter spring season will provide perfect conditions for spread of *P. hordei* and infection earlier in the growing season. This will allow more time for the pathogen to establish a successful infection and infect flag leaves, which has a detrimental impact on grain development.

Main management strategies for *P. hordei* include the use varieties containing resistance genes, eradicating volunteers, and applying fungicides, however these pose challenges. None of the recommended barley cultivars grown in the UK have a full resistance to *P. hordei*, with highest resistance rating of 6.2 (out of 10) for winter varieties and 4.2 for spring barley cultivars (AHDB, 2025). Additionally, the strategy to eradicate volunteers to prevent the formation of a green bridge is not always possible, especially in regions with intense agriculture and poor soil conditions, which prevents cultivation of other crops. Adding to this the need of reducing the use of fungicides it becomes clear that it is crucial to develop management strategies and resistant varieties to decrease the impact of *P. hordei* on barley cultivation.

1.2.3 Resistance

Resistance to *P. hordei* has been associated with genes conferring both seedling resistance and adult plant resistance (Park et al., 2015). These can be further divided into incompatible genes, or those that confer a partial resistance associated with reduction in compatibility (Clifford, 1985).

To date, 25 genes conferring resistance to *P. hordei* (*Rph* genes) were identified from *Hordeum vulgare, Hordeum vulgare ssp. Spontaneum* and *Hordeum bulbosum* (Kavanagh et al., 2015). The majority of the genes, however are not used in barley breeding programmes, as they do not confer resistance to the new pathogen strains (Park et al., 2015). Genes *Rph3, Rph7, Rph9 and Rph12* have been shown to be present in European commercial accessions, with only *Rph7* conferring effective resistance across Europe (Niks et al., 2000). The high mutation rates in *P. hordei* therefore pose a major challenge in developing resistant varieties (Park, 2003).

Understanding the molecular mechanism underlying the resistance conferred by the *Rph* genes is important for developing more targeted resistance. Two of the *Rph* genes have been cloned: *Rph1 and Rph3*. *Rph1* was the first identified and cloned *Rph* gene. It was found to be a novel coiled-coil NLR (nucleotide-binding domain and C-terminal leucine-rich repeats) receptor protein which is a part of a complex resistance locus (Dracatos et al., 2018). *Rph3* on the other hand, was identified as a transmembrane protein without homology to any known disease resistance proteins in plants and specific to *P. hordei* strains expressing *AvrRph3* gene (Dinh et al., 2022). Expression of *Rph3* was specific to infected tissue and transient expression in *Nicotiana benthamiana* and barley induces cell death. All this suggests, that *Rph3* could be an executor gene. Executor genes are plant resistance genes that are expressed when pathogen effectors bind to the promoter region to activate expression of plant susceptibility genes (Ji et al., 2022).

More molecular and genetic studies are needed to understand the nature of barley resistance against *P. hordei* and develop commercial varieties with robust and long-lasting resistance.

1.3 The plant immune system

Understanding of the plant immune system is essential in developing targeted crop protection strategies. Although much of our current knowledge of plant immunity derives from studies in *Arabidopsis thaliana*, many core immune mechanisms are conserved in monocot species, including cereals such as barley and rice (Jones and Dangl, 2006). Studies in barley and other monocots show that, while the fundamental components of the immune system are broadly conserved between monocotyledonous and dicotyledonous plants, differences exist in specific signalling pathways and immune-related genes (Balmer et al., 2012). Therefore, where possible, examples from monocot systems are highlighted below. As most studies of plant immune responses have been conducted in model dicot plants, fundamental studies of immune mechanisms in cereals are needed to support the development of more resilient crops.

Plants protect themselves against pathogens using a sophisticated multilayer immune system (Jones and Dangl, 2006). The first layer recognises the presence of the pathogen on the plant surface and triggers the transcriptional changes that switch the plant priorities from growth to defence. The second layer responds specifically to the pathogen-secreted proteins. Both layers of immunity are needed for a successful protection against pathogens.

1.3.1 Pattern triggered immunity

The first layer of defence is composed of surface pattern recognition receptors (PRRs) which recognise conserved microbial-associated molecular patterns (MAMPs) like bacterial flagellin-derived flg22 peptide (Gómez-Gómez et al., 1999) or fungal chitin (Kaku et al., 2006). In *Arabidopsis* fungal chitin is recognised by conserved lysine motif (LysM) PRR CERK1. In rice however, chitin perception is also dependent on another protein, a plasma membrane glycoprotein CEBiP, which binds chitin oligosaccharides and forms a heterodimer with CERK1 (Shimizu et al., 2010). It is not known if this mechanism is also present in barley, however studies show that the homologue of OsCEBiP is present on the plasma membrane in barley cells (Okada et al., 2002) and the HvCEBiP contributes to basal resistance in barley (Tanaka et al., 2010). This suggests that barley chitin perception might also require a receptor complex formation.

MAMP perception triggers a cascade of signalling events resulting in pattern triggered immunity (PTI), which aims to prevent pathogen colonisation (Jones and Dangl, 2006). The signalling cascade is mediated by phosphorylation of mitogen-activated protein kinases (MAPKs) and calcium-dependent protein kinases (CDPKs), which trigger calcium influx, production of Reactive Oxygen Species (ROS) and induce changes in the localisation of various transcription factors and transcriptional co-activators (Bigeard et al., 2015). These drive the expression of defence genes that induce callose deposition around the recognition site, production of antimicrobial compounds, and synthesis of immune hormones.

1.3.2 Effector triggered immunity

The second layer of the immune response is activated when pathogens successfully evade the plant immune system and colonise plant tissues (Jones & Dangl, 2006). It involves recognition of pathogen-derived proteins called effector proteins, hence the name – effector-triggered immunity (ETI). Successful pathogens can evade ETI and remain unrecognised by the host (Jones & Dangl, 2006). However, plants can evolve resistance (R) proteins, which recognise specific pathogen effectors (Tameling and Takken, 2008). This recognition makes the pathogen avirulent by triggering an ETI response, which is associated with the amplification of PTI response and often results in programmed cell death (Morel & Dangl, 1997). Most of the R genes encode nucleotide binding and leucine rich repeat (NB-LRR) proteins. An example from barley is a group of coil-coil (CC) NB-LRR encoded by the *MLA* locus, which confer resistance against various biotrophic and hemibiotrophic fungal pathogens (Bettgenhaeuser et al., 2021; Maekawa et al., 2018; Lu et al., 2016).

1.3.3 Plant immune hormones

Plant phytohormones are small organic molecules synthesised in response to both biotic and abiotic stimuli. They regulate a wide range of physiological processes, including growth, development, and stress responses, and play a central role in transcriptional reprogramming upon activation. Among these, salicylic acid (SA) and jasmonic acid (JA) are the primary phytohormones involved in plant immunity. They usually act antagonistically, by inducing immune responses against biotrophic and necrotrophic pathogens, respectively (Bari and Jones, 2008). Ethylene (ET) is another immune phytohormone as it regulates responses to necrotrophic pathogens and

association with beneficial microorganisms (Berrocal-Lobo et al., 2002; Camehl et al., 2010). Auxin, primarily a growth hormone, has also been implicated in mediating immune responses, by enhancing resistance against necrotrophs and acting as a susceptibility factor exploited by biotrophic pathogens (Tiryaki and Staswick, 2002; Djami-Tchatchou et al., 2020).

1.3.3.1 Salicylic acid pathway

Salicylic acid is essential for promoting immune defence responses against biotrophic pathogens by inducing basal defence and establishment of the systemic acquired resistance (SAR) (Gaffney et al., 1993). In plants, salicylic acid is synthesised from chorismate via two pathways – isochorismate synthase (ICS) and phenylalanine ammonia lyase (PAL). It is thought that the ICS pathway is more critical in plants; however, studies have shown that the PAL pathway is likely more important in the monocots (Zhang et al., 2021; Qin et al., 2019; Xu et al., 2022). Indeed, in rice it has been proved to be the primary SA biosynthesis pathway, and overexpression of key biosynthesis genes induces resistance against a biotrophic fungal pathogen *Xanthomonas oryzae pv. Oryzae* (*Xoo*) (Zhu et al., 2025). In the ICS pathway, chorismite is converted into isochorismate by ICS in the chloroplast and then transported into the cytosol via ENHANCED DISEASE SUSCEPTIBILITY 5 (EDS5) transporter protein (Nawrath et al., 2002). Then the avrPphB SUSCEPTIBLE3 (PBS3) protein converts isochorismate into isochorismate-9-glutamate and finally ENHANCED PSEUDOMONAS SUSCEPTIBILITY 1 (EPS1) converts it into SA (Torrens-Spence et al., 2019).

In the PAL pathway, chorismate is converted into phenylalanine, which is then converted by PAL into Trans-cinnamic acid (tCA) (Rohde et al., 2004). ABNORMAL INFLORESCENCE MERISTEM1 (AIM1) then catalyses the conversion of tCA to benzoic acid, which is then finally converted into salicylic acid (SA) (Zhu et al., 2025). While barley possesses a single copy of the ICS gene, it contains ten copies of PAL genes, compared to two ICS and four PAL genes in Arabidopsis (Lefevere, Bauters & Gheysen, 2020). The expansion of the PAL gene family in barley is attributed to its involvement in the phenylpropanoid pathway, which leads to the biosynthesis of various defence-related compounds, including lignins and flavonoids (Dong & Lin, 2020).

Upon an increase in salicylic acid (SA) levels, the cellular redox state shifts, resulting in the reduction of NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (NPR1) from its oligomeric form in the cytosol to a monomeric form through the breaking of intermolecular disulfide bonds. The monomeric NPR1 then translocates to the nucleus, where it interacts with TGA transcription factors to activate the expression of defence-related genes (Mou, Fan & Dong, 2003; Spoel et al., 2009). In the nucleus, NPR1 functions as a transcriptional coactivator, promoting the expression of SA-responsive genes such as PATHOGENESIS RELATED (PR) gene family (Kinkema & Dong, 2000). In addition to NPR1, SA can also bind to its homologues, NPR3 and NPR4, which act as negative regulators of systemic acquired resistance (SAR) (Fu et al., 2012). Interestingly, basal level of SA in rice is much higher than that in Arabidopsis and, unlike in Arabidopsis, it undergoes only a subtle increase upon pathogen infection (Silverman et al., 1995). This suggests that, in monocots, defence signalling may rely less on the large SA bursts, and more on changes in signalling sensitivity and downstream transcriptional regulation (De Vleesschauwer et al., 2014).

Salicylic acid (SA) is a key phytohormone involved in the defence against biotrophic pathogens, such as *Puccinia hordei* (Mengiste & Liao, 2025). However, studies investigating the role and mechanisms of SA-mediated immune responses in barley remain limited.

1.3.3.2 Jasmonic acid pathway

Jasmonic acid is synthesised in plants in response to herbivores and necrotrophic pathogens. This, as well as the SA-JA antagonism is also true for monocots (De Vleesschauwer et al., 2014). Interestingly however, it has been implicated that in monocots JA signalling could be responsible for inducing resistance against pathogens with different lifestyles (De Vleesschauwer et al., 2014). For example, in rice, JA has been shown to induce resistance against hemibiotrophic pathogens *Xoo* and *Magnaporthe oryzae* (Yamada et al., 2012; Riemann et al., 2015).

The biosynthesis of JA starts in the chloroplast, where galactolipids from chloroplast membrane are degraded into linoleic acid, which is then converted into cis-12-oxophytodienoic acid (OPDA). This is catalysed by 13-lipoxygenase (LOX), allene oxide synthase (AOS) and allene oxide cyclase (AOC) enzymes. Next, OPDA is exported from the chloroplast via a JASSY protein and imported into the peroxisome

via an ABC transporter COMATOSE (CTS) (Theodoulou, et al., 2005; Guan et al., 2019). There, it is converted into JA by OPDA reductase 3 (OPR3) and β-oxidation enzymes (Wasternack & Hause, 2019). JA is then transported into the cytoplasm and converted into bioactive jasmonoyl-L-isoleucine by Jasmonate Resistant 1 (JAR1) protein (Staswick and Tiryaki 2004).

Perception of JA depends on its bioactive JA-Ile form, which binds to the nuclear receptor F-box protein CORONATINE INSENSITIVE 1 (COI1). In the presence of JA-Ile, COI1 interacts with the Skp1/Cul1/F-box (SCF) complex (SCF^{COI1}) which then ubiquitinates a subfamily of TIFY-domain proteins termed JASMONATE-ZIM DOMAIN (JAZ) proteins, targeting them for degradation (Xu et al., 2002; Sheard et al., 2010). JAZ proteins are repressors of MYC transcription factors and their degradation induces MYC-mediated transcription of JA-responsive genes (Fernández-Calvo et al., 2011).

1.3.3.3 Ethylene signalling

Ethylene is a gaseous hormone produced in plants during developmental processes and responses to biotic and abiotic factors (Singh et al., 2022). In the absence of ethylene, ethylene receptors release CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1) protein which phosphorylates and therefore inhibits ETHYLENE INSENSITIVE 2 (EIN2) (Ju et al., 2012). This prevents EIN2 translocation into the nucleus and blocks downstream signalling. Upon synthesis, ethylene diffuses throughout the plant and binds to the ethylene receptors, which causes inactivation of CTR1 (Ju et al., 2012). This results in cleavage and dephosphorylation of EIN2 which releases its C-terminus for nuclear translocation, prevents proteasomal degradation of ETHYLENE INSENSITIVE 3 (EIN3) transcription factor, and leads to activation of ethylene-responsive transcription factors (ERFs) (Wen et al., 2012).

1.3.3.4 SA-JA/ET Cross-talk

Salicylic acid (SA) is primarily associated with defence responses against biotrophic pathogens, whereas ethylene and jasmonic acid (JA) are typically involved in resistance to necrotrophic pathogens and play essential roles in interactions with beneficial microorganisms. This functional divergence implies that tight cross-regulation among these hormonal pathways is necessary to ensure the appropriate immune response is activated. Indeed, in Arabidopsis and rice, several SA-induced

WRKY transcription factors have been shown to simultaneously promote SA-responsive gene expression while repressing JA-responsive genes, illustrating a mechanism of mutual antagonism (Li et al., 2006; Mao et al., 2007; Qiu et al., 2007). On the other hand, mitogen activated protein kinase 4 (MPK4) protein has been identified as a negative regulator of SA-signalling, but positive regulator of ethylene and JA signalling (Brodersen et al., 2006). Recent studies have shown that SA-induced NPR1 binds to JA-responsive promoters and co-occupies chromatin with MYC2 and the MED25 Mediator subunit. NPR1 physically interacts with MYC2 and displaces JAZ repressors, disrupting MYC2–MED25 interactions and repressing JA-regulated genes (Nomoto et al., 2023). These findings highlight NPR1's central role in mediating SA–JA crosstalk and shaping immune outcomes through transcriptional interference. The mechanisms underlying this cross-talk remain poorly understood in monocots, with some studies suggesting a positive regulation, indicative of major overlap in the SA and JA- upregulated genes (Tamaoki et al., 2013; Ding et al, 2016).

1.3.4 Induced systemic resistance

Plants encounter various microorganisms, also the non-pathogenic ones. As those also contain MAMPs and can interact with the plant, they can induce certain aspects of the plant immune response and provide systemic resistance via immune priming. Systemic resistance triggered by non-pathogenic microbes is referred to as induced systemic resistance (ISR) (Kloeper, Tuzun & Kuc, 2008). It has been shown that beneficial microorganisms can promote ISR by activating both SA and JA/ET signalling pathways (Yu et al., 2022). In barley, root colonisation with a basidiomycete fungus *Piriformospora indica* was found to induce systemic resistance against a biotrophic fungal leaf pathogen *Blumeria graminis f. sp. hordei* causing barley powdery mildew, by inducing faster and stronger expression of PR and heat-shock protein-coding genes (Molitor et al., 2011). Colonisation of plant roots with beneficial microbes can also have other advantages, like promoting growth by improving nutrient uptake and photosynthesis (Ortíz-astro et al., 2009).

1.4 The Plant ubiquitin system

The dynamic nature of host-pathogen interactions requires a high degree of immune plasticity from the host. This is achieved through controlled modulation of protein homeostasis (proteostasis), which regulates the synthesis, folding, localisation, and

degradation of proteins to maintain functional protein networks within the cell. These processes are facilitated by molecular chaperones and protein degradation pathways such as the ubiquitin–proteasome system (UPS) and autophagy.

Proteostasis enables dynamic regulation of immune signalling by controlling the stability and turnover of key regulatory proteins, including immune receptors, signalling components, and transcription factors (Zhao and Wang, 2024). For example, autophagy is involved in PRR homeostasis, by selectively degrading non-active FLAGELLIN-SENSIN2 (FLS2) (Yang et al., 2019). The importance of proteostasis is also underscored by the fact that pathogens actively target host proteostatic pathways (Leary et al.,2017; Langin et al., 2023). For example, an oomycete pathogen of potato, *Phytophthora infestans*, evolved an effector (PexRD54) which can bind host autophagy protein ATG8CL to deplete the cargo receptor Joka2 and counteract host defences (Dagdas et al., 2016).

The proteostasis is also largely dependent on posttranslational modifications of immune proteins, where the contribution of ubiquitin is crucial. Ubiquitination is one of the most abundant protein modification processes in the cell, involving covalent attachment of the highly conserved small protein ubiquitin to substrate proteins (Khoury et al., 2011; Kaminskaya et al., 2024; Akutsu et al., 2016). Modifications by ubiquitin and ubiquitin-like proteins provide dynamic modulation of protein function in response to both abiotic and biotic stress (Callis, 2014).

1.4.1 Ubiquitination cascade

Ubiquitin is a small 8kDa protein consisting of a β-grasp fold (with five antiparallel β-strands and one α-helix) and a flexible C-terminal tail (Vijay-kumar et al., 1987). Ubiquitination involves a covalent attachment of the ubiquitin monomer through its C-terminal glycine residue to the, most commonly, lysine residue of the target protein. This reaction is catalysed by three enzymes: E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme and E3 ubiquitin ligase and results in monoubiquitination (Kommander and Rape, 2012). Subsequently, another ubiquitin molecule can be added to one of the lysine residues (K6, K11, K27, K29, K33, K48 and K63) or the N-terminal methionine residue (M1) of the previous ubiquitin, resulting in polyubiquitination. This can lead to the formation of homotypic (one linkage type) and

heterotypic (mixed linkages) ubiquitin chains and the linkage architecture is defined by the E2 enzyme (Zhou and Zeng, 2017).

The topologies of ubiquitin modifications on target proteins form the ubiquitin code, which can be “read” by reader proteins containing topology-specific ubiquitin-binding domains (UBDs). With the complexity of the ubiquitin code comes the diversity of UBDs, with more than 100 identified in barley (Zhou et al., 2017). Reader proteins decide on the fate of the target protein by initiating the ubiquitin-signalling cascade. In turn, the ubiquitin mark can be “erased” by specific deubiquitinase enzymes (DUBs), further modulating the protein fate (Vogel and Isono, 2024).

The ubiquitin code is most widely associated with the activity of the ubiquitin-proteasome system (UPS), which is responsible for the removal of damaged or unnecessary proteins via the proteasome-mediated degradation pathway, primarily associated with K48- and K11- linked ubiquitination. More studies, however, emerge, which show the non-proteolytic role of ubiquitin in regulating protein signalling (Zhou and Zeng, 2017).

1.4.2 Ubiquitin ligases

E3 ligases make the most diverse group of enzymes involved in ubiquitination, with over 1500 potential proteins in Arabidopsis (Vierstra, 2012). This diversity reflects their role in capturing the substrate protein, providing substrate specificity for the ubiquitination machinery. E3 ligases can be divided into five subfamilies, based on the structure and mechanistic function of their catalytic domain. HECT (Homologous to E6-associated protein 7 C-Terminus), RING (Really Interesting New Gene), U-Box and RBR (RING between RING), are subfamilies containing members of single-subunit E3 ligases, while CRL (Cullin-RING ligases) subfamily contains members of multi-subunit ligase complexes (Zhou & Zeng, 2017). The activity and mechanisms of action differ between E3 ligase families. RING and U-box non-covalently interact with E2 enzymes during transfer of the ubiquitin molecule to the target protein (Deshaies and Joazeiro, 2009), while HECT and RBR ligases form a covalent bond with the ubiquitin before the transfer to the substrate protein (Zhou & Zeng, 2017). Additionally, the difference between RING and U-box ligases lies in the structure of the catalytic domain, which in RING ligases relies on the presence of zinc ions to stabilise the RING domain (Barlow et al., 1994). CRL ligase complexes consist of an RBX1 (RING-box 1)

protein, and a CULLIN (CUL) scaffold, usually containing adaptor and receptor proteins conferring substrate specificity. There are six CUL proteins in Arabidopsis, which can pair with different adaptors and receptors to form CRL complexes, for example SCF complex containing SKP1 (S-PHASE KINASE ASSOCIATED PROTEIN 1), CUL1 (CULLIN1) and an F-box protein (Choi et al., 2014). There is high evidence for the involvement of different classes of E3 ligases in regulating plant development, hormone signalling and immune responses (Ban and Estelle, 2021; Zhou, Zeng 2017).

1.4.3 Ubiquitin and immunity

The success of a plant's survival following pathogen invasion relies on the rapid and effective activation of specific immune responses, starting from pathogen perception and the subsequent downstream signalling cascade. This requires a "wired" immune system, which relies on high proteomic plasticity, provided by ubiquitination. As ubiquitination appears to play a central role in regulating plant environmental responses, it has been extensively studied in the context of plant immunity (Marino et al., 2012; Gao et al., 2022).

Ubiquitin is a crucial protein in plants, and mutations in ubiquitin genes have significant developmental and functional consequences. For example, the introduction of a K48R mutant of the ubiquitin gene, which causes an inability to form K48-linked ubiquitin chains, resulted in the induction of cell death, ROS production, and changes in the expression of immune genes in Arabidopsis (Schlögelhofer et al., 2006). Associated with this, the plant proteasome system has also been implicated in mediating defence responses by facilitating targeted protein degradation (Dielen et al., 2009). For example, RPN1 acts as a regulatory subunit of the 26s proteasome and is required for basal defence and R protein-mediated defence in Arabidopsis (Yao et al., 2012).

Ubiquitination is also involved in the sequential regulation of the immune master coactivator NPR1. Initial ubiquitination by the Cullin-RING Ligase 3 (CRL3) E3 ligase promotes its association with chromatin and the expression of immune genes, while further ubiquitination with the E4 UBE4 enzyme, which adds ubiquitin to the previous chains, targets NPR1 for proteasomal degradation (Skelly et al., 2019). Moreover, proteasome-associated DUBs UBP6/7 can remove some ubiquitin to prolong NPR1

activity (Skelly et al., 2019). This highlights the sophisticated multi-step ubiquitin-regulation mechanism that controls plant immune signalling.

Many other ubiquitin E3 ligases have also been shown to regulate immune signalling events (Zhou and Zeng, 2017). Members of the RING family, the ARABIDOPSIS TOXICOS EN LEVADURA (ATL) proteins have been implicated in mediating immune responses, as expression of *ATL2 and ATL9* is induced in Arabidopsis in response to chitin treatment (Salinas-Mondragón et al., 1999; Berrocal-Lobo et al., 2010), and ATL6 has been shown to positively regulate BIK-mediated immunity in Arabidopsis (Liu et al., 2022). Also worth mentioning are the Plant U-box (PUB) members of E3 ligases, which have been associated with the negative regulation of flagellin perception (Lu et al., 2011; Trujillo et al., 2008). Due to the diversity of E3 ligases, they have the potential to exhibit a wide range of immune functions. Although we know that many of them are involved in mediating immune responses, their substrates and the mechanisms underlying their function remain largely unknown.

Interestingly, pathogens also recognised the importance of ubiquitin in mediating immune response and evolved strategies that sequester the plant ubiquitin system to evade recognition and defence. They achieve this by targeting host proteins for degradation, modulating hormone signalling, stabilising negative immune regulators, or mimicking/sequestering plant E3 ligases (Langin et al., 2023).

1.5 Strategies for improving crop health

With the growing demand for improving farming efficiency and with the climate change increasing abiotic and biotic pressures on crop production, we need to develop new strategies for improving crop health. This can be done by incorporating favourable genetic traits into crops, or following beneficial agricultural practices.

1.5.1 Engineering resistance

Incorporation of genetic resistance can be one the most sustainable methods of protection against pathogens, however discovery of effective and durable resistance traits is often challenging. This is due to the fast-evolving nature of plant pathogens and resistance-associated trade-offs (de Vries et al., 2020; He et al., 2022). As the ubiquitin system controls many agronomic plant traits, it could serve as a potentially useful tool for selective induction of plant immune responses (Linded and Callis, 2020).

Indeed, several studies showed that modulation of the E3 ligase genes can induce pathogen resistance. An example in rice shows, that overexpression of OsRING13 E3 ligase enhances broad-spectrum resistance and results in improved response against *Magnaporthe oryzae* and *Xanthomonas oryzae pv. Oryzae* without the growth trade-off (Zhang et al., 2024). Similarly, constitutive expression VriATL156 protein in transgenic grapevine induced resistance against *Plasmopara viticola* (Downy mildew) (Vandelle et al., 2021). This shows, that the approach of using E3 ligases in improving immune responses can be effectively applied to crops.

1.5.2 Symbiont-mediated resistance

A different approach of improving crop defence responses takes into account the soil health and aims to utilize the rhizosphere to improve plant growth and manage disease. As described before, rhizobacteria and mycorrhizal fungi can enhance plant defence responses by activating the induced systemic resistance (ISR). Arbuscular mycorrhizal fungi (AMF) have been shown to provide not only protection against a variety of soil and foliar pathogens, but also improve growth and help cope with environmental stress (Wahab et al., 2023). Additionally, several field studies show, that AMF inoculation can be effective in providing protection against biotic stress (Alaux et al., 2018; Jamiolkowska et al., 2020). If efficient against *P. hordei*, AMF could therefore provide a multitude of benefits to barley cultivation.

1.6 Aims and objectives

Given the need of increasing agricultural output through improving crop health, this study aims to explore the ways of enhancing barley resistance against a biotrophic fungal pathogen *P. hordei*. In order to explore novel approaches and provide a knowledge base for studying barley-pathogen interactions, I will use omics approaches to address three objectives.

First, I explore the use of arbuscular mycorrhizal fungi (AMF) in providing pathogen protection and boosting plant health. The soil health is important for plant productivity and ensuring long-term agricultural capabilities. Even though AMF has been associated with positive regulation of plant immune responses, most studies focus on necrotrophic and hemibiotrophic pathogens, with little understanding of the interaction during infection with a biotrophic pathogen. Therefore Chapter 3 uses

a transcriptomics approach to dissect the effects of AMF on barley defence responses against *P. hordei*.

Ubiquitination proved important in mediating various aspects of plant biology, including regulation of growth-defence response. With reports of discrepancies between defence mechanisms in dicot and monocot plant, and the scarcity of studies looking at immunity in barley, I found it necessary to investigate the role of ubiquitin in mediating barley immune responses. In Chapter 4, I aim to establish a comprehensive barley immune ubiquitome, to understand the extent of ubiquitin regulation during activation of immune responses, and identify barley immune proteins regulated by ubiquitin.

In order to engineer efficient resistance in crops, we need to understand the molecular mechanism behind the function of given immune proteins. Therefore, in Chapter 5 I attempt to decipher the roles of proteins identified through ubiquitome profiling, in mediating barley immune responses. Due to the lack of availability of molecular techniques for barley as a model organism, I use Arabidopsis as an alternative plant platform and utilise in-vitro assays together with proteomics approaches to gain insight into ubiquitin-mediated regulation.

This study uses a barley-*P. hordei* plant-pathogen system to study the interactions that are relevant to the agricultural context. By using barley as a model crop, we contribute to a broader understanding of plant immunity while enabling meaningful comparisons with established model systems. This approach aims to support the wider research community through the development of protocols and experimental workflows that facilitate functional studies in barley.

Overall, this thesis offers novel insights into the regulation of immune responses in barley and highlights the crucial role of ubiquitination in plant defence. I anticipate that this knowledge will inform future strategies to engineer ubiquitin-mediated immune regulation, to enhance resistance to *P. hordei* and other pathogens in cereal crops.

Chapter 2

Materials and Methods

2.1 Plant materials and growth conditions

2.1.1 *Hordeum vulgare subsp. vulgare* (Barley)

Barley seeds (cv. *Bowman*) were germinated on plates in between two rounds of filter paper (Whatman Filter Paper No.1, 9.0cm) soaked with 20 ml of ddH_2O for 3 days at 4 °C, followed by incubation for 3 days at 20℃ day/17℃ night long day cycle (16h light, 8h dark), 70 % humidity with light supplied by fluorescent lamps (FL40SSENW37) at an average intensity of 150 $\mu mol.m^{-2}.s^{-1}$. Plants were then transferred to soil and grown at the same conditions until required.

2.1.2 *Arabidopsis thaliana*

Arabidopsis plant material are all in the Col-0 ecotype background. The *bd1, ef1x3, cam5* plants are SALK T-DNA insertion lines (Alonso et al., 2003; Sessions et al., 2001) (Table 2.1). The *npr1-1* and *cat2-1* lines have been previously described (Cao et al., 1994; Bleu, 2023).

Table 2.1 Arabidopsis SALK/SAIL lines.

Locus	Allele	SALK/SAIL line
AT5G49430	*bd1*	SALK_093473
AT1G07940, AT1G07930, AT1G07920	*ef1ax3*	SAIL_565_A09
AT2G27030	*cam5*	SALK_138758

Arabidopsis seeds were sterilised by incubating with 70% ethanol for 5 min on a rotator, followed by 10% bleach for 5 min on a rotator. Seeds were washed 3 times with sterile ddH_2O and sown either on soil or plates. Seeds were stratified for 2 days at 4°C before transferring to a growth chamber at 21°C and 100 $\mu mol.m^{-2}.s^{-1}$ light on a 16-h-light/8-h-dark light cycle at 65% day/55% night humidity.

2.1.3 *Nicotiana benthamiana*

Tobacco seeds were stratified for 2 days at 4°C before transferring to soil in a growth chamber at 21°C and 100 $\mu mol.m^{-2}.s^{-1}$ light on a 16-h-light/8-h-dark light cycle at 65% day/55% night humidity.

2.2 Plant treatment and infection

2.2.1 Immune hormone treatment

21-day old plants were infiltrated with 0.1 mM or 0. 5 mM sodium salicylate and 0.1 mM of 0.5 mM methyljasmonate in 10 mM $MgSO_4$. Leaf samples (n=3) were collected after 6h and frozen in liquid nitrogen.

2.2.2 *Puccinia hordei* infection

14-day old plants were infected with *Puccinia hordei* isolate BBR 20-003 (NIAB) by brushing urediniospores in a 1:10 spores:talk powder mixture on the second leaf of each plant (~1.3×10^4 spores per plant). Control plants were brushed with talk powder only. Plants were bagged and left in darkness for 15 h. For subsequent analysis, leaf tissue was collected (n=3) at different timepoints and frozen in liquid nitrogen.

2.2.3 Field trial sample collection

Barley samples of 24 commercial barley cultivars infected with *P. hordei* from the field trial were provided by Dr Neil Havis and Dr Francois Dussart (SRUC). Commercial barley cultivars were grown in the field. A susceptible variety (Laureate) was chosen as a spreader of *P. hordei* spores and also served as a control. Spreaders were infected in the nursery and moved to the field in May 2021. Leaf samples (n=3) were collected in June (winter barley cultivars) and July (spring barley cultivars) 2021 and frozen in liquid nitrogen. Seven spring barley cultivars and four winter barley cultivars were chosen randomly from the supplied sample set for the analysis. Cultivar rating and percentage (%) *P. hordei* infection data was provided by Dr Neil Havis. Disease was assessed based on the visual disease symptoms on a scale of 1-50 based on the disease chart (Figure 2.1).

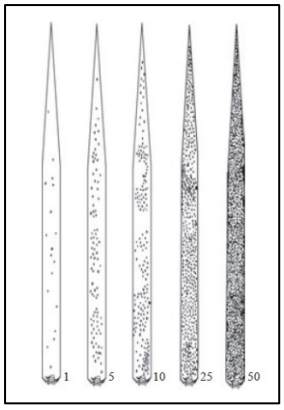

Figure 2.1 *Puccinia hordei* on barley; percentage of leaf area affected. © Crown copyright 1976. Ministry of Agriculture, Fisheries and Food (GB).

2.2.4 AMF media preparation, inoculation and *P. hordei infection*

AMF media preparation and inoculation was performed by Dr Claire Moulton-Brown (University of Edinburgh).

The growth media has been optimised to ensure efficient AMF colonisation without negative impacts caused by nutritional availability on barley growth. Together with Dr Claire Moulton-Brown, we tested different growth media:

1) 1:1 (v:v) mixture of coarse builder's silica sand and Terragreen® (Oil Dri, UK) supplemented with 0.25 g/l bonemeal.
2) 1:1 (v:v) mixture of soil and Terragreen® supplemented with 0.5 g/l bonemeal.
3) 1:1 (v:v) mixture of coarse builder's silica sand and Terragreen® with 0.5 g/l bonemeal.
4) 1:1 (v:v) mixture of coarse builder's silica sand and Terragreen® (Oil Dri, UK) with 0.5 g/l bonemeal and with implementation of 25 ml/plant of modified P-free Hoagland's complete nutrient solution (Hoagland and Arnon, 1950) twice a week from two weeks after planting.

Media 4 allowed for easy root cleaning and supplemented enough nutrients for barley plants to grow without nutrient deficiency symptoms, therefore we decided to use this media onwards.

Inoculum control with bacterial washout was prepared by washing the *Rhizophagus irregularis* (Plantworks, UK) inoculum in dH_2O and filtering the washout through a 11 µm filter paper (Whatman filter paper grade 1, Whatman, UK) to remove AM propagules.

Hordeum vulgare (barley) cultivar Bowman seeds were sterilised by soaking in 50 ml of sterilising solution (30% bleach and 0.02% Triton X-100), vortexing for 15 s and incubating at RT for 6 minutes. Vortexing and incubating was repeated for a total of 3 times. Seeds were washed 3 times with dH_20.

Two seeds were placed in 1 l pots containing autoclaved 1:1 (v:v) mixture of coarse builder's silica sand and Terragreen® (Oil Dri, UK) with 0.5 g/l bonemeal and, depending on the treatment, bacterial washout solution with autoclaved 20 g/l *Rhizophagus irregularis* (Plantworks, UK) (-AMF), or 20 g *Rhizophagus irregularis* inoculum (+AMF).

Plants were grown at 20℃ day/17℃ night long day cycle (16h light, 8h dark), 70 % humidity with light supplied by fluorescent lamps (FL40SSENW37) at an average intensity of 150 $\mu mol.m^{-2}.s^{-1}$. 7 days after planting, plants were thinned to 1 plant per plant. Two weeks after planting, plants were fed twice a week with 25 ml/plant of modified P-free Hoagland's complete nutrient solution (Hoagland and Arnon, 1950). 27 days after a sacrificial plant was harvested and AM colonisation was confirmed by qPCR and microscopy. Next day plants were infected with *Puccinia hordei* as described in 2.2 2. 7 days post infection 1 g of infected leaf tissue was collected, frozen in liquid nitrogen and stored at -80 ℃.

2.2.5 Pseudomonas infection and quantification

Pseudomonas syringae pv. maculicola ES4326 (*Psm)* culture was grown at 28 ℃ in a shaking incubator overnight in LB liquid media supplemented with 50 mg/ml Streptomycin and 10mM $MgSO_4$. Next day culture was centrifuged at 4000 rpm for 10 min and resuspended in 10 mM $MgSO_4$. The OD of the culture was measured and diluted to OD = 0.002. Arabidopsis leaves were infiltrated with the solution and tissue was collected three days later. 5 mm diameter discs from infected leaves were each ground in 500 µl $MgSO_4$ and diluted to 10^{-1} and 10^{-2} original concentration. 10 µl of each concentration was plated in a streak pattern on LB agar plates supplemented with 50 mg/ml Streptomycin and 10mM $MgSO_4$ and incubated at 28 ℃ for two days. After this time, the colonies were counted and cfu/leaf disc and standard error calculated.

2.3 Generation of plasmid constructs and expression

2.3.1 Constructs for *in planta* expression

To obtain the HvHSP70-2-GFP, HvHSP70-2-T70A-GFP, HvPAL1-GFP and HvPAL1-K238R-GFP constructs, *HvHSP70-2* and *HvPAL1* gene-coding DNA sequences were cloned from barley cDNA (RNA extraction and cDNA synthesis was performed as described in sections 2.6 and 2.7 respectively) using gene-specific primers (Table 2.2). Mutant versions of the gene were created using a target-specific primers with a single base pair mutation (Table 2.2). Constructs were cloned into Gateway® pDONR207™ vector (Invitrogen) then recombined into pEarlyGate103 using ™ LR Clonase™ II Enzyme (Invitrogen).

Vectors were transformed into *Agrobacterium tumerfaciens* strain GV3101 (pMP90) using a freeze-thaw method (Weigel & Glazenbrook, 2006).

2.3.2 Transient expression in *Nicotiana benthamiana*

Agrobacterium tumerfaciens carrying a specific construct were collected from an overnight culture by centrifuging at 4000 rpm for 10 min and resuspended in a buffer containing 10 mM $MgSO_4$ to final OD600 = 0.5. *N. benthamiana* leaves were infiltrated with Agrobacterium suspension, harvested after 3 days and stored at -80°C.

2.3.3 Constructs for bacterial expression

SynRGLG construct (4xUbiquilin-FLAG-HvRGLG2) was synthesised by Twist Biosciences (NGS ProLab). It was then cloned into PET28a(+) vector using NEBuilder® HiFi DNA Assembly Master Mix (New England Biolabs) and specific primers according to manufacturer's instructions (Table 2.2).

Vectors were transformed into *E.coli* strain BL21 (DE3) using a freeze-thaw method (Weigel & Glazenbrook, 2006).

2.3.4 SynRGLG2 expression and protein purification

E.coli BL21 (DE3) cells carrying a SynRGLG2 construct were incubated in a liquid LB media until OD=0.5 before 1 mM IPTG was added and culture incubated at 21℃ for 6h. Culture was divided into 50 ml aliquots and cell culture was harvested by brief centrifugation at 4000 rpm for 30 min, 4°C. Pellet was frozen and subsequent protein purification was performed with 2 ml of protein purification buffer (1x BugBuster

(Millipore), 1x PBS, 150 mM NaCl, 10 mM DTT, protease inhibitor cocktail, 1 ul/ml benzonase nuclease). Bacterial suspension was incubated at RT for 20 min and centrifuged at 16 000 x g for 15 min at 4°C. The supernatant was transferred to a new tube on ice, combined with 50 µl of HisPur™ Ni-NTA Magnetic Beads (Thermo Scientific™) and incubated on a rotator for 2h at 4°C. The beads were washed two times with cell lysis buffer and two times with 1xPBS. Protein was eluted in 100 µl of 250 mM imidazole for 15 min with shaking at 900 rpm. The elution was repeated one more time for 10 min. Sample was dialysed using Micron ® 10 kDa columns and 1 x PBS as the exchange buffer, according to the manufacturer's instructions (Millipore). Purified protein was stored in 50% glycerol at -20°C.

Table 2.2 Primers used for cloning

Target	Primer sequence (5'→3')
HvHSP70-2	F: GCTTCCTTCTCGGCCGATCTAG R: GTCGACCTCCTCGATCTTGGG
HvHSP70-2TA	F: GAACCCCATCAACGCCGTCTTC R: GAAGACGGCGTTGATGGGGTTC
HvPAL1	F: GATACGCATACACCTGCCGGC R: GCAGATAGGCAGGGGCTCAC
HvPAL1-KR	F: GCAGAGGCATTTAGGATCGCCG R: CGGCGATCCTAAATGCCTCTGC
SynRGLG	F: CAGTGGTGGTGGTGGTGGTGGTACAACTTGATTCTGG-TTGTGATGGG R: CTTTAAGAAGGAGATATACCATGCGGTCAGGCGGG
pET28	F: ACGCCCCCGCCTGACCGCATGGTATATCTCCTTCTTAA-AGTTAAACAAAATTATTTCTAGAGG R: CAACCAGAATCAAGTTGTACCACCACCACCACCACCACT

2.4 DNA extraction and genotyping of T-DNA insertion lines

One 5mm in diameter disc of plant tissue was homogenised in 100 µl of DNA extraction buffer (20 mM Tris pH7.5, 25 mM NaCl, 2.5 mM EDTA, 0.05% SDS), and 1 µl of extract was used for the PCR reaction. For screening, gene primers specific to

T-DNA flanking regions (Table 2.3) and a T-DNA left border primer (LBb1.3 for SALK and LB1 for SAIL) were used for PCR reactions (O'Malley et al., 2017).

Table 2.3 Primers used for genotyping of SALK/SAIL lines.

Target	Primer sequence (5'→3')
ef1ax3	LP: TTGGGTCCTTCTTGTCAACAC RP: ACCATTGACATTGCTCTCTGG
bd1	LP: TCCAGTATGTCCACGGAGAAC RP: ACCTGGTGGAGCTTCTAAAGC
cam5	LP: TATGATTTGGCCCATTCTTTG RP: TCGAAAAGGCTAAAAGCTTCC

2.5 Barley root staining for AMF colonisation confirmation

Protocol adaptation from Plant Mineral Nutrients: Methods and Protocols (Schultze, 2013).

Plant was removed from the pot and the growth medium was gently shaken off from the roots. Roots were stored in 40% ethanol at 4℃ until needed. 0.1 g of root tissue was washed 3 times with 1ml of dH_20. Washed roots were incubated in 1 ml of 10% KOH solution in 70 ℃ for 50 min. Roots were rinsed three times with dH_20. 0.5 ml of ink staining solution was added to the roots (5% pelican brilliant black ink, 5% acetic acid, 90% dH2O) and incubated on a shaker for 30 min. Roots were rinsed three times with 1 ml of 1% acetic acid. Roots were left in the solution of 1% acetic acid and rinsed again next day until the solution remained clear. Root sample was mounted onto a glass slide, and examined with an Olympus CX21 brightfield microscope using 10×–40× objectives (≈100–400× total magnification).

2.6 Barley resource allocation measurements

These measurements were performed by Dr Claire Moulton-Brown (University of Edinburgh).

Plants were cleaned and dried, shoot and root fractions were separated. Each fraction was dried at 60℃ until they reached a constant weight and the weight was recorded.

2.7 DNA extraction from barley roots

This was performed by Dr Claire Moulton-Brown (University of Edinburgh).

DNA extraction was performed using the DNeasy Plant Mini Kit (Qiagen) following the user handbook.

0.1g fresh root tissue was added to a 2ml tube with one sterile tungsten carbide bead and 400 µl Buffer AP1 and 4 µl RNase A stock solution (100 mg/ml). Tissue was lysed using the Tissue Lyser III (Qiagen) two times for 2 min at 30Hz. Mixture was incubated for 10 min at 65°C. 130 µl of buffer P3 was added to the lysate, mixed and incubated for 5 min on ice. The lysate was centrifuged for 5 min at 20,000 x g. The lysate was pippeted into the QIAshredder Mini spin column and centrifuged for 2 min at 20,000 x g. The flow-through was transferred into a new tube. 1.5 volumes of buffer AW1 was added to the cleared lysate and mixed by pipetting. The mixture was transferred into the DNeasy Mini spin column and centrifuged for 1 min at 6000 x g. 500 µl of buffer AW2 was added to the column and centrifuged for 1 min at 6000 x g. 500 µl of buffer AW2 was added again to the DNeasy Mini spin column, and centrifuged for 2 min at 20,000 x g to dry the membrane. DNA was eluted by adding 100 µl buffer AE directly onto the DNeasy membrane, incubated for 5 min at room temperature, and then centrifuged for 1 min at 6000 x g.

2.8 RNA extraction from barely leaves

Plant material was ground at -20°C and 0.5 ml of warm phenol:chloroform:isoamylalcohol mixture (25:24:1) mixture was added. Samples were vortexed and centrifuged at 13 000 rpm for 5 min at 4°C. The aqueous phase was transferred to a new tube and mixed with 0.5 ml of cold chloroform:isoamylalcohol by vortexing vigorously. Samples were centrifuged as earlier. This wash was repeated once more. The aqueous phase was transferred to a new tube and 1/3 volume of 8 M LiCl was added. Samples were incubated at 4°C overnight. Next day, samples were centrifuged at 13 000 rpm at 4°C for 15 min. Supernatant was removed and the pellet was washed 2 times with ice-cold 0.5 ml of 70% ethanol. The pellet was dried for 1 min at room temperature in the SpeedVac. The pellet was rehydrated with 400 µl ddH_2O and kept on ice for 30 min. The pellet was dissolved and mixed with 40 µl NaAc and 1 ml of ice cold 96% ethanol. The samples were incubated for 60 min at -20°C. Samples were centrifuged at 13 000 rpm at 4°C for 15min. Supernatant was removed

and the pellet was washed with 70 % ethanol. Remaining ethanol was removed and the pellet was dried in the SpeedVac at room temperature for 1 min. Pellet was rehydrated with 25 µl ddH_2O and left on ice for 30 min before resuspending. RNA was stored at -80°C until required.

2.9 cDNA synthesis

1 µq of RNA was used when possible. cDNA was generated using SuperScript™ III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. The synthesized cDNA was diluted 10 times with ddH_2O, and stored at -20°C until required.

2.10 Quantitative real-time PCR

Quantitative RT-PCR was performed using SYBR™ Green Universal Master Mix (Applied Biosystems). Each reaction was performed in technical triplicates and contained 2 µl of cDNA and gene-specific primers (Table 2.4). The reaction was run on a Quanstudio 5 system according to the manufacturer's instructions (Applied Biosystems).

Table 2.4 Primers used for genotyping of recombinant constructs.

Target	Primers (F-forward, R- reverse)	Reference
18S rRNA (AMF)	F: ATAGGGATAGTTGGGGGCAT	(Hewins et al., 2015) (Helgason et al., 1998)
	R: GTTTCCCGTAAGGCGCCGAA	
HvEMF1-α	F: GAAGATGATTCCCACCAAGC	(Hua et al., 2015)
	R: TGACACCAACAGCCACAGTT	
HvACT	F: GCCGTGCTTTCCCTCTATG	This study
	R: GAAGGAGTAACCTCTCTCGG	
HvLOX2	F: CGCACCAGTGTCTCCAAG	This study
	R: GAGGAAATCACGAACCCCGG	
Wrky28	F: CATGTGTTTCAACCCGTTCCAG	Adapted from Meng & Wise, 2012
	R: GAAGGCAGAAATGTCGAAGTTGG	
HvPR1b	F: GCTAGCCATCTTGCTCGCC	Adapted from Gao et al. 2018
	R: GCTTGCAGTCGTTGATCCTC	
HvPR2	F: GATGTTGCCTCCATGTTTGCAG	Adapted from Gao et al. 2018
	R: GCATGCCGTTGATGCCCTTG	

HvPR3	F: GTTCCAGGCTACGGTGTAATC	Adapted from Gao et al. 2018
	R: GTTCCGTTGGGTGTAGCAGT	
PhEF2	F: CCCTGAAAATGCTTTGGGTGG	This study
	R: TTGACGGTGTACATGGGAGTG	

2.11 RNA sequencing, processing and analysis

RNA sequencing was performed at Novogene (UK) Company Limited using Illumina Novaseq Platform. The quality of the raw data was assessed using FASTQC (Andrews, 2010). Adapter sequences were trimmed using Trimmomatic (Bolger et al., 2014) aligned, and annotated against barley genome (MorexV3) using Histat2 (Kim et al., 2019). Gene count data was quantified and standardised using HTseq2 (Putri et al., 2022). Differential analysis was performed using edgeR (Chen et al. 2025) in R version 4.3.0 (R Core Team, 2024) by filtering out cpm < 1 and normalising using a TMM method (Robinson and Oshlack, 2010). DEGs were defined as: FDR< 0.05, log2FC > ± 1, logCPM > 2.

2.12 Protein extraction from plant tissue

For SDS-PAGE analysis with subsequent immunoblotting: Frozen plant tissue was ground and 2x volume of protein extraction buffer (20 mM Tris pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.5% NP40, 0.5% SDS, 0.5% Sodium deoxycholate, 20 µM MG132, 10 mM NEM, protease inhibitor cocktail (50 µg/ml N-p-Tosyl-L-phenylalanine chloromethyl ketone (TPCK), 50 µg/ml Nα-Tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), 0.6 mM phenylmethylsulfonyl fluoride (PMSF))) was added. Samples were vortexed and incubated on ice for 10 min. Samples were centrifuged at 10 000 rpm at 4°C for 15 min and the supernatant was saved.

2.13 SDS PAGE and western blot analysis

Protein extracts were incubated in 1 X SDS sample buffer with 50 mM DTT at 80°C for 10 min and separated on an SDS-PAGE. Separated proteins were transferred to a nitrocellulose membrane (BioRad) using a wet transfer at 20 V o/n. Transfer efficacy was assessed by incubating the membrane with Ponceau S solution (Biotum). The membrane was incubated for 2 h with 5% milk in 1 x PBS and 0.1 % Tween (PBST). Subsequently the membrane was incubated with the primary antibody (Table 2.5) for

1.5 h, washed 3 times for 5 min with 1 x PBST and secondary antibody (Table 2.5) for 1 h. The membrane was washed 3 times for 5 min with 1 x PBST and incubated with chemiluminescent substrate (SuperSignal™ West Pico PLUS, ThermoFisher Scientific) using Azure 300 instrument (Cambridge Biosciences Limited).

Table 2.5 Primary and secondary antibodies

Target	Concentration	Make
Poly-ubiquitin (P4D1)	1: 2500	Santa Cruz Biotechnology
Actin	1:10 000	Agrisera
FLAG	1:5000	Merck
His	1:5000	Rockland
HA	1:5000	Invitrogen
GFP	1:5000	Roche
Anti-Mouse HRP-linked	1:10 000	Cell Signalling
Anti-Rabbit HRP-linked	1:10 000	Cell signalling

2.14 Ubiquitin-associated and ubiquitinated proteins immunoprecipitation for LC-MS/MS analysis

As there were time gaps between designing the experiments and processing samples for different datasets used for the immune barley ubiquitome project, the experimental workflow differs slightly for each dataset (Figure 2.2). Datasets included: Immune hormones treated, field trial samples infected with *P. hordei*, and *P. hordei* infected in the lab (at day 1, day 4 and day 7). Samples for all datasets were subject to ubiquilin immunoprecipitation. *P. hordei* infection time course samples were additionally subject to second step of immunoprecipitation with a Lysine remnant (DiGly) antibody to enrich for ubiquitinated peptides. This was done to improve discovery of ubiquitination sites. Subsequent mass spectrometry methods are described in section 2.16. To achieve a higher coverage of identified peptides and better reproducibility, we opted for Data Independent Acquisition (DIA) when possible. For field trial data, due to the large number of samples DDA method was used. For DiGly samples, due to the novelty of the approach (no previous studies in barley ubiquitin proteomics) and as I already enriched for ubiquitinated peptides, I opted for DDA approach. The exemption is day 7, for which DIA approach proved more effective, likely due to the large protein

population in this sample. Samples from ubiquilin IP were subject to Label Free Quantification (LFQ) and downstream differential analysis.

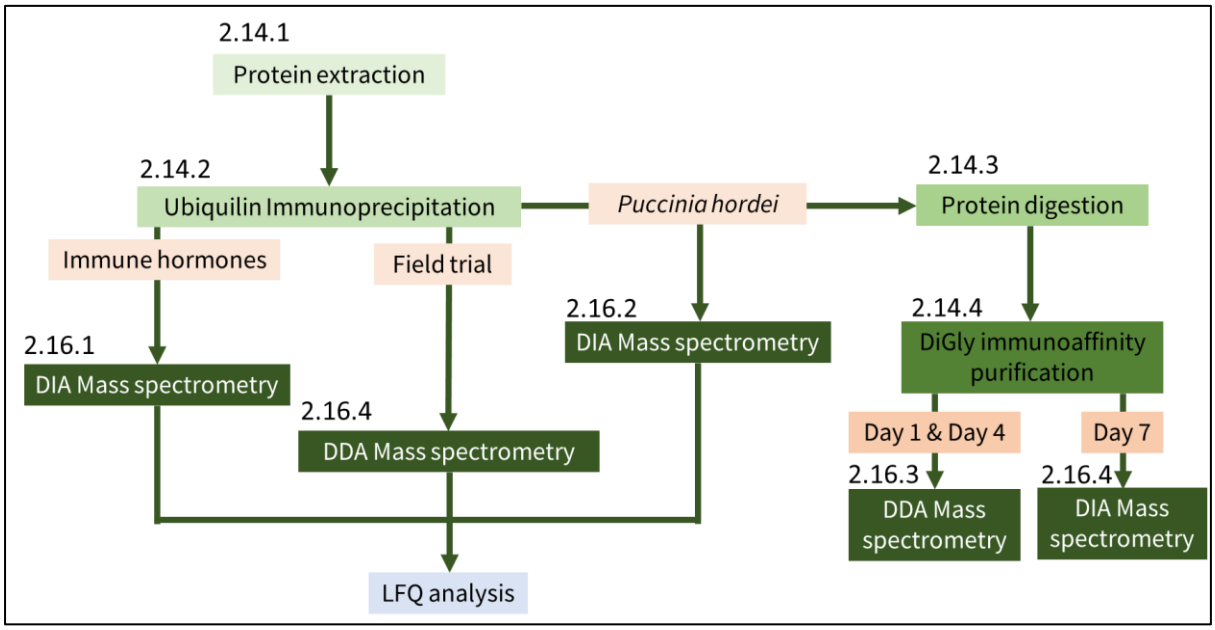

Figure 2.2 **Proteomics workflow.** Diagram shows steps undertaken for each dataset (light orange box) in order to generate proteomics data for the barley immune ubiquitome. A number next to each step (green box) indicates section of the methods chapter where the description of the method can be found.

2.14.1 Protein extraction

Frozen plant tissue was ground in 2x volume of plant protein extraction buffer (0.1% NP-40, 0.1% Triton, 1x PBS, 10 mM NEM, 40 µM MG132, 1x PPI3, 20 µM NSC, protease inhibitor cocktail) and centrifuged for 12 min at 8 500 rpm. The supernatant was collected and spun again for 2 min at 10 500 rpm, supernatant collected and stored on ice. Protein was quantified using Protein Assay kit (Bio-Rad) according to the manufacturer's instructions.

2.14.2 Ubiquilin Immunoprecipitation

HALO-P-Ubiquilin tubes x4 construct (Medical Research Council Protein Phosphorylation and Ubiquitylation Unit, University of Dundee) was expressed in *E.coli* strain BL21 (DE3) with 1 mM IPTG at 24℃ 6h. 50 ml of cell culture was harvested by brief centrifugation at 4000 rpm for 30 min, 4°C. Pellet was frozen and

subsequent protein purification was performed with 2 ml of protein purification buffer (1x BugBuster (Millipore), 1x PBS, 150 mM NaCl, 10mM DTT, protease inhibitor cocktail, 1 ul/ml Benzonase® Nuclease (Millipore)). Cell suspension was incubated on a rotation mixer at RT for 20 min and then spun at 16 000 x g for 14 min at 4°C. Supernatant was collected and combined with washed HaloLink™ magnetic resin (Promega) and incubated on a rotation mixer for 2 hours at 4°C. Resin was washed 4 times with 1 ml of plant protein extraction buffer, combined with 10 mg of plant protein extract and incubated on a rotation mixer for 3 hours at 4°C. Resin was washed 2 times with 1 ml of plant protein extraction buffer and 2 times with 1 ml of 1xPBS. For immune hormone treatment beads were frozen at -80℃ and further processed by the Proteomics Facility of the Institute of Genetics and Cancer, University of Edinburgh. Field trial samples were eluted in 50 µl of 5% SDS in 50 mM TEAB pH 8.5 for 15 min at 70°C, 1200 rpm, then frozen and further processed by the Proteomics Facility at the Roslin Institute, University of Edinburgh. For *P. hordei* time course experiment samples were eluted in 50 µl of 8 M Urea in 20 mM HEPES, pH 8.0 for 30 min at 80°C, 1200 rpm and processed as described in 2.12.3.

2.14.3 Protein digestion

Urea eluted protein samples were incubated with 5 µl of 10 mM DTT in 20 mM HEPES for 30min at room temperature. Then 5 µl of 55 mM chloroacetamide in 20 mM HEPES, pH 8.0 was added and incubated as previously, in darkness. Then 1 µg LysC solution/50 µg protein was added to the samples and incubated overnight at room temperature. 0.5 µg LysC solution/50 µg protein was added to the samples and incubated for another 6 h. Then samples were diluted 4 times with 20 mM HEPES, pH 8.0 and incubated with 1 µg Trypsin solution/50 µg protein at room temperature overnight. The digestion was stopped by acidifying the sample to pH<2.5 with 10% TFA. Peptides were dried in a SpeedVac at room temperature for 2h and then resuspended in 50 µl of 0.15% TFA. Samples were desalted by passing through Peptide Desalting C18 StageTips and washed twice with 10 µl of 0.1% TFA. Samples were eluted in 25 µl of 50% acetonitrile, 0.05% TFA, elution repeated and sample divided into 20% (for mass spectrometry) and 80% (for DiGly purification) fractions. Samples were dried in a SpeedVac at room temperature overnight and further processed as described in 2.12.4. Samples for mass spectrometry were frozen at -

80℃ and further processed by the Proteomics Facility of the Institute of Genetics and Cancer, University of Edinburg.

2.14.4 DiGly immunoaffinity purification

PTMScan® Ubiquitin Remnant Motif kit (Immune Signalling) protocol was followed as per manufacturer's instructions:

Dried peptides from the DiGly fraction were resuspended in 0.5 ml of 1 x HS IAP Bind buffer and pH of the solution was adjusted to pH ~ 7 with 1 M Tris solution. 5 µl of bead slurry/sample was washed 4 times with 1 ml PBS and resuspended in 4 times the original volume of 1x PBS. Beads were combined with the sample and incubated on a rotator for 2 h at 4℃. Supernatant was removed and beads were washed three times with 0.5 ml of chilled HS IAP Wash Buffer and then three times with 0.5 ml of ice-cold 1x PBS. Peptides were eluted with 50 µl of IAP Elution Buffer for 10 min at room temperature, 500 rpm. Elution was repeated one more time. Samples were desalted using Peptide Desalting C18 StageTips (as described previously), eluted in 50% acetonitrile, 0.05% TFA and dried overnight in a SpeedVac at room temperature. Samples were stored at -80 ℃ and further processed by the Proteomics facilities as described in 2.14.

2.15 Ubiquitination assays

2.15.1 *In vitro* ubiquitination assay

SynRGLG2 expression and protein purification was carried as described in 2.3. until the beads washing step. Ubiquitination reaction was set up in 30 µl with 1.5 µl 20x reaction buffer (1M Tris-HCl, pH 7.4, 200 mM $MgCl_2$, 100 mM ATP, 40 mM DTT), 50 ng UBE1 (BioVision), 200 ng Ubc5hc (Ubiquigent), 5 µg ubiquitin (Boston Biochem) and 5 µl SynRGLG2 bound on beads (2.5 µg). 20 mM EDTA was added to the inactive SynRGLG2 control sample. Tubes were incubated for 1.5 h at 30°C with agitation at 900 rpm. The reaction was stopped by adding 10 µl of 4xSDS samples buffer and incubating samples at 95°C for 5 min.

2.15.2 *In vitro* ubiquitination assay with plant protein extract

Plant protein purification was performed as described in 2.12.1. SynRGLG2 expression and protein purification was carried as described in 2.4. Purified SynRGLG2 was then immobilised using Pierce™Anti-DYKDDDDK beads (Thermo

Scientific) by incubating at 4°C overnight. Beads were then washed with 1xPBS and resuspended in plant protein buffer. Ubiquitination reaction was set up in 150 µl with 7.5 µl 20x reaction buffer, 50 ng UBE1 (BioVision), 200 ng Ubc5hc (Ubiquigent), 5 µg HA-ubiquitin (Boston Biochem), 500 µg plant protein extract and 5 µl SynRGLG2 bound on beads (4 µg). The volume was made up to 150 µl with plant protein extract buffer. 20 mM EDTA was added to the inactive SynRGLG2 control sample. Tubes were incubated for 1.5 h at 30°C with agitation at 900 rpm. Beads were washed four times with 1xPBS before resuspending in 30 µl of 10x SDS sample buffer and incubating samples at 95°C for 5 min.

2.15.3 Target trapping

Reaction was carried out as for semi-in vitro assay described in 2.1.2 until the washing step. Beads were frozen at -80°C and handled to the proteomics facility.

2.15.4 Ubiquitination of target proteins

Targets from the *N. benthamiana* frozen tissue from 2.3.2 were immobilized on GFP-Trap beads (Chromotek) using a following protocol. Frozen plant tissue was ground and 2x volume of protein extraction buffer (20 mM Tris pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.5% NP40, 0.5% SDS, 0.5% Sodium deoxycholate, 100x MG132, 100x NEM, protease inhibitor cocktail) was added. Samples were vortexed and incubated on ice for 10 min. Samples were then centrifuged at 10 000 rpm at 4°C for 15 min and the supernatant was saved. The supernatant was combined with 10 µl GFP Trap beads and incubated at 4°C for 1h. Beads were washed two times with protein extraction buffer and two times with DUB buffer (50 mM Tris pH 7.5, 1 mM DTT) and resuspended in 18 µl of DUB buffer. To remove native ubiquitination from target proteins, 2 µl of 10 µM USP2 (R&D Systems) was added to each sample and incubated for 2h at 37°C. Beads were washed 2 times with DUB buffer.

Ubiquitination reaction was set up in 30 µl with 1.5 µl 20x reaction buffer (1M Tris-HCl, pH 7.4, 200 mM $MgCl_2$, 100 mM ATP, 40 mM DTT), 50 ng UBE1 (Biovision), 200 ng Ubc5hc (Ubiquigent), 5 µg HA-ubiquitin (Boston Biochem), 2 µl of purified SynRGLG2 and 5 µl of GFP-bead bound target. Tubes were incubated for 2h at 30°C with agitation at 900 rpm. The reaction was stopped by adding 10 µl of 4xSDS samples buffer and incubating samples at 95°C for 5 min.

2.16 LC – Mass spectrometry

Samples from different datasets were analysed at different times and at separate proteomics facilities. This was primarily due to the experiments being conducted at different stages of the project, as well as differences in instrument availability and technical expertise across the facilities. Consequently, samples were processed and analysed at the facility best suited to support the specific requirements of each dataset at the time of analysis. Key information about the proteomics processing is summarised in Table 2.6. Protein samples from all biological replicates, belonging to the same dataset, were processed at the same time and using the same digestion protocol without any deviations. They were subjected for MS analysis under the same conditions. Samples from ubiquilin immunoprecipitation were later subjected to Label-Free Quantification (LFQ) method. To make up for the differences between datasets, I followed the same data analysis protocol (described in 2.16.7) for all datasets.

Table 2.6 Comparison of the applied mass spectrometry approach for each dataset.

Dataset	Elution buffer	Digestion	Approach	LC	MS instrument	Software
Immune hormones	Not eluted prior to digestion	At the proteomics facility; Trypsin	DIA	Aurora Column	Fusion Lumos	DIANN
Field Trial	5% SDS in 50 mM TEAB pH 8.5	At the proteomics facility; Trypsin	DDA	Aurora column	timsTOF FleX	PEAKS Xpro and SpectroMine

Puccinia (ubiquilin)	8 M Urea in 20 mM HEPES, pH 8.0	Done by me; LysC & Trypsin	DIA	Aurora Column	Fusion Lumos	DIANN
Puccinia (DiGly day 1 and day 4)	8 M Urea in 20 mM HEPES, pH 8.0	Done by me; LysC & Trypsin	DDA	Self-packed analytical uChrom column	Fusion Lumos	MS-Fragger v. 22.0
Puccinia (DiGly day 7)	8 M Urea in 20 mM HEPES, pH 8.0	Done by me; LysC & Trypsin	DIA	EASY-Spray column	Fusion Lumos	MaxQuant

2.16.1 Immune hormones samples

Samples were processed at the Western General mass spectrometry facility using a DIA approach. Beads were digested using 2M Urea, 50mM Tris-HCl, 1mM DTT and 0.1 µg/µL LC-MS grade trypsin. The digestion buffer was left on the beads for 30 mins at 37℃after which it was removed from the beads and left to digest overnight at 37℃. Once the digestion was complete, the samples were alkylated using 20 µL of a 5 mg/ml solution of Iodoacetamide and were incubated in the dark for 30 minutes. Once the incubation was completed, the samples were acidified to 1% final concentration using 10% TFA (Trifluoroacetic acid). The samples were desalted using C18 tips that were prepared in-house using P200 tips. They were activated with washes of methanol and 0.1% TFA respectively. The samples were then added to them and the tips the washed with 0.1% TFA once again. The samples were eluted using 50% ACN and 0.05% TFA and dried in a vacuumfuge after which it was reconstituted using 0.1% TFA.

Desalted peptides were then loaded onto 25cm Aurora Columns (IonOptiks, Australia) using a RSLC nano uHPLC systems connected to a Fusion Lumos mass spectrometer. Peptides were separated by a 70 min linear gradient from 5% to 30% acetonitrile, 0.5% acetic acid. The mass spectrometer was operated in DIA mode, acquiring a MS 350-1650 Da at 120k resolution followed by MS/MS on 45 windows with 0.5 Da overlap (200-2000 Da) at 30 k with a NCE setting of 28. The samples were searched against Uniprot *Hordeum vulgare subsp. vulgare* proteome (UP000011116) database including ubiquitin modifications using DIA-NN 1.9.

2.16.2 Puccinia Day 1, Day 4, Day 7 ubiquilin IP

Samples were processed at the Western General mass spectrometry facility using a DIA approach. Desalted peptides were loaded onto 25cm Aurora Columns (IonOptiks, Australia) using a RSLC nano uHPLC systems connected to a Fusion Lumos mass spectrometer. Peptides were separated by a 70 min linear gradient from 5% to 30% acetonitrile, 0.5% acetic acid. The mass spectrometer was operated in DIA mode, acquiring a MS 350-1650 Da at 120k resolution followed by MS/MS on 45 windows with 0.5 Da overlap (200-2000 Da) at 30 k with a NCE setting of 28. Protein discovery was performed against Uniprot *Hordeum vulgare subsp. vulgare* proteome (UP000011116) database including ubiquitin modifications using DIA-NN 1.9.

2.16.3 Puccinia Day 1 and Day 4 DiGly samples

Puccinia Day 1 and Day 4 DiGly samples were processed at the Proteomics Facility of the Institute of Genetics and Cancer, University of Edinburg using a DDA approach. 5 µl of the resuspended peptides was analysed by reversed-phase nano–LC-MS/MS using a nano-Ultimate 3000 LC system and a Lumos Fusion mass spectrometer (Thermo Fisher Scientific). Flow rates were 400 nl/min. Peptides were loaded onto a self-packed analytical column (uChrom 1.6, 0.075 mm by 25 cm) using a 67-min gradient buffer A (2% acetonitrile, 0.5% acetic acid) and buffer B (80% acetonitrile, 0.5% acetic acid); 0 to 16 min: 2% buffer B, 16 to 56 min: 3 to 35% buffer B, 56 to 62 min: 99% buffer B; 62 to 67 min 2% buffer B. Full-scan spectra recording in the Orbitrap was in the range of m/z 350 to m/z 1,400 (resolution: 240,000; AGC: 7.5e5 ions). MS2 was performed in the ion trap, with an isolation window of 0.7, an AGC of 2e4, an HCD collision energy of 28, rapid scan rate, a scan range of 145 to 1450 m/z, 50-ms maximum injection time, and an overall cycle time of 1 s. Protein discovery was

performed against Uniprot *Hordeum vulgare subsp. vulgare* proteome (UP000011116) database including ubiquitin modifications using MS-Fragger v. 22.0.

2.16.4 Puccinia Day 7 DiGly samples

Puccinia Day 7 DiGly samples were processed at the Proteomics Core of the DRP-HCB using a DIA approach. Peptides were eluted in 40 µl of 80% acetonitrile in 0.1% TFA and concentrated down to 1 µl by vacuum centrifugation (Concentrator 5301, Eppendorf, UK). They were then prepared for LC-MS/MS analysis by diluting it to 5 µl by 0.1% TFA.

LC-MS-analyses were performed on an Orbitrap Fusion™ Lumos™ Tribrid™ Mass Spectrometer (Thermo Scientific, UK) coupled on-line, to an Ultimate 3000 RSLCnano Systems (Dionex, Thermo Fisher Scientific, UK). Peptides were separated on a 50 cm EASY-Spray column (Thermo Scientific, UK), which was assembled on an EASY-Spray source (Thermo Scientific, UK) and operated at 50°C. Mobile phase A consisted of 0.1% formic acid in LC-MS grade water and mobile phase B consisted of 80% acetonitrile and 0.1% formic acid. Peptides were loaded onto the column at a flow rate of 0.3 µl min-1 and eluted at a flow rate of 0.25 µl min-1 according to the following gradient: 2 to 40% mobile phase B in 150 min and then to 95% in 11 min. Mobile phase B was retained at 95% for 5 min and returned back to 2% a minute after until the end of the run (190 min). FTMS spectra were recorded at 120,000 resolution (scan range 350-1500 m/z) with an ion target of 7.0×105. MS2 was performed in the ion trap with ion target of 1.0×104 and HCD fragmentation (Olsen et al. 2007) with normalized collision energy of 27. The isolation window in the quadrupole was 1.4 Thomson. Only ions with charge between 2 and 6 were selected for MS2.

The MaxQuant software platform (Cox and Mann, 2008) version 1.6.1.0 was used to process the raw files and search was conducted against the Uniprot *Hordeum vulgare subsp. vulgare* proteome (UP000011116), using the Andromeda search engine (Cox et al. 2011). For the first search, peptide tolerance was set to 20 ppm while for the main search peptide tolerance was set to 4.5 pm. Isotope mass tolerance was 2 ppm and maximum charge to 7. Digestion mode was set to specific with trypsin allowing maximum of two missed cleavages. Carbamidomethylation of cysteine was set as fixed modification. Oxidation of methionine, and the DiGly residue on lysine were set as variable modifications. Absolute protein quantification was performed as described

in Schwanhüusser et al. 2011. Peptide and protein identifications were filtered to 1% FDR.

2.16.5 Field trial samples

Field trial samples were processed by the Proteomics Facility at the Roslin Institute using a DDA approach. Protein samples (10 µg each), quantified by BCA assay, were digested using S-Trap micro columns (Protifi, USA) following the standard protocol. Lysates were clarified by centrifugation at 15,000 × g for 15 min. Disulfide bonds were reduced with 10 mM TCEP (30 min, 25 °C, 900 rpm) and alkylated with 10 mM chloroacetamide (45 min, 25 °C, dark). Proteins were acidified with 12% phosphoric acid (1:10 ratio) and precipitated using 6× volume of S-Trap buffer (90% methanol, 100 mM TEAB). Samples were loaded onto S-Trap columns and washed four times by centrifugation at 4,000 × g. On-column digestion was performed with trypsin (1:25 enzyme-to-substrate) in 50 mM TEAB at 47 °C for 2 h. Peptides were eluted sequentially with 50 mM TEAB, 0.1% formic acid, and 50% ACN/0.1% FA, pooled, and quantified by microBCA assay.

Peptides were separated over a 90 min gradient on a 25 cm Aurora column (IonOpticks) using an UltiMate RSLCnano LC system (Dionex) coupled to a timsTOF FleX mass spectrometer (Bruker) via CaptiveSpray ionization. Flow rate was 200 nL/min (gradient) and 500 nL/min (washout), with the column at 50 °C. DDA-PASEF mode was used, scanning 100–1700 m/z and 1.45–0.65 Vs/cm² ($1/K_0$), acquiring up to 10 MS/MS frames per cycle, excluding singly charged ions. Raw mass spectrometry data were processed using PEAKS Xpro (Studio 10.6, Bioinformatics Solutions Inc., Canada) for de novo sequencing, database searching, and protein identification for identification of DiGly peptides and with SpectroMine (Biognosys) for Label-free quantification (LFQ). Spectra were searched against the Uniprot *Hordeum vulgare subsp. vulgare* proteome (UP000011116) database. Search parameters included trypsin digestion with one missed cleavage, a parent mass error tolerance of 20 ppm, and a fragment mass error tolerance of 0.06 Da. Carbamidomethylation of cysteine and lysine diglycine (+114.04) were set as a fixed modification. FDR was controlled at 1% at PSM, peptide, and protein levels. Label-free quantification (LFQ) was performed with ID transfer enabled.

2.16.6 SynRGLG target trapping samples

Beads were digested using 2M Urea, 50mM Tris-HCl, 1mM DTT and 0.1 ug/uL LC-MS grade trypsin. The digestion buffer was left on the beads for 30 mins at 37 degrees after which it was removed from the beads and left to digest overnight at 37 degrees. Once the digestion was complete, the samples were alkylated by using 20 µlof a 5 mg/ml solution of Iodoacetamide and were incubated in the dark for 30 minutes. Once the incubation was completed, the samples were acidified to 1% final concentration using 10% TFA (Trifluoroacetic acid).

The samples were desalted using C18 tips that were prepared in-house using P200 tips. They were activated with washes of methanol and 0.1% TFA respectively. The samples were then added to them and the tips the washed with 0.1% TFA once again. The samples were eluted using 50% ACN and 0.05% TFA and dried in a vacuumfuge after which it was reconstituted using 0.1% TFA.

Desalted peptides were then loaded onto 25cm Aurora Columns (IonOptiks, Australia) using a RSLC nano uHPLC systems connected to a Fusion Lumos mass spectrometer. Peptides were separated by a 70 min linear gradient from 5% to 30% acetonitrile, 0.5% acetic acid. The mass spectrometer was operated in DIA mode, acquiring a MS 350-1650 Da at 120k resolution followed by MS/MS on 45 windows with 0.5 Da overlap (200-2000 Da) at 30 k with a NCE setting of 28. The samples were searched against the Uniprot *Hordeum vulgare subsp. vulgare* proteome (UP000011116) with the addition of protein sequences of enzymes used in the reaction (SynRGLG, UBE1, Ubc5hc) using DIA-NN 1.9.

2.16.7 Data analysis

For label free quantification, proteins that appeared as at least two different peptides in the same sample were only considered as real. Differential protein abundance analysis was performed using LFQ intensities to identify proteins significantly altered between control and treatment conditions. Data preparation was done according to the protocol described by Aguilan et al. (2020): LFQ intensity values were Log2 transformed and normalised by the average and the slope. Missing values were imputed by assigning a random number with the distribution comparable to the sample distribution. Fold change and p-value were then calculated comparing treated vs control conditions.

Protein information regarding associated biological processes and protein function were accessed through UniProt (The UniProt Consortium, 2025).

Promoter analysis for WRKY- and ERF-binding motifs was performed using FIMO software (Grant et al., 2011).

2.17 Data visualisation and analysis

The majority of visualisations were performed in R using ggplot2 package (Wickham et al., 2016). Venn diagrams were created using ggVennDiagram (Gao et al., 2024), heatmaps using pheatmap package (Kolde, 2025) and PCA using PCAtools (Blighe and Lu, 2025). Upset plots were created in Python using upsetplot package (Lex et al., 2014). Motif analysis was performed using PTMphinder (Wozniak and Gonzalez, 2019) and visualised using ggseqlogo package (Wagih, 2017).

Gene ontology (GO) enrichment analysis was performed using g:Profiler (Kolberg et al., 2023) and visualised using ggplot2 package in R.

Protein interaction networks were created in Cytoscape (Shannon, 2003) Using STRING database for protein-interaction and Gene Ontology enrichment data (Szklarczyk et al., 2023). Build-in ClusterMaker app was used for MCL clustering of protein interaction nodes (Morris et al., 2011).

Pathway enrichment and visualisation were performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) resource (Kanehisa & Goto, 2000). Rice (*Oryza sativa subsp. Japonica)* orthologues of gene of interest were first identified using g:Orth g:Profiler tool (Kolberg et al., 2023) and then queried against the KEGG Mapper tool of chosen pathways. For visualisation, pathways were downloaded directly from KEGG as annotated pathway maps.

Chapter 3

Arbuscular Mycorrhizal Fungi (AMF) prime immune response against *P. hordei* and modulate barley ubiquitin system

Parts of this chapter have been published in:

Moulton-Brown, C., Brzezinska, K., Orosa-Puente, B. & Helgason, T. 2026. AMF primes immune genes against *Puccinia hordei* (Brown rust) in *Hordeum vulgare* but does not reduce pathogen burden. FEMS Microbiology Letters, 373.

3.1 Introduction

Current management strategies for *P. hordei* involve fungicide application, removal of volunteer hosts and relying on partially-resistant cultivars. Breeding for resistance, however has proven challenging in the past (Park, 2003). With climate change increasingly creating conditions that favour *P. hordei* epidemics, there is an urgent need to develop more reliable and sustainable strategies to reduce the spread of this pathogen. Such approaches include the use of arbuscular mycorrhizal fungi (AMF), which not only provide growth and nutrition benefits but have also been proven to be successful in providing protection against various plant pathogens (Boyno et al., 2024).

AMF are symbiotic fungi belonging to the phylum Glomeromycota (Schüßler et al., 2001), comprising 332 known and many more unknown species (Wijayawardene et al., 2020). They form a symbiotic relationship with roots of more than 70% of plant species (Brundrett & Tedersoo, 2018), including many cereal crops, and are present in soils throughout the globe (Větrovský et al., 2023). AMF can provide a multitude of benefits to the host plants, including increasing accessible soil volume and therefore nutrient availability, reducing the need for fertilizers (Mimmo et al., 2015); increasing drought tolerance, by improving plant's water economy and creating stable soil aggregates (Tang et al., 2022); improving soil health; and most importantly, protecting against pests and diseases, by providing a broad-spectrum systemic resistance, termed mycorrhiza-induced resistance (MIR) (Cameron et al., 2013).

AMF colonisation requires the activation of processes that closely resemble those triggered during immune responses. To initiate colonisation, AM fungi release chitooligosaccharides (COs), which are recognised by the plant and initiate the symbiosis (Feng et al., 2019). However, these same chitin-derived COs can also act as pathogen-associated molecular patterns (PAMPs), which, when detected by the plant, activate PAMP-triggered immunity (PTI). One of the first detectable responses to AM fungi is the initiation of calcium oscillation, which is also important in the early stages of the immune response (Luginbuehl and Oldroyd, 2016). Unlike pathogen-induced resistance, however, mycorrhiza-induced resistance (MIR) leads to immune priming, which remodels host immune signalling networks to enable faster and more effective activation of defence responses (Cameron et al., 2013).

It is thought that the suppression of the SA-mediated immune response pathway during the early colonisation phase of the AM symbiosis formation results in MIR by activating JA- and ethylene-dependent immune response (Cameron et al., 2013). This is why AMF have traditionally been associated with enhanced resistance against necrotrophic pathogens and herbivores, rather than biotrophic pathogens (Pozo & Azcón-Aguilar, 2007). However, recent studies show that MIR can amplify both SA- and JA- responses and provide protection against foliar biotrophic pathogens (Mustafa et al., 2016; Fujita et al., 2022). For example, colonisation of wheat with *Funneliformis mosseae* reduced susceptibility to powdery mildew (*Blumeria graminis*) by upregulating pathogenesis-related (PR) and phenylpropanoid biosynthesis genes. Similarly, colonisation of barley with the basidiomycete fungus *Piriformospora indica* induced systemic resistance against the biotrophic leaf pathogen *Blumeria graminis f. sp. hordei* (Molitor et al., 2011).

Overall, there is a scarcity of studies investigating the effect of AM symbiosis in mediating resistance against barley pathogens and most of the studies look at hemibiotrophic and necrotrophic pathogens (Farhaoui et al., 2025). However, evidence from other cereal species, together with the demonstrated effectiveness of AMF against hemibiotrophic pathogens, which require a flexible immune response able first to suppress the biotrophic phase and then combat the transition to necrotrophy, highlight the potential of AMF to enhance resistance against biotrophic pathogens in barley.

In this chapter, I investigate how symbiosis with the arbuscular mycorrhizal (AM) fungus *Rhizophagus irregularis* influences barley immune responses and disease development during infection with the biotrophic pathogen *Puccinia hordei.* I hypothesised that AM symbiosis with *R. irregularis* would alter barley responses to *P. hordei*. To test this, together with collaborators, we established a controlled tripartite system including the host (*Hordeum vulgare subsp. vulgare cv. Bowman*), the AM fungus (*R. irregularis*), and the pathogen (*P. hordei*), applied either individually or in combination. Results presented here show that *P. hordei* infection had a stronger negative effect on barley growth than AM symbiosis could compensate for, and AM association did not prevent *P. hordei* infection.

However, AMF was found to prime SA-responsive PR genes and regulate immune signalling pathways by modulating expression of WRKY transcription factors, calcium-dependent protein kinases (CDPKs) and proteins involved in immune hormone pathways. Notably, AMF also primed the expression of ubiquitination machinery genes, particularly members of the ATL and PUB families, while reducing global ubiquitination levels. Taken together, these results indicate that *R. irregularis* primes the barley immune system by reprogramming immune gene expression and reshaping the cellular proteome through the ubiquitin pathway, but this priming is not sufficient to confer MIR.

This work was conducted in collaboration with Dr Claire Moulton-Brown and Prof Thorunn Helgason, who are experts in the study of arbuscular mycorrhizal fungi (AMF).

3.2 Results

3.2.1 AMF colonise barley roots without a growth trade-off

Association with Arbuscular Mycorrhizal Fungi (AMF) can provide a multitude of benefits to the colonised plant such as increased biomass, better nutrition and protection against environmental and biotic stresses. However, because the relationship between the plant and its symbionts is nutrient-dependent, it was important to establish an optimal growth environment that promotes efficient AMF colonisation while maintaining adequate nutrient availability to prevent nutrient deficiency and support normal barley growth. Together with the collaborators, we optimised the growth media (see methods section 2.2.4) and developed a method for successful AMF colonisation in barley under controlled conditions. We found that, after five weeks of growing barley plants with inoculum of an AM fungus *Rhizophagus irregularis*, barley plants were colonised by Rhizophagus with a median of 15 AMF DNA copy numbers in the roots (Figure 3.1A). I also observed established colonisation in the roots with visible arbuscules and vesicles (Figure 3.1B). To determine whether colonisation had a detrimental effect on barley growth, we measured shoot and root dry weight as indicators of growth performance. Analysis of resource allocation (Figure 3.1C) showed no significant differences between control and colonised plants, although the root:shoot ratio was slightly higher in inoculated plants. These results

demonstrate that a successful AMF-barley association can be established without negatively impacting plant growth.

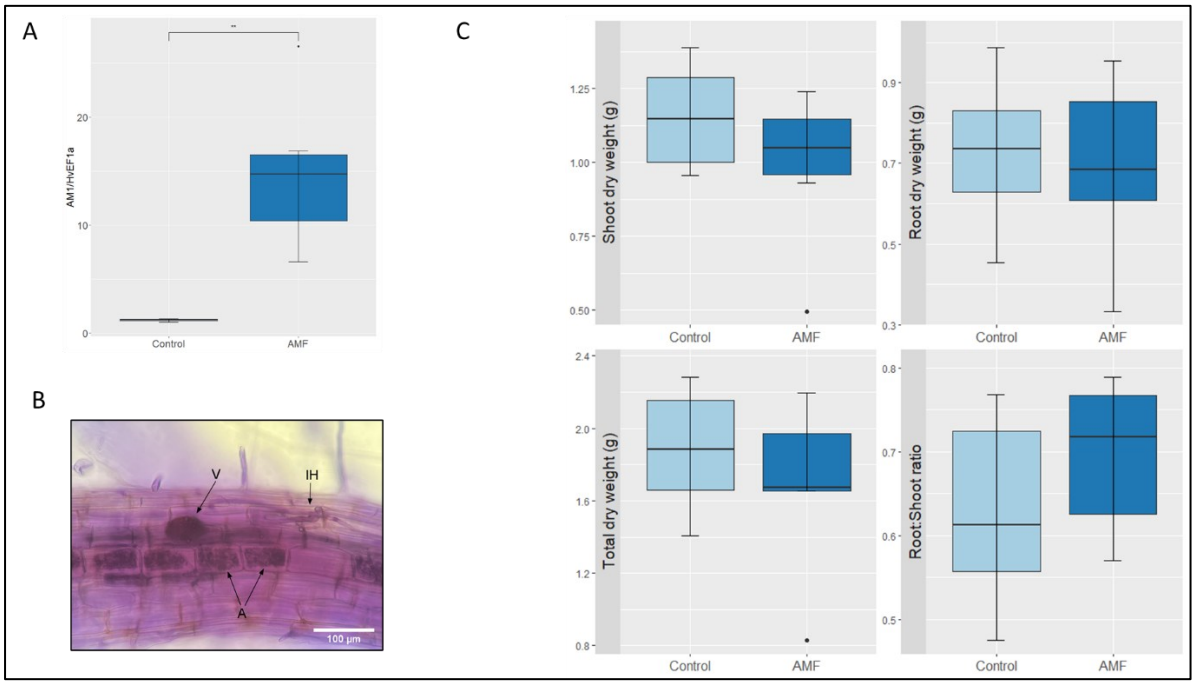

Figure 3.1 AMF colonise barley roots. (A) Quantification of the relative AMF DNA abundance in barley roots, quantified as AMF DNA copy number normalised to *HvEMF1α* in control and AMF inoculated barley plants after 4 weeks from inoculation; (B) Staining of the roots colonised with AMF; V – vesicle, IH – intraradical hyphae, A – arbuscules; (C) Resource allocation measurements for shoot, root and total dry weight, and the root:shoot ratio (t-test; n=6).

To study the effect of AM fungi on barley fitness and disease resistance, we established a model system in which barley plants were grown with Rhizophagus inoculum for four weeks before being infected with the biotrophic fungal pathogen *Puccinia hordei* for one week, a time point previously established in our lab as corresponding to the decline of immune activation and the substantial accumulation of fungal biomass within the leaf.

Our results showed that there is no significant difference in AM fungal colonisation between healthy and infected plants (Figure 3.2A). Infected plants showed higher range of colonisation, with a lower median AMF copy number than healthy plants, however it cannot be concluded whether this difference was due to the infection or

inconsistency in the colonisation. I observed a slight, although not significant, increase in the infection load in AMF-colonised infected plants when compared to AMF-free plants (Figure 3.2B). Additionally, we there is no difference between AMF-colonised infected plants and infected plants in all four metrics of resource allocation measurements (Figure 3.2C). As expected, we can observe a striking difference in resource allocation when comparing control and *P. hordei* infected plants, with shoot, root and total dry weight significantly lower than control plants, which was not reversed by AMF. Root:shoot ratio was not affected, which shows that *P. hordei* infection has a uniformly negative effect on plant fitness. Altogether, these results show that, in this experimental setup, AMF does not have a significant effect on growth or *P. hordei* infection in barley plants.

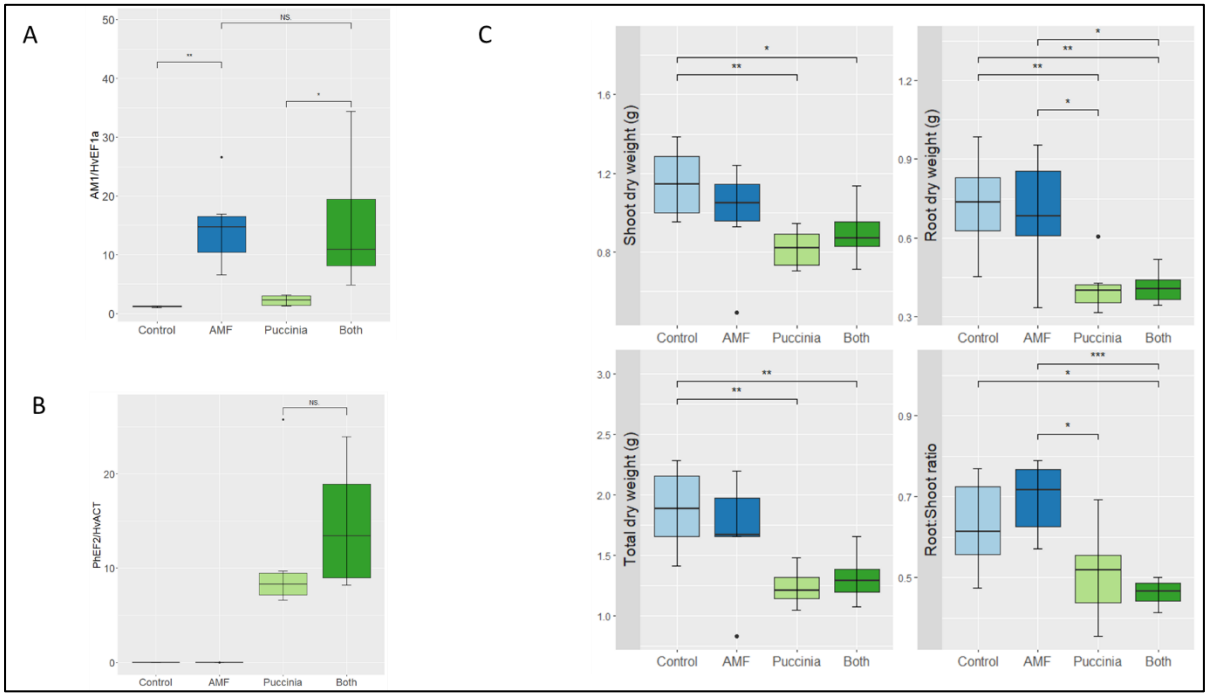

Figure 3.2 *P. hordei* infection has a larger effect on growth than AMF colonisation does. (A) Quantification of the relative AMF DNA abundance in barley roots, quantified as AMF DNA copy number normalised to *HvEMF1α* in control and AMF inoculated barley plants infected with *P. hordei*. (B) Relative quantification of *P. hordei* infection. (C) Resource allocation measurements for shoot, root and total dry weight, and the root:shoot ratio for control and infected, AMF inoculated plants (t-test; n=6).

3.2.2 AMF have an effect on expression of barley immune genes

Despite not observing a clear phenotype of MIR in this system, previous studies have reported protective effects of AMF in crops against several pathogens. We therefore decided to investigate whether AMF colonisation induces molecular changes in barley and whether these could modulate host responses during *P. hordei* infection. Because plants undergo extensive transcriptional reprogramming upon pathogen detection, we chose gene expression as a sensitive approach to capture even subtle changes. I analysed the expression of pathogenesis-related (*PR*) genes, which are typically induced in response to *P. hordei*, as well as an immune-associated WRKY transcription factor and a jasmonic acid-induced lipoxygenase (*LOX*) gene. These results show that AMF alone did not induce *PR* genes or *WRKY28* expression, but enhanced their induction during *P. hordei* infection (Figure 3.3A–D). Interestingly, AMF alone upregulated *LOX2*, but this induction was reversed upon pathogen infection (Figure 3.3E).

Together, these findings indicate that AMF colonisation does not trigger immune gene expression in the absence of challenge, avoiding potential fitness costs, but may enhance the amplitude of immune responses once *P. hordei* infection occurs.

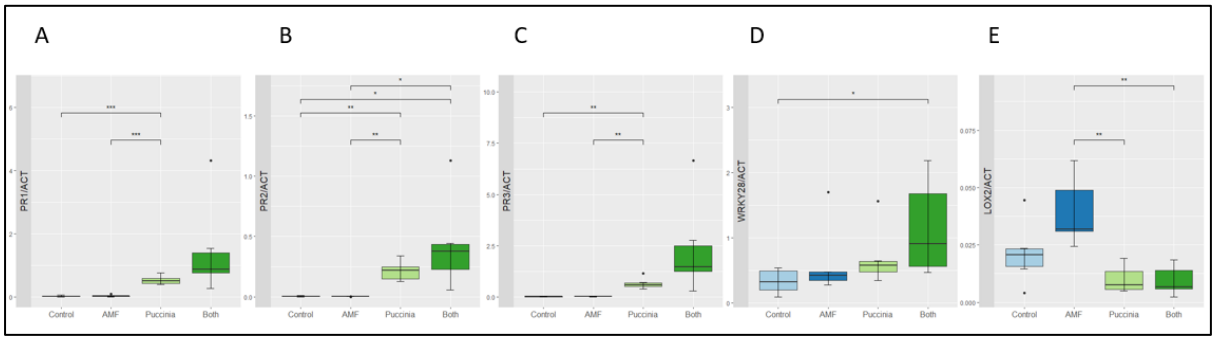

Figure 3.3 AMF boost expression of immune genes during *P. hordei* infection. Relative expression of immune marker genes (A) *PR1*, (B) *PR2*, (C) *PR3*, (D) *WRKY28*, (E) *LOX2* (t-test, n=6).

To further unravel the complex relationship between the symbiont, host plant, and pathogen, and gain a comprehensive view of the transcriptomic changes induced by AMF, I performed RNA sequencing on leaf tissue under the same treatment conditions. Three samples were chosen for each treatment, representative for the within-treatment variability (Figure S1). Principal component analysis performed on the treatment-specific transcriptomes revealed a large difference between the control and AMF-colonised samples (Figure S2). In total, 526 genes were found to be differentially expressed between control and AMF-colonised samples, with 344 genes being upregulated, and 182 downregulated by AMF (Figure 3.4A). Gene ontology enrichment analysis revealed a major effect of AMF colonisation on various biological processes (Figure 3.4B). AMF was found to induce expression of genes involved in sugar and amino-acid metabolism and, more importantly, regulation of specific defence responses. Interestingly, AMF-colonised plants had upregulated genes involved in protein-modification processes, which could suggest that AMF induces changes by targeting plant proteome.

Additionally, I observed a downregulation in proline biosynthesis, an amino acid involved in stress tolerance, cellulose metabolism, and response to antioxidant stress, which suggests that AMF may enhance stress tolerance in colonised plants.

Altogether, these data show, that AMF has a significant impact on barley transcriptional profile in healthy plants and could be involved in priming immune genes for improved resistance against pathogens. Interestingly, this effect in transcriptional reprogramming does not have a growth trade-off.

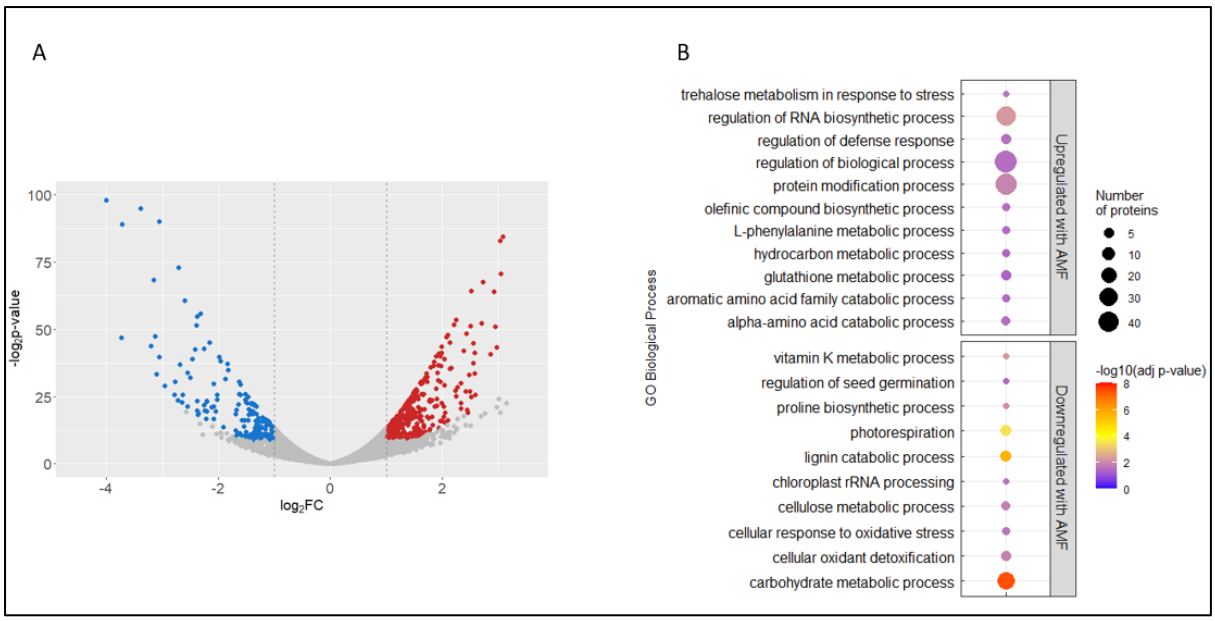

Figure 3.4 AMF induce transcriptional changes in barley leaves. (A) 526 genes were differentially expressed between control and AMF colonised plants (FC>2, FDR<0.05, logCPM >2). (B) Gene Ontology enrichment analysis of Biological Processes enriched in healthy plants grown in the presence of AMF (based on g:Profiler two-stage hybrid term list filtering algorithm and Benjamini-Hochberg method applying significance threshold of 0.05, term size >2).

3.2.3 AMF modulate expression of immune genes during *Puccinia hordei* infection

To understand how AMF regulates gene expression during immune response, I looked at genes that were differentially expressed between treatments. The analysis showed a synergistic expression behaviour between control and AMF-colonised plants upon *P. hordei* infection, with several gene clusters exhibiting interesting behaviours (Figure 3.5A). To see whether this gene behaviour correlates with gene function, I performed a Gene Ontology (GO) enrichment analysis on the Clusters from Figure 3.5A (Figure 3.5B). I found that Cluster 1, which showed upregulation in healthy and downregulation in infected barley leaves, independently on AMF colonisation, was involved in response to light and regulation of translation. Cluster 2, which contains genes highly induced by infection, and induced to a lesser extent in AMF-colonised plants, was shown to be involved in protein phosphorylation and metabolism of cinnamic acid and oxoacids. Interestingly, as showed by Clusters 5 and 6, during infection, AMF also

dampens the expression of genes involved in protein phosphorylation. This antagonistic behaviour of protein phosphorylation genes could be explained by regulation on different levels of phosphorylation-dependent signalling pathways. Cluster 3 revealed that during infection AMF amplifies expression of genes involved in defence response: response to wounding, biotic stimulus and glutathione metabolism. This confirms the observation made in Figure 3.3, that AMF is able to boost certain aspects of the immune response during *P. hordei* infection, potentially corresponding to the well-documented phenomenon of Mycorrhiza-induced resistance (MIR).

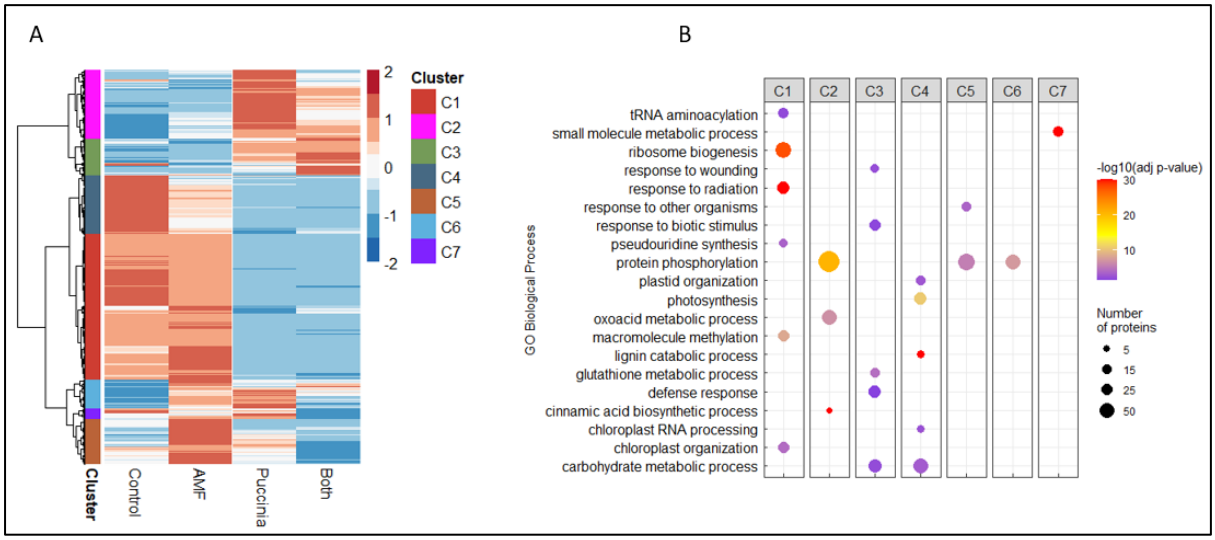

Figure 3.5 AMF modulate expression of barley immune genes. (A) Scaled expression of differentially expressed genes in barley leaves in control, AMF-colonised, *P. hordei*-infected and AMF-colonised *P. hordei* infected plants (FC>2, FDR<0.05, logCPM >2; AMF/Control, AMF/Puccinia, Both/Puccinia, Puccinia/Control, Both/Control, AMF/Both). Genes were clustered into 7 clusters based on behaviour (Ward's method was used for clustering and Euclidean method for calculating row distances; Euclidean distance dis-similarity = 20). (B) Gene Ontology enrichment analysis showing Biological Processes enriched in each cluster of genes from A (based on g:Profiler g:SCS multiple testing correction method applying significance threshold of 0.05).

As shown in Figure 3.5, the symbiotic association with AMF influences gene expression during *P. hordei* infection. Most of the transcriptional changes observed in AMF-colonised healthy plants are not maintained once infection occurs. Moreover, *P.*

hordei-induced transcriptional reprogramming appears less pronounced in AMF-colonised plants, with only a subset of genes being further up- or downregulated compared to *P. hordei*-infected plants without AMF. This intriguing observation prompted us to further analyse the transcriptome changes in AMF-colonised infected plants (Figure 3.6). Only 163 genes were found to be upregulated and 68 downregulated by AMF during infection (Figure 3.6A). Additionally, when comparing the number of genes upregulated by *P.hordei* infection in control plants (Puccinia/Control) and AMF-inoculated plants (Both/AMF) – there is 15% less upregulated and 30% less downregulated *P. horde*-induced genes in the presence of AMF. Taken together, these results suggest that AMF dampens the extent of the typical transcriptional response to *P. hordei* while also inducing additional transcriptional changes. Alternatively, it is possible that infection progression, and consequently the timing of immune activation, does not follow the same trajectory in AMF-colonised plants.

To assess whether AMF regulates specific pathways, I analysed GO Biological Processes associated with all genes up- or downregulated in AMF-colonised plants during infection compared to infected controls (Figure 3.6B). The most common processes among upregulated genes were linked to targeted immune responses, including defence against fungi, chitin catabolism, response to wounding, and salicylic acid (SA) and jasmonic acid (JA) metabolism. In contrast, most downregulated genes were associated with broader defence responses (e.g., cell surface receptor signalling, response to other organisms, vesicle-mediated transport) as well as metabolic processes. This could be caused by the AMF-induced priming of broad-spectrum defence responses resulting in the variability in the timing of immune activation.

Importantly, relying solely on gene expression patterns and GO-term enrichment to assess AMF contributions to barley immunity risks overlooking key pathways. Pathways represented by only a small number of differentially expressed genes may escape enrichment analysis, yet still play critical roles in mediating the tripartite interaction. Therefore, I next explored the regulation of specific immune pathways that might be modulated by AMF.

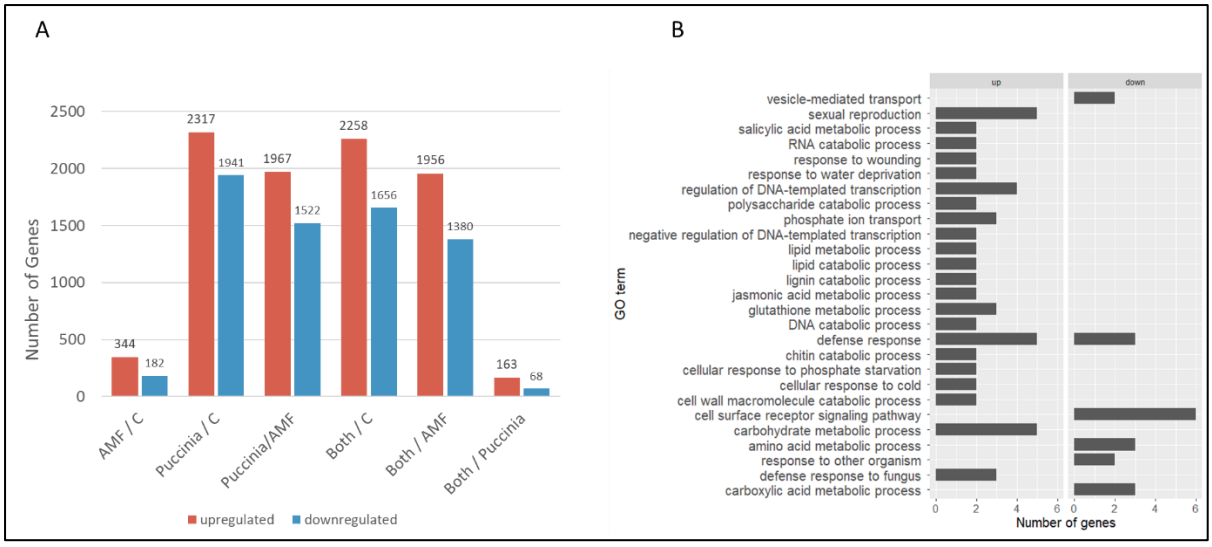

Figure 3.6 AMF induce changes in the expression of immune genes during *P. hordei* infection. (A) Genes identified to be differentially upregulated (red) and downregulated (blue) in AMF-colonised, *P. hordei* infected, or AMF-colonised *P. hordei*-infected plants compared to respective controls (FC>2, FDR<0.05, logCPM >2). (B) GO Biological Processes associated with proteins upregulated and downregulated in AMF-colonised *P. hordeu* infected plants compared to *P. hordei*-infected control (≥2 genes).

3.2.4 AMF modulate immune signalling pathways in barley.

To investigate the crosstalk between AMF and the pathogen during barley infection, and their respective contributions to the host immune response, I analysed key immune signalling pathways and their components at the pathway level. Since transcriptional reprogramming is a hallmark of immune activation, and AMF colonisation was found to broadly alter transcription factor expression, I examined this group in more detail. I identified presence of many WRKY transcription factors in the list of differentially expressed genes, which are thought to be involved in regulating both biotic and abiotic stress responses. Almost half of all identified barley WRKY transcription factors were found to be differentially expressed in this study (Figure 3.7A). I found that AMF induced the expression of many WRKY genes in healthy plants, but generally downregulated infection-induced WRKYs in infected plants. Notable exceptions included *WRKY4/106* in healthy plants and *WRKY27, WRKY63,* and *WRKY1/38* in infected plants. Interestingly, while AMF had no effect on *P.hordei*-

induced WRKYs in healthy plants, they reduced their expression during infection. Most differentially expressed WRKYs belonged to Class II or III, which are predominantly associated with biotic stress responses. Given their strong enrichment in this dataset, it is reasonable to assume that the transcriptional reprogramming I observed is at least partially mediated by these WRKY factors and their downstream targets. To test this, I scanned for the WRKY-binding W-box motif (TTGACY) within 500 bp promoter regions of the differentially expressed genes. Strikingly, 80% of DEGs contained a W-box motif, and GO analysis revealed that over 400 of these genes are associated with phosphorylation, alongside other enriched immune-related processes such as response to biotic stimulus, stress responses, and olefinic compound biosynthesis (Figure 3.7B).

Because WRKY transcription factors are regulated through phosphorylation by calcium-dependent protein kinases (CDPKs), and given that calcium-binding proteins were induced in AMF-colonised plants, I next examined the expression of genes involved in calcium signalling (Figure 3.7C). I found several Calcium-Dependent Protein Kinases (CDPKs), Calmodulin and Calmodulin-like proteins unregulated in AMF-colonised healthy plants, suggesting that they might be involved in WRKY-mediated transcriptional reprogramming occurring upon mycorrhization. Additionally, expression of a cluster of Calmodulin-like proteins induced by *P. hordei* infection was found to be maintained in AMF-colonised infected plants.

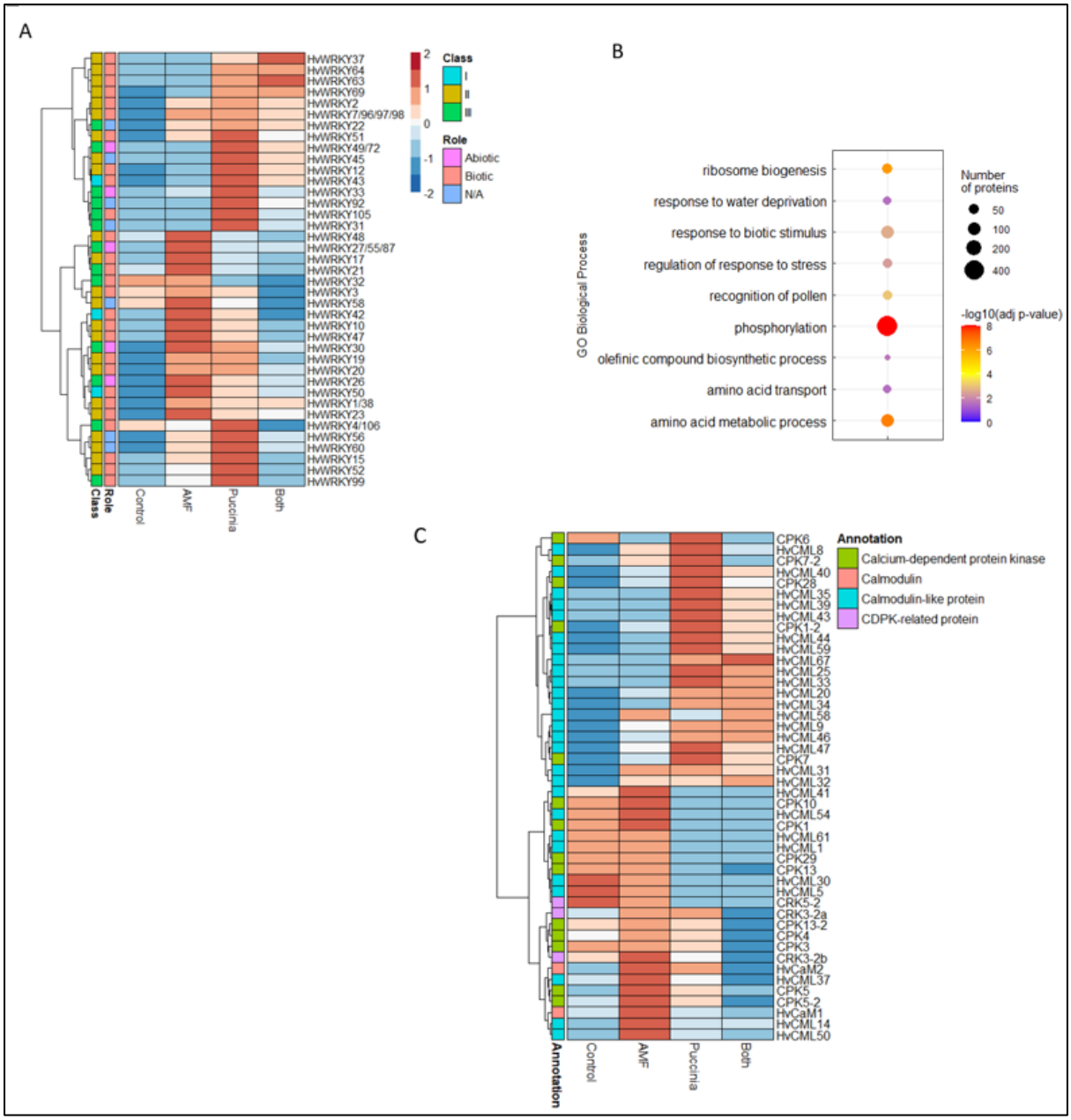

Figure 3.7 AMF modulate expression of WRKY transcription factors. (A) Scaled expression of differentially expressed WRKY genes in barley leaves in control, AMF-colonised, *P. hordei*-infected and AMF-colonised *P. hordei* infected plants (FC>2, FDR<0.05, logCPM >2; AMF/Control, AMF/Puccinia, Both/Puccinia, Puccinia/Control, Both/Control, AMF/Both). Genes were annotated based on Class (Based on Kan et al., 2021) and Role in mediating response to biotic or abiotic factors (Table S1). (B) GO analysis of 4416 of DEGs found to contain a WRKY-binding motif in their promoter region (FIMO, 500 bp, p<0.001). (C) Scaled expression of differentially expressed genes involved in calcium signalling in barley leaves in control, AMF-colonised, *P. hordei*-infected and AMF-colonised *P. hordei* infected plants (FC>2, FDR<0.05,

logCPM >2; AMF/Control, AMF/Puccinia, Both/Puccinia, Puccinia/Control, Both/Control, AMF/Both). Calmodulin and calmodulin-like genes were annotated based on Cai et al. (2022) and calcium depended protein kinase (CDPK) and CDPK-like genes were annotated based on the closest Arabidopsis homologue (Table S2).

To understand how transcriptional changes initiated by AMF affect downstream immune responses, I looked at the effect of AMF on major immune hormone pathways – Ethylene, Salicylic and Jasmonic acid. As ethylene is mostly involved in the response to necrotrophic pathogens, activation of the ethylene response could hinder response to *P. hordei* infection. I found that expression of EIN3 (Ethylene Insensitive 3), an ethylene-activated transcription factor, and multiple downstream ethylene responsive factors is induced in healthy AMF-colonised plants (Figure 3.8A). However, AMF does not seem to alter the expression of immune-induced ethylene responsive genes (not present in the DEG list). Additionally, the analysis of the ERF DNA binding motif, GGC-box (AGCCGCC), in the promoter regions of DEGs from the study identified 1321 genes that contain a GGC-box motif and are associated with phosphate and amino acid metabolism (Figure 3.8B). This further confirms that the ethylene response induced by AMF and infection is likely targeting the metabolic pathways and not involved directly in the biotic stress responses.

Jasmonic and salicylic acid are two major plant hormones mediating response to herbivores/necrotrophic and biotrophic pathogens respectively. As AM fungi are known to use the jasmonic acid (JA) signalling pathway during colonisation, induction of the JA pathway could have a negative effect on the immune response to *P. hordei*. During infection, AMF caused downregulation of immune-induced *LOX* (lipoxygenase) genes, peroxisomal genes and *COI1* (Coronate insensitive 1), which acts as a positive regulator of JA-mediated signalling (Figure 3.8C). Ultimately, however, AMF did not change the expression of *TIFY* genes. The effect on JA-synthesis genes could therefore be unlinked from the JA-signalling in this context.

Unlike JA, salicylic acid (SA) signalling is beneficial during infection with a biotrophic pathogen. We already saw in Figure 3.3, that AMF can have a positive effect on SA-responsive *PR* genes. I investigated whether it can also affect the upstream components of the pathway. AMF had no effect on the ICS part of the SA synthesis

pathway, however it caused downregulation of CM (Chorismate mutase) and most *PAL* (Phenylalanine ammonia lyase) genes during *P. hordei* infection (Figure 3.8D). Additionally, presence of AMF had no effect on expression of *NPR1*, however it induced expression of *NPR4* in healthy plants and reduced in infected plants.

Altogether, these results indicate that AMF reshapes the transcriptional profile of immune hormone pathways without exerting a detrimental effect on defence responses. The changes are concentrated at the level of transcription factors and protein modifications, while alterations in hormone biosynthetic genes likely reflect broader metabolic adjustments rather than direct modulation of the immune response.

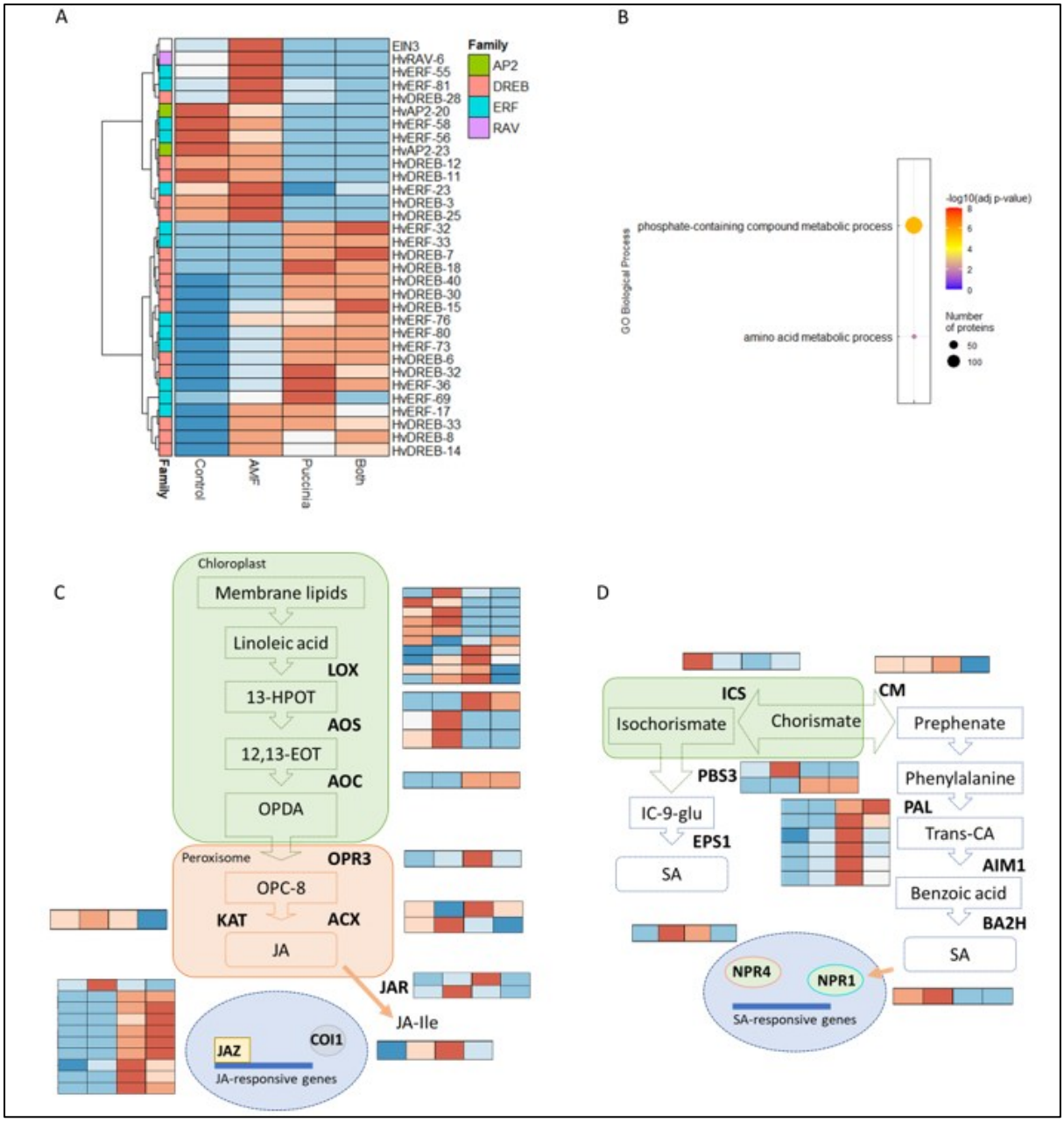

Figure 3.8 AMF modulate expression of genes involved in the immune hormone pathways. (A) Scaled expression of differentially expressed ethylene-signalling genes - Ethylene Insensitive 3 (*EIN3*), Ethylene Response Factors (*ERFs*), Dehydration-responsive element binding (*DREB*), Related to ABI3/VP1 (*RAV*) and APETALA2 (*AP2*) - in barley leaves in control, AMF-colonised, *P. hordei*-infected and AMF-colonised *P. hordei* infected plants (FC>2, FDR<0.05, logCPM >2; AMF/Control, AMF/Puccinia, Both/Puccinia, Puccinia/Control, Both/Control, AMF/Both). Genes are annotated based on Family (Annotation based on Ding et al., 2021). (B) GO analysis of 1321 of DEGs found to contain a ERF-binding motif in their promoter region (FIMO, 500 bp, p<0.0001). (C) Jasmonic acid synthesis and signalling pathway and (D)

Salicylic synthesis pathway annotated with heatmaps showing expression of barley genes in bold (from left) Control, AMF-colonised, *P. hordei*-infected, AMF-colonised *P. hordei* infected plants. LOX – Lipooxygenase, AOS – Allene oxide synthase, AOC – Allene oxide cyclase, OPR3 - Oxophytodienoate reductase 3, KAT - Ketoacyl-CoA thiolase, ACX - Acyl-CoA oxidase, JAR – Jasmonate resistant, COI1 – Coronate insensitive 1; ICS – Isochorismate synthase, CM – Chorismate mutase, PBS3 - Avrpphb susceptible 3, EPS1 – Enchanced pseudomonas susceptibility 1, PAL – Phenylalanine ammonia lyase, AIM1 – Abnormal inflorescence meristem 1, BA2H - Benzoic acid 2-hydroxylase, NPR – Non-expressor of pathogenesis-related 1.

3.2.5 AMF induce expression of ubiquitination machinery genes

Our analysis revealed that GO terms associated with protein modifications were enriched among AMF-regulated genes in both healthy and infected samples. These were predominantly linked to phosphorylation and ubiquitination, processes central to pattern-triggered immunity and to the regulation of many transcription factors. Ubiquitination, in particular, plays a pivotal role in activating plant immune responses. Genes encoding ubiquitin-system enzymes are often upregulated during immune activation to support the rapid and complex protein modifications required for signal transduction, proteostasis, and fine-tuning of immune pathways (Zhou et al., 2016; Lewis et al., 2015). This includes E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, E3 ubiquitin ligases, and deubiquitinases (DUBs), which collectively provide dynamic control of the ubiquitin–proteasome system during defence signalling. Guided by this, I examined the expression patterns of differentially expressed ubiquitination-related enzymes in this dataset. I identified four clusters of proteins with distinct expression profiles (Figure 3.9A). The smallest cluster comprised proteins upregulated in AMF-colonised plants under both healthy and infected conditions, and notably included E3 ligases from the ATL and PUB families. In contrast, the largest cluster contained proteins that were modestly upregulated in AMF-colonised healthy plants but strongly downregulated in AMF-colonised infected plants compared with infected controls. These contained the E1 enzyme UBA1 (Ubiquitin-activating enzyme 1) and a E2 ubiquitin conjugating enzyme UBC26 associated with formation of K48-linked chains. This suggests the increase in

ubiquitination in heathy AMF plants and decrease in proteasomal-associated degradation during infection. Third cluster contains genes that are not regulated by AMF and are downregulated during infection. These contain proteins involved in autophagy and endocytosis (ATG3, ATG7, ELC) and developmental E3 ligases (e.g., BRIZ1, BB). The last cluster included genes upregulated in AMF-colonised healthy plants, downregulated during infection and further downregulated in AMF-colonised infected plants. This cluster contains mostly E3 enzymes with various functions from immune (RHF2A), proteotoxic stress (MPSR1) or abiotic stress (RDUF2) response. Interestingly, all clusters contained many members of the ATL and PUB families.

To assess whether transcriptional changes in ubiquitination-related genes were reflected at the protein level, I examined global ubiquitination patterns in control and *P. hordei*-infected plants grown with or without AMF. AMF consistently reduced overall ubiquitination levels in both healthy and infected plants (Figure 3.9B). Although this reduction was not statistically significant, a clear decrease in ubiquitination signal in healthy plants was visible in the western blot.

Altogether, these results indicate that AMF lowers the abundance of ubiquitinated substrates during infection, consistent with the reduced expression of several E1, E2, and E3 enzymes. However, in healthy AMF-colonised plants, the decrease in global ubiquitination did not align with transcriptome data, as many E3 ligases showed induced expression. Interestingly, AMF preferentially regulated members of two large E3 ligase families, ATL and PUB, both of which have diverse roles in stress responses and developmental processes.

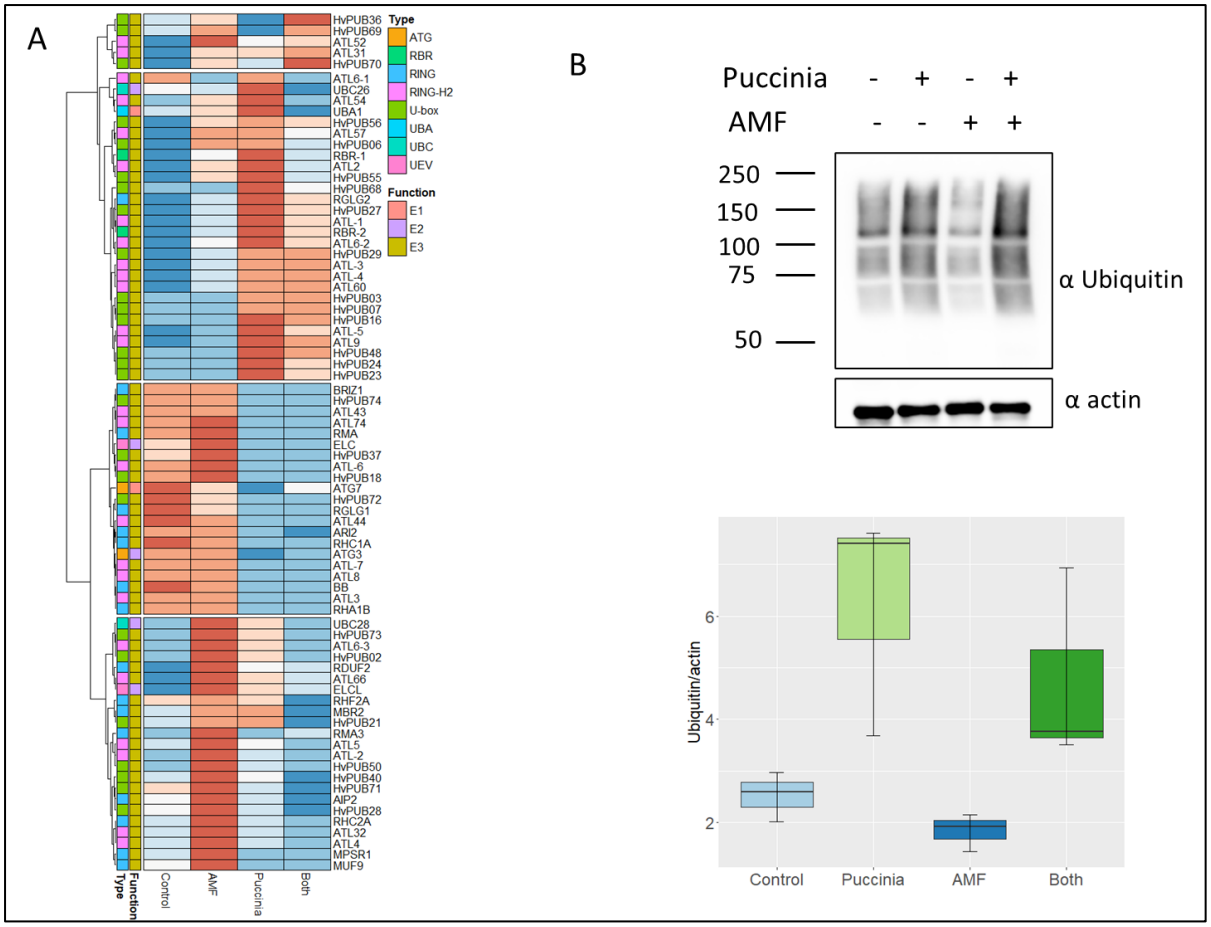

Figure 3.9 AMF modulate ubiquitination at gene and protein level. (A) Scaled expression of DE ubiquitin-machinery genes (FC>2, FDR<0.05, logCPM >2; AMF/Control, AMF/Puccinia, Both/Puccinia, Puccinia/Control, Both/Control, AMF/Both). Genes are annotated based on the Function of the ubiquitin enzyme. PUB genes were annotated based on Young Ryu et al. 2019, and the rest based on the closest Arabidopsis homologue (Table S3). (B) Western blot and quantification of the ubiquitin signal normalised by actin in Control, *P. hordei*-infected, AMF-colonised and AMF-colonised *P. hordei* infected plants (n=3).

3.3 Discussion

AMF has been proven to induce resistance against various pathogens of crop plants. Here, I examined how *R. irregularis* modulates barley immune responses during infection with *P. hordei*.

In this study, together with collaborators, we developed a method for successful and consistent barley colonisation with *R. irregularis.* Although we have not observed the

growth benefit usually associated with AM symbiosis (Figure 3.1 C), this could be due to low compatibility between the host-symbiont chosen for this study, as it has been shown, that the growth effect on the host plant can vary significantly even between different AMF isolates (Munkvold et al., 2004; Hong et al., 2012, Koch et al., 2017). Likewise, the response to AMF can be cultivar specific, as the plant genotype can affect the success of the colonisation. Plant responsiveness to AMF has been shown to vary significantly between different cultivars and under different levels of nutrient deprivation (Sawers et al., 2017; Lehnert et al., 2017; Chu et al., 2013). As there are no other published studies using cv. Bowman – *R. irregularis,* this study provides the first insight into the relationship between these two organisms and further studies could shed some light onto the underlying mechanisms of the growth results.

Observed increased expression of PR genes and *WRKY28* in AMF colonised infected plants is characteristic of MIR, where the plant exhibits an enhanced immune response under pathogen challenge (Cameron et al., 2013). Transcriptional profiling confirmed that AMF reprogrammes the leaf transcriptome of healthy barley plants towards enhanced immune readiness, by upregulating genes involved in defence responses, secondary metabolism and signalling pathways (Figures 3.4 and 3.5). Concurrent downregulation of photorespiration and carbohydrate metabolism is consistent with the growth-to-defence shift during MIR (Cameron et al., 2013).

During *P. hordei* infection, AMF was found to attenuate the response to the pathogen and induce additional transcriptional changes. Downregulation of genes involved in immune signalling and redox homeostasis, and upregulation of specific defence response genes suggests a dampening of broad-spectrum resistance in favour of specific immune responses. This can also be a result of induced immune priming, which allowed for a faster initiation of broad-spectrum responses. An experiment looking at earlier timepoints of infection could allow us to test this hypothesis.

We also observed the maintenance of a positive regulation of abiotic stress response, by upregulating genes involved in water deprivation, phosphate transport or response to cold (Figure 3.6B), which could have a potential negative effect on the immune response. I have not observed any significant upregulation of abiotic WRKY TFs, however some of the ethylene responsive genes (*ERF32, DREB7, DREB15, ERF76, DREB8*) showed upregulation. *DREB7* is an orthologue of rice *DREB1H*, which has

been shown to mediate response to cold (Ji et al., 2025), while *HvDREB15* (*AtDREB1a*) is known to mediate drought, cold, and salinity response in Arabidopsis (Liu et al., 1998). This highlights the importance of understanding the cross-talk between the biotic and abiotic stress response pathways under AM symbiosis.

GO enrichment and protein function analysis allowed us to identify WRKY transcription factors as the key regulators of the primed immune state. This phenomenon has been observed previously: in potato WRKY TFs were found to be upregulated in the root during AMF colonisation (Gallou et al., 2012). Similarly, studies in tomato leaves report AMF-triggered upregulation of WRKY TFs (Guadalupe Cervantes-Gámez et al., 2015) and in apple overexpression of *WRKY40* improved the resistance to *F. solani* (Wang et al., 2022). However, none of the studies showed such a large-scale reprogramming as observed in this study, where nearly half of the annotated WRKYs were differentially expressed. Differential regulation of WRKYs initiated by AMF suggests that AM fungal colonisation results in fine-tuning of the stress and growth regulators. Additionally, regulation of calcium dependent protein kinases (CDPKs) and calmodulin genes (Figure 3.7), which work both upstream and downstream of WRKY TFs, and are important in establishing AM symbiosis, supports the role of calcium-WRKY signalling in MIR, consistent with other studies in different plant systems (Gu et al., 2025; Vangelisti et al., 2018, Guadalupe Cervantes-Gámez et al., 2015).

Classically, later stages of MIR prime JA and ethylene defences (Cameron et al., 2013), which can compromise resistance against biotrophic pathogens. Interestingly, in this study AMF colonised infected plants showed little difference in the expression profile of ethylene signalling genes (Figure 3.8 A), downregulation in many components of the JA biosynthesis pathway, and upregulation of TIFY genes, which are repressors of JA signalling pathway (Figure 3.8B), indicating that the immune response is prioritised over the canonical MIR effects. Simultaneously, AMF downregulated most SA biosynthesis and signalling genes (Figure 3.8D), yet SA-responsive markers were induced and defence was amplified upon infection. Although contradictory, this might have been an implication of AMF-induced priming.

Altogether, the hypothesis that *R. irregularis* will induce MIR against *P. hordei* was partially proven. Even though we did not observe a significant reduction in pathogen infection in AM colonised plants, we observed a systemic priming of SA-dependent

responses and cell wall defences, characteristic of the second phase of MIR establishment (Cameron et al., 2013).

This study highlighted the importance of protein modifications in regulating plant defence responses. Phosphorylation was the primary suspect, however surprisingly, I also identified many ubiquitination enzymes present in the list of DEGs. The finding that AMF decreases global ubiquitination levels while upregulating many ubiquitination enzymes (Figure 3.9) would be contradictory if it was not for the fact that higher expression of ubiquitination machinery could result in faster protein turnover and in turn lower overall ubiquitination. To my knowledge, this is the most comprehensive indication of AMF-mediated reprogramming of the host ubiquitin system, extending beyond specific examples such as the *Rhizophagus irregularis*- mediated interference with histone 2B (H2B) monoubiquitination (Wang et al., 2021). Finally, two of the E3 ligase families highlighted in this study, PUBs and ATLs, are known to regulate diverse stress responses (Trujillo, 2017; Guzmán, 2012). In fact, MtPUB1 is known to negatively regulate AM symbiosis (Vernié et al., 2016). While ATLs have not been linked directly to AMF signalling, members of this family control immune outputs like callose deposition (ATL6/ATL31) and chitin-triggered signalling (ATL9) (Maekawa et al., 2012; Berrocal-Lobo et al., 2010), aligning with the observed defence priming.

Altogether, this study showed the extensive transcriptional and post-transcriptional reprogramming that occurs in AM colonised plants and how it changes upon pathogen infection. These changes may confer a delayed or context-dependent protection, which this experiment did not capture. These findings support the broader view that arbuscular mycorrhizal symbiosis can function as a natural immune enhancer contributing to reduced dependence on chemical inputs and improved yield stability under stress. It also adds to the literature, as we exploit a barley – *P. hordei* pathosystem, which has not been described in this context before. Observation of the effect of AMF on the plant ubiquitin system is specifically interesting, as it links the regulation observed at the transcriptional level to the translational effect required to initiate these changes.

Chapter 4

Ubiquitination functions as a major regulator of barley immune responses

4.1 Introduction

Ubiquitination is a key post-translational modification in plants that regulates protein localisation, activity, and turnover, thereby coordinating a wide range of biological processes (Sadanandom et al., 2012). This regulation becomes particularly critical during pathogen attack, when plants must undergo rapid and dynamic reprogramming from growth to defence. As shown in the previous chapter, ubiquitination is strongly modulated in response to both AMF and *P. hordei*, likely contributing to proteome remodelling through adjustments in protein activity and stability. Importantly, polyubiquitination has been shown to be essential for basal host resistance in barley: partial depletion of cellular ubiquitin pools results in extreme susceptibility to the biotrophic fungal pathogen *Blumeria graminis f. sp. hordei* (powdery mildew) (Dong et al., 2006). Previous studies have shown that ubiquitination regulates plant immune responses, with numerous reports showing direct control of key components of both PTI and ETI (Gao et al., 2022). Elucidating how ubiquitin signalling contributes to immune regulation in barley could reveal opportunities to fine-tune this pathway and engineer resistance to *P. hordei*. However, the role of ubiquitination in barley immunity remains largely unexplored, with most mechanistic insights to date derived from studies in model species (Gao et al., 2022).

Advances in mass spectrometry have enabled detailed investigations of proteome-wide changes in plants responding to environmental stress (Hu et al., 2015). Such approaches provide a comprehensive overview of protein dynamics and serve as powerful platforms for identifying molecular targets for focused studies. Given the economic and agricultural importance of barley, the development of robust proteomic workflows and multi-omics resources holds considerable potential for improving key agronomic traits. Indeed, proteomics has already been successfully applied to assess the impact of environmental conditions on barley growth and to inform downstream processes such as malting, brewing, and evaluation of nutritional quality (Bahmani et al., 2021). Proteomics approaches have also been used to decipher the molecular mechanisms underlying protein function, for example, identifying the role of PR5 Thaumatin-Like Protein TLP5 in mediating susceptibility to barley powdery mildew (Lambertucci et al., 2019).

Proteomics approaches have also been successfully applied to characterise the ubiquitome—the complete set of ubiquitinated proteins within a cell. Plant ubiquitomes have been mapped in several species, both in an organ- or developmental stage-specific context and in response to diverse biotic and abiotic stresses. It has also been used to detect proteins regulated by ubiquitin during immune response in Arabidopsis (Ma et al., 2021), Tobacco (Yang et al., 2025), Rose (Li et al., 2023) or Rice (Chen et al., 2018). To my knowledge, no previous studies have performed barley ubiquitome profiling or established the barley proteome in response to *P. hordei*.

The aim of this study is to generate a comprehensive barley immune ubiquitome. This dataset will provide a resource for identifying immune-related proteins, uncovering biological processes regulated by ubiquitination under different immune pressures, and mapping novel ubiquitination sites in barley proteins. Beyond its descriptive value, the barley immune ubiquitome represents a powerful platform for understanding and ultimately modulating immune responses against pathogens. To gain insight into the changes that occur upon pathogen perception, I first investigated the effects of the immune hormones salicylic acid (SA) and jasmonic acid (JA) on the ubiquitin-regulated immune ubiquitome. As expected, both hormones altered the barley ubiquitome, and importantly, I uncovered evidence of ubiquitin-mediated cross-talk between the two pathways. I then characterised the *P. hordei*-induced ubiquitome and highlighted dynamic differences in ubiquitin regulation across the course of infection. Finally, I addressed the question of cultivar-dependent resistance by analysing the extent to which host genotype modulates the barley immune ubiquitome. Ultimately, I demonstrate that the dynamic barley ubiquitome responds to various immune factors and regulates diverse biological processes to orchestrate plant immune responses.

4.2 Results

4.2.1 Immune hormone treatment alters barley global ubiquitin profile

Immune hormones are essential in activating and regulating plant signalling pathways. Two main immune hormones in plants are salicylic acid (SA) and jasmonic acid (JA), which are produced in plants in response to biotrophic and necrotrophic pathogens, respectively. It has been shown that exogenous application of these immune hormones triggers immune responses in various plant species (Thomma et al., 2000; Duan et al., 2014; Feng et al., 2021; Shimono et al., 2007). Additionally, both SA and

JA signalling, as well as the components that allow for crosstalk between those two pathways, have been shown to be regulated by ubiquitin (Miricescu et al., 2018).

Even though immune hormone signalling pathway in *Arabidopsis* is well understood, few studies have looked at it in barley. To test the hypothesis that salicylic acid (SA) and jasmonic acid (JA) can activate immune transcriptional reprogramming and modulate the immune ubiquitome in barley, I examined the effect of exogenous hormone application on immune gene expression and the accumulation of ubiquitinated targets. As a first step, I conducted a dose–response experiment using different concentrations of SA and JA to determine the minimum effective dose required to elicit a measurable immune response. Barley plants were sprayed or infiltrated with different concentrations of SA (0 mM, 0.5 mM, 1 mM) and JA (0 mM, 0.1 mM, 0.5 mM) and I monitored the expression of immune marker genes: *Pathogenesis-related protein 1* (*PR1*), *Lipoxygenase 2* (*LOX2*) and *WRKY-transcription factor 28* (*WRKY28*), as well as accumulation of ubiquitinated targets (Figure 4.1). Spraying was discarded as an application method, because it produced high variability and inconsistent immune activation, likely due to treatment of plants grown under communal conditions optimised for Arabidopsis with high air flow. Changes in protein ubiquitination after SA treatment (Figure 4.1A) and JA treatment (Figure 4.1 C) occurred earlier than the activation of *PR1* (Figure 4.1B) and *LOX2* (Figure 4.1 D), which were only visible 24 h after treatment. This suggests that ubiquitination is initiated before the expression of immune genes and can be used as an early response marker to pathogen infection. Additionally, lower concentrations of both hormones were sufficient to trigger ubiquitination changes and induce expression of marker genes.

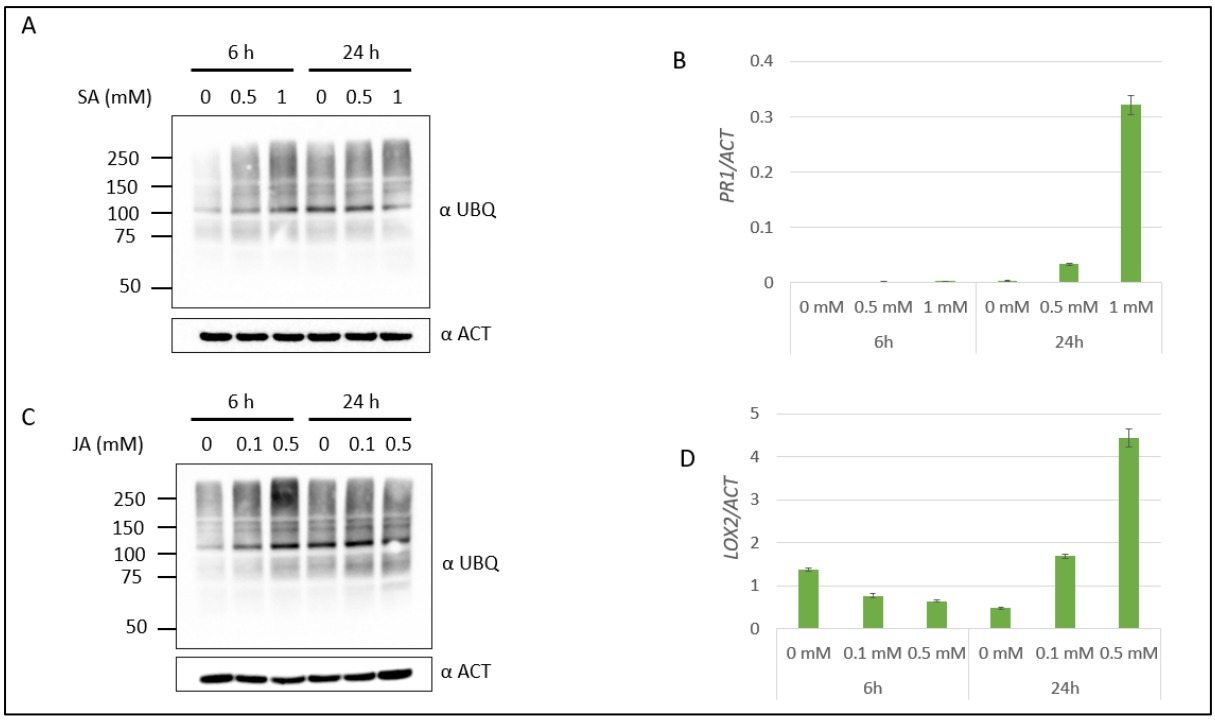

Figure 4.1 Hormone treatment induces expression of immune genes and accumulation of ubiquitinated targets in barley leaves. Barley plants were infiltrated with varying concentrations of immune hormones: Salicylic acid (A, B) and Jasmonic acid (C, D) and samples collected after 6h and 24h. Leaf tissue was subject to protein extraction and ubiquitinated proteins detected by immunoblotting against poly-ubiquitin and actin (loading control) (A, C). Expression of SA-induced gene *PR1* (B) and JA-induced gene *LOX2* (D) was measured and normalised to *ACT*; error bars represent mean ± SD, n = 3.

As the treatment with immune hormones induced changes to barley global ubiquitin profile, I decided to perform a proteomics study to identify barley immune ubiquitome in response to the immune activation with SA and JA. I used lower concentrations of both hormones (0.5 mM for SA and 0.1 mM for JA), as these were able to induce changes in both the proteome and transcriptome, and collected samples after 6 h, to capture the early changes to the ubiquitome. To capture proteins regulated by ubiquitin, I used tandem-repeated ubiquitin-binding entities (TUBEs) based on ubiquitin-associated (UBA) domain from Ubiquilin1 protein to isolate ubiquitinated and ubiquitin-associated proteins (Hjerpe et al., 2009). Ubiquilin1 contains two C-terminal UBA domains that bind ubiquitin with high affinity and preferentially recognise

polyubiquitin chains with high affinity and broad specificity for linkage type (Zhang et al., 2008). This allows for immunoprecipitation of poly-ubiquitinated and associated proteins, providing a comprehensive coverage and facilitating the characterisation of the plant ubiquitin-regulated proteome. After immunoprecipitation with ubiquilin 1 TUBEs from plant protein extract, I used liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) to identify isolated proteins. Label-free quantification (LFQ) was used to compute protein intensities, followed by normalization and differential analysis to identify proteins with significant abundance changes between treated and untreated conditions. The results revealed a strong ubiquitin-mediated response to both immune hormones. 808 proteins (30.4%) were identified to be differentially enriched (treated/untreated, $p < 0.05$) after SA treatment, with 383 proteins showing positive and 425 proteins negative fold change compared to untreated controls (Figure 4.2A). Treatment with JA had a lower overall impact on the immune ubiquitome, with 308 proteins (11.5%) differentially enriched after treatment, but 21 proteins showing strong positive enrichment ($Log_2FC>2.5$) after treatment (Figure 4.2B), including proteins involved in JA biosynthesis (Allene Oxide Synthase – AOS) and JA signalling (Jasmonate Induced Protein – JIP). A comparison between datasets revealed substantial crosstalk between SA and JA signalling. 46% of JA and 18% of SA ubiquitome was found to be differentially associated with ubiquitin after both treatments (Figure 4.2C). The majority of these proteins behave similarly in both SA and JA datasets (Figure 4.2D), suggesting shared downstream ubiquitin regulation for both hormones. Gene ontology analysis revealed that the overlapping proteins are involved in lipid oxidation, pigment biosynthesis, plastid organisation, protein catabolism and response to heat (Figure 4.2E). A synergistic regulation of these processes might be beneficial for both SA – and JA – mediated response.

Despite sharing common downstream pathways, when comparing the hormone-specific enriched GO Biological Processes (BPs), it is clear that SA induces a broader response than JA. SA induces changes in ubiquitin-signalling involved in transcription, protein processing and sorting (Figure 4.2E). Ubiquitin directly regulates JA – induced response and downstream response to wounding (Figure 4.2E). Moreover, these results indicate that the ubiquitin-dependent regulation of both hormones induces regulation of protein catabolism, response to oxidative stress, translation and metabolic processes, but targeting different proteins in the pathways (Figure 4.2E).

These data demonstrate that both SA and JA induce changes in the barley ubiquitome, underscoring the role of ubiquitination in mediating immune responses. The extent of ubiquitin signalling observed upon SA treatment, together with the broad nature of the enriched GOBPs, suggests that ubiquitination regulates a distinct set of pathways, enabling the plant to tailor its immune response to specific pathogen cues.

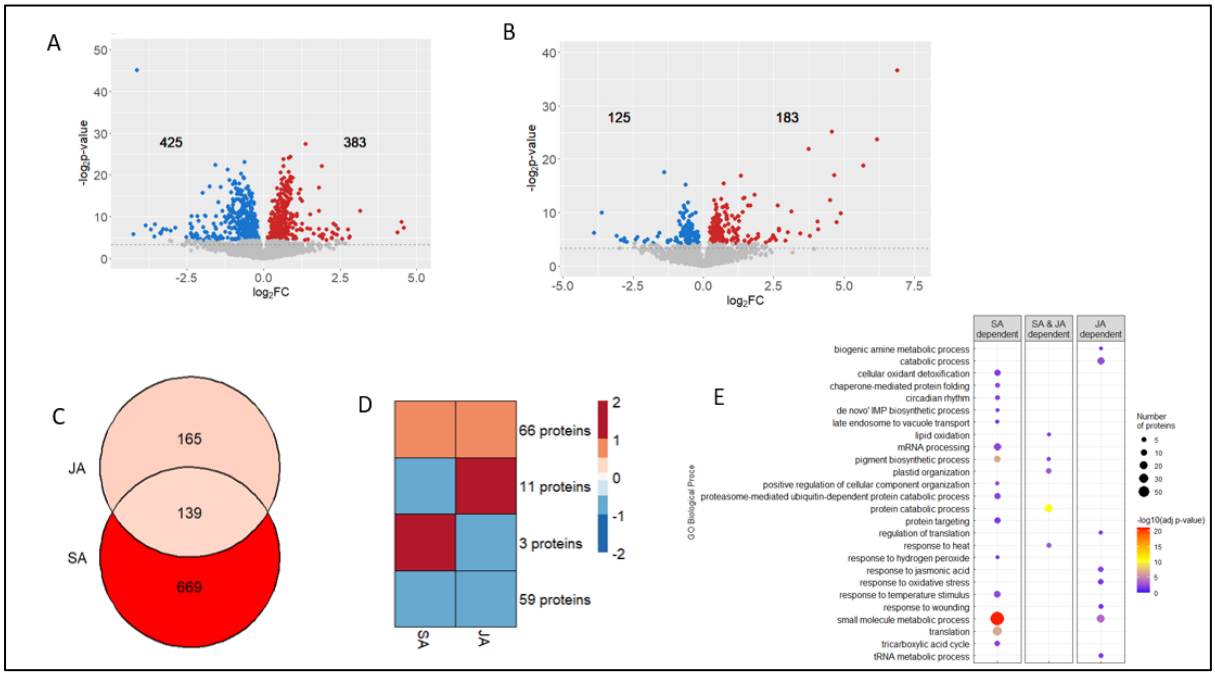

Figure 4.2 SA and JA cross-regulate ubiquitin signalling. Barley plants were infiltrated with 0.5 mM SA and 0.1 mM JA and samples collected after 6h. A ubiquitome profiling identified induced (red) and repressed (blue) by SA - (A) and JA (B) treatment ubiquitinated proteins and their interactors (treated/untreated, $p < 0.05$; n=3, N=2). (C) Significant proteins from (A) and (B) show overlap between treatments. (D) 139 overlapping proteins grouped based on FC. (E) GO analysis revealed BP terms dependent on hormone signalling (based on g:Profiler two-stage hybrid term list filtering algorithm and Benjamini-Hochberg method applying significance threshold of 0.05, term size >2, GO BPs for JA dataset were based on Arabidopsis orthologue IDs).

4.2.2 The barley ubiquitome undergoes dynamic changes during *P. hordei* infection

SA-induced immune ubiquitomics allowed us to identify ubiquitin and ubiquitin-associated proteins that accumulate upon activation of defence against biotrophic pathogens, independent of direct pathogen interference. Building on this knowledge of extensive ubiquitome reprogramming under SA treatment, I next asked whether similar changes occur during actual pathogen challenge. To address this, I infected barley plants with the biotrophic fungal pathogen *Puccinia hordei* and monitored ubiquitination dynamics alongside immune marker gene expression. Both global ubiquitination (Figure 4.3A) and PR gene expression (Figure 4.3B) exhibited oscillatory patterns throughout disease progression, indicating a highly dynamic regulation of the immune response during infection. Ubiquitination levels increase rapidly, becoming detectable as early as day 1, reaching a first peak at day 4, then decreasing slightly before rising again by day 7. In parallel, expression of the *P. hordei* marker gene (*EF2*) revealed a slow increase in fungal biomass until day 4, followed by a sharp acceleration thereafter. This pattern suggests that elevated ubiquitination may initially help to restrict pathogen proliferation, but once ubiquitination levels decline, potentially due to pathogen interference, *P. hordei* biomass increases rapidly. A similar trend was observed for immune marker genes: *PR* gene expression peaked at day 2, dropped markedly at day 4, and then increased again at later stages. Together, these results indicate an antagonistic interaction between host defences and pathogen progression, in which strong early immune activation slows infection but is eventually overcome, allowing *P. hordei* biomass to accumulate. To better understand how ubiquitination contributes to the dynamics of the immune response, I next established a barley-*P. hordei* immune ubiquitome at multiple stages of infection: inoculation/penetration (Day 1), pathogen colonisation (Day 4) and reproduction (Day 7). I performed an immunoprecipitation experiment with ubiquilin binding domain, followed by enrichment of ubiquitinated peptides with a DiGly remnant antibody, and used liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) to identify ubiquitinated and ubiquitin-associated proteins. In this section, I focus on the analysis of the data from ubiquilin immunoprecipitation only and the DiGly data will be discussed in 4.2.5.

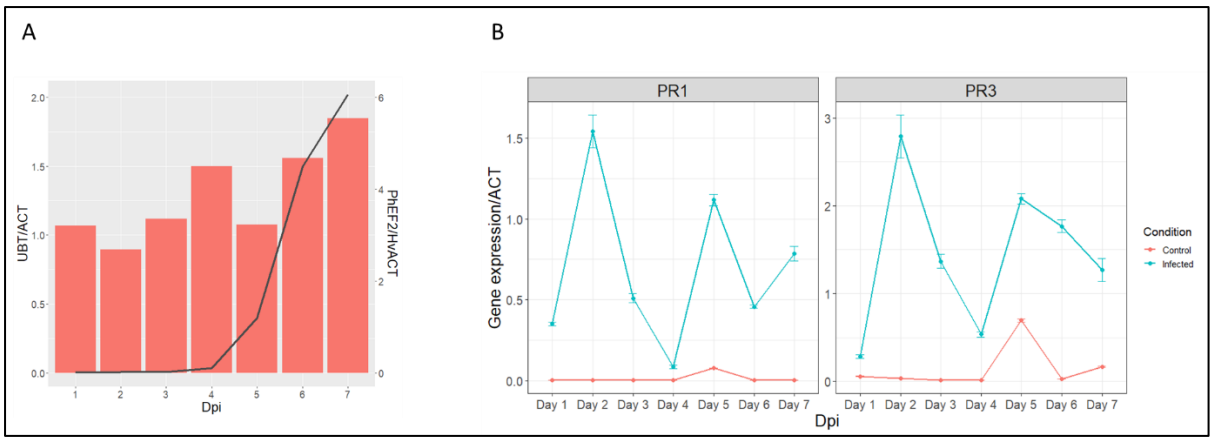

Figure 4.3 Ubiquitination regulates immune response in barley throughout *P. hordei* infection. Barley plants were infected with *P. hordei* and leaf samples were collected each day until 7 dpi. (A) Ubiquitination levels (left) in plants based on a quantified western blot signal using antibodies against poly-ubiquitin and expression of *P. hordei* marker gene (right) quantified using RT-QPCR; ubiquitin signal normalised against signal from untreated plants; Western blot signal normalised against Actin, n = 2, *PhEF2* expression normalised against *HvACT*. (B) Expression of barley pathogenesis related (*PR*) genes in infected and untreated plants throughout the infection; error bars represent mean ± SD, n = 3; expression normalised against *HvACT*.

Using a proteomics approach, 1,934 proteins were identified as differentially ubiquitinated or associated with ubiquitin during *P. hordei* infection (compared to a healthy control), with 460 proteins significant at day 1, 574 at day 4 and 1 398 at day 7 (Figure 4.4A). The Venn diagram shows a moderate overlap between datasets, suggesting a selective ubiquitin regulation at each stage of infection. GO analysis indeed showed enrichment of specialised BP terms at different days of infection (Figure 4.4B). At day 1, ubiquitin was found to regulate proteins involved in central metabolic pathways, such as energy production and precursor biosynthesis, suggesting that the pathogen was perceived and the plant initiated a physiological reprogramming required to combat the pathogen. This is also evidenced when looking at proteins depleted from the ubiquitin-regulated pool at day 1, which include terms associated with photosynthesis (TCA cycle, pigment biosynthesis, chloroplast organisation, carbon fixation).

At day 4, we can observe an overall decrease in enriched terms, with only a small group of processes, such as small molecule metabolism and photosynthesis, remaining consistently regulated from day 1. Enriched ubiquitin-regulated processes were associated with translation and response to salt stress, whereas depleted ubiquitin-associated processes included chaperone-mediated protein folding, glycine biosynthesis, and biogenic amine metabolism. Enrichment of translation could be due to the proteome reprogramming and increased protein translation, while responses to salt, glycine biosynthesis and biogenic amine metabolism suggest activation of pathways involved in stress response. This suggests that day 4 represents a transitional phase between early and late stages of infection.

At Day 7, processes involved in stress response (response to hydrogen peroxide, protein folding, K63-linked ubiquitination, vesicle-mediated transport) as well as translation-related processes are positively associated with ubiquitin. Stress-related processes were also enriched among depleted proteins, these include protein repair, protein folding, lignin biosynthesis, oxidative stress and cell redox homeostasis. In contrast, processes associated with metabolism (TCA, ATP synthesis) and response to abiotic stress (temperature stimulus, cadmium ion) are not regulated by ubiquitin at this stage. This could mean, that, even though the infection cycle of the pathogen is finishing, the plant is still focused on regulating defence-response genes. Additionally, at this stage, I identified GO terms related to pathogen responses (including response to biotic stimulus, bacteria, and interspecies interactions) that were not enriched in the other two datasets. This suggests that at days 1 and 4, barley activates broad-spectrum immune responses, whereas by day 7, the response becomes more specifically targeted against biotrophic pathogens.

As GO term enrichment provides only an overview of functional categories across time points, but not insight into specific pathways regulated by ubiquitination during infection, I refined my analysis by examining enrichment of individual proteins across the infection timeline (Figure 4.4C). Early in infection, proteins associated with biosynthetic processes (Cluster 2) were enriched but became depleted at later stages. Positive regulation of responses to biotic stimuli and wounding (Cluster 12) was enriched at days 1 and 7, but notably absent at day 4, suggesting suppression of this regulatory pathway during the transition phase of infection. A similar transient suppression was observed at day 4 for proteins involved in translation and protein

transport (Cluster 5) and for regulators of the phenylpropanoid pathway (Cluster 11). These patterns suggest that *P. hordei* may actively suppress key immune regulatory pathways during mid-infection to evade host defences, before pathogen biomass increases to levels that are no longer concealed or the host immune system reasserts control. By contrast, proteins enriched at later stages were mainly associated with stress responses, including responses to abiotic stress and glutathione metabolism (Cluster 7), lipid oxidation and regulation of gene expression (Cluster 9), and responses to fungal infection (Cluster 13). Together, these findings highlight the dynamic and stage-specific regulation of barley metabolism and immunity through ubiquitin signalling during *P. hordei* infection.

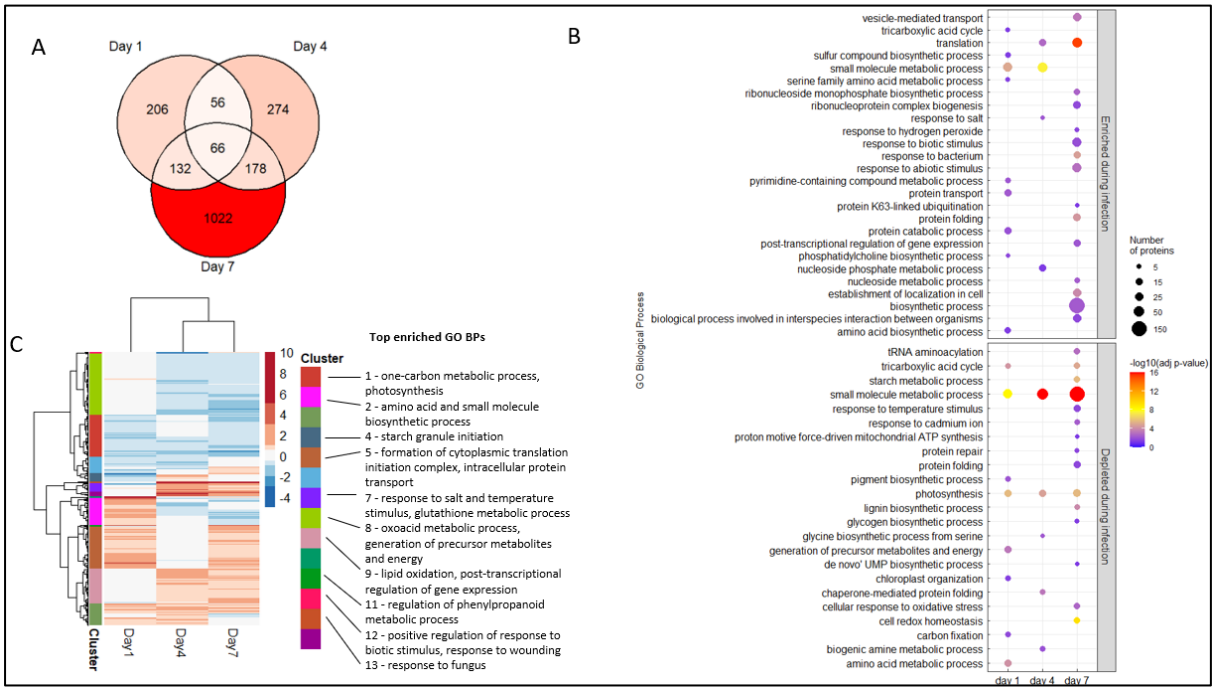

Figure 4.4 Ubiquitin selectively regulates biological processes at different stages of infection. Barley plants were infected with *P. hordei* and samples collected at 1, 4 and 7 dpi. (A) A proteomics study identified proteins differentially regulated by ubiquitin at different stages of infection (treated/untreated, $p < 0.05$; Log2FC ± 0.5, n=3). (B) GO analysis revealed BP terms regulated by ubiquitin at different stages of infection (based on g:Profiler g:SCS multiple testing correction method applying significance threshold of 0.05, term size >2). (C) Enrichment of proteins overlapping between treatments (>2 timepoints). Colour scale represents Log2FC. Proteins were clustered into 14 clusters based on behaviour (Ward's method was used for clustering and Euclidean method for calculating row distances; Euclidean distance dis-similarity

= 6) and each cluster assessed based on protein function; clusters 3, 6, 10 and 14 did not have enriched GO BP terms associated with them (based on g:Profiler g:SCS multiple testing correction method applying significance threshold of 0.05).

While GO analysis and functional protein clustering are helpful in identifying overrepresented biological processes, they do not provide insight into the spatial distribution or cooperative interactions of the identified proteins. A protein interaction network analysis allows for the identification of functional nodes involved in ubiquitin signalling. I used a STRING v12 database to retrieve interaction data for each significant protein identified throughout *P. hordei* infection, and developed a protein interaction network based on physical and functional interaction data (Figure 4.5). I obtained a dense network of interconnected nodes, with 1619 nodes and 44853 edges. I performed an MCL clustering method to isolate strong functional nodes. Here, we can still observe interactions between nodes, but it allows us to isolate strongly connected proteins which likely share a function. Indeed, a GO enrichment analysis performed on each node revealed biological processes associated with connected proteins. The most significant clusters contain proteins involved in metabolism, but I also isolated large nodes related to protein processing and responses to stress. Most clusters contain proteins identified at different stages of infection, which suggests regulation of all functional clusters throughout the infection.

Altogether, these data provide an insight into the ubiquitin-mediated protein regulation during *P. hordei* infection and allows for the identification of biological processes and functional networks critical during the immune challenge.

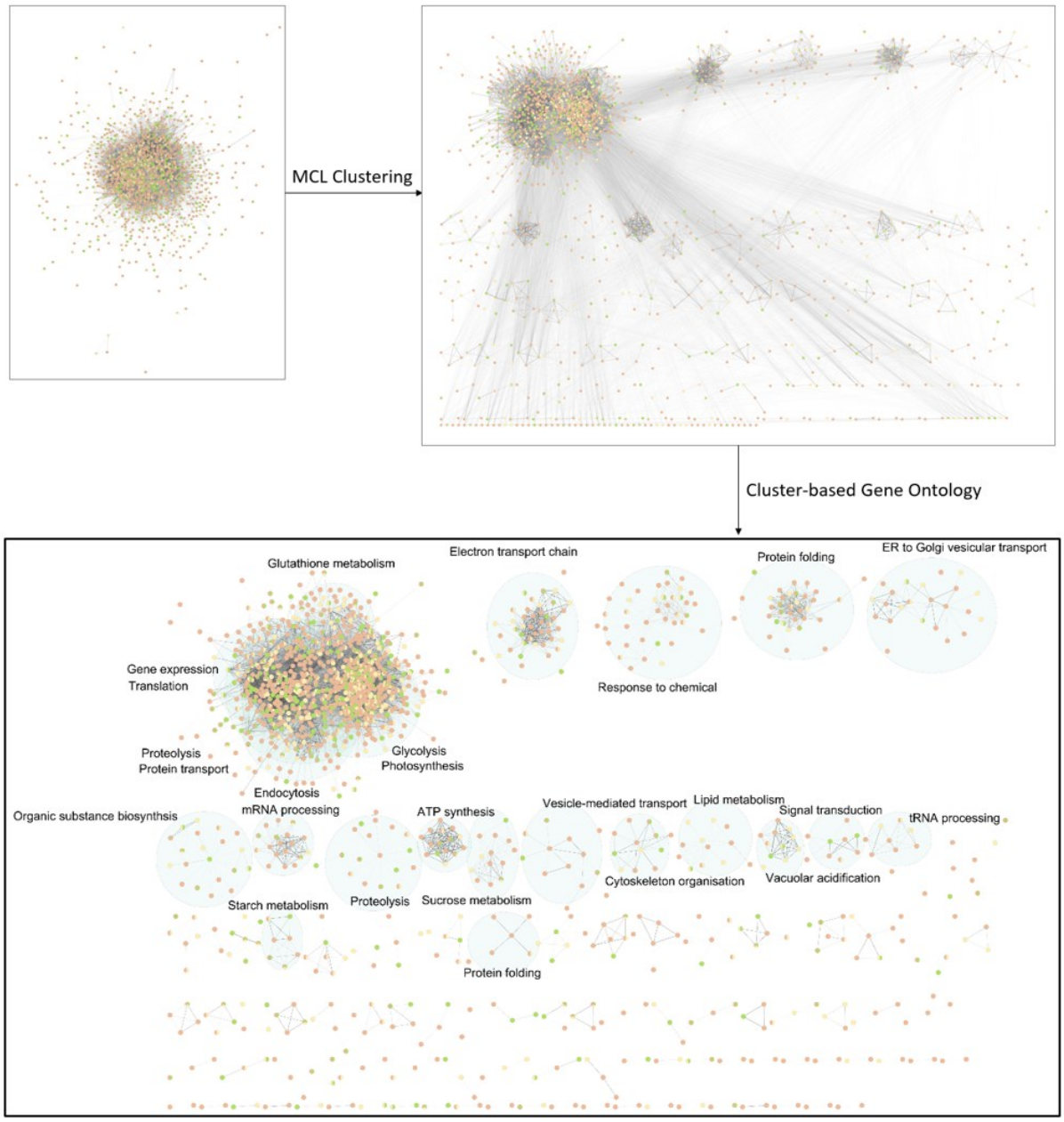

Figure 4.5 Ubiquitin-regulated proteins form functional network hubs. (A) Proteins regulated by ubiquitin throughout *P. hordei* infection were used to create a protein interaction network using STRING v12 (based on >90% sequence overlap); nodes represent proteins, coloured based on appearance (Day 1 – green nodes, Day 4 – yellow nodes, Day 7 – orange nodes), while edges represent STRING full interaction score (edge cut-off >0.5). (B) MCL clustering based on the interaction scores was used to extract functional nodes (Granularity parameter = 2.5; (C) GO enrichment analysis was performed on each node and the top BP annotated to each node; nodes without annotation did not have enriched GO BP terms; Network generated using Cytoscape V 3.10.3

4.2.3 Barley immune ubiquitome is cultivar-specific and linked to the cultivar's resistance against *P. hordei*

As highlighted in Figure 4.4B, the activation of the immune response by P*. hordei* induces not only well-established immune pathways but also pathways typically associated with abiotic stress. This suggests that barley may engage a broader stress response network, potentially co-opting shared signalling components to respond to both biotic and abiotic challenges. Moreover, the genetic diversity of commercial barley cultivars makes it even more difficult to develop universal resistance strategies. This study was conducted in the Bowman cultivar, a parental line widely used in barley breeding research, particularly for the development of near-isogenic lines (NILs) and genetic studies involving morphological markers, due to its genetic stability and well-characterised background (Franckowiak et al., 1985; Druka et al.,2011). To strengthen the relevance of these findings, I deemed it essential to validate them in commercial cultivars through field trials conducted under natural *P. hordei* infection conditions.

To address this issue, I analysed the immune ubiquitome of a panel of commercial barley cultivars challenged with *P. hordei* under field conditions. To ensure uniform infection pressure, spreader plants, artificially inoculated in the laboratory, were introduced into the field trials (in collaboration with SRUC). Leaf samples were collected from plants where *P. hordei* was the predominant pathogen. The study included seven spring barley cultivars (Cadiz, Fairing, LG Diablo, KWS Sassy, SY Bronte, Prospect, and Propino) and three winter barley cultivars (KWS Orwell, LGBU16-6889, and SY Thunderbolt). The non-infected cultivar Laureate, which was used as a natural barrier in the field trial, served as the control. Since it was not possible to guarantee the complete absence of infection in any cultivar despite the lack of visible symptoms, all cultivars were compared against Laureate to establish a threshold, and comparative analyses were also performed between cultivars. Samples were subject to protein purification and western blotting for detection of ubiquitination levels, as well as expression of immune marker genes. Combining this data with the quantification of *P. hordei* visible infection symptoms (unpublished data, SRUC) showed a varying correlation between ubiquitination levels, PR1 expression and cultivar susceptibility (Figure 4.6A). While high levels of ubiquitination were generally associated with high levels of infection, this did not necessarily correlate with the resistance status. These findings suggest that ubiquitination may reflect the intensity

of the host response or pathogen activity, rather than serving as a direct marker of resistance. As spring barley is more economically important and prone to *P. hordei* infection, I focus on spring barley for the rest of the study.

Since ubiquitination may reflect the intensity of the host response, I aimed to study the immune ubiquitome of susceptible and more resistant spring barley cultivars. I performed an immunoprecipitation experiment as described in 4.2.1. When looking at the ubiquitination profile of each cultivar compared to the negative control (non-infected Laureate samples) we can observe a clustering of cultivars based on resistance (Figure 4.6B). Cadiz exhibits a more extensively enriched ubiquitome compared to other cultivars, which affects row clustering and analysis of enriched GO terms. The clustering revealed enrichment in proteins involved in nuclear import in Cadiz ubiquitome. Analysis of GO term enrichment across different cultivars revealed consistent enrichment of methylation-related processes, while proteins involved in threonine biosynthesis were predominantly depleted, particularly in susceptible cultivars. Both of these GO terms are important for immunity, during transcriptional reprogramming and signalling (López Sánchez et al., 2016; Afzal et al., 2006).

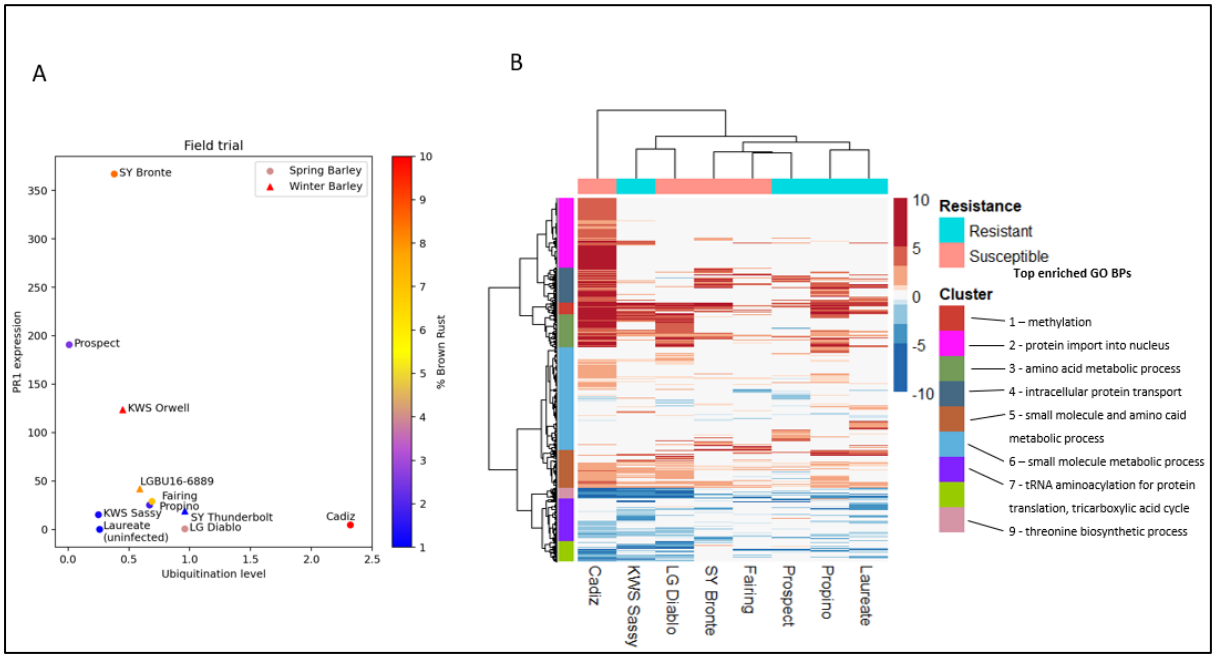

Figure 4.6 There is a correlation between the ubiquitination levels and cultivar susceptibility to *P. hordei*. Leaf samples from different spring and winter barley cultivars were processed to extract RNA and total proteins. (A) PR1 expression and

ubiquitination levels for each cultivar; marker colour represents the average percentage of Brown Rust covering the leaves (measured by SRUC in the field); circular markers correspond to spring barley cultivars and triangular markers to winter barley cultivars. (B) A proteomics study of spring barley cultivars identified 1 973 proteins differentially associated with ubiquitin upon *P. hordei* infection (infected cultivar/uninfected control – Laureate cultivar; $p < 0.05$; Log2FC ± 0.5, n=2). Colour scale represent Log2FC. Cultivars are divided into susceptible and resistant based on SRUC scoring (% Brown rust > 2 indicates susceptible cultivars and % Brown rust <2 resistant cultivars). Proteins were clustered into 9 clusters based on behaviour (Ward's method was used for clustering and Euclidean method for calculating row distances; Euclidean distance dis-similarity = 40) and each cluster assessed based on protein function; cluster 8 did not have enriched GO BP terms associated with it (based on g:Profiler g:SCS multiple testing correction method applying significance threshold of 0.05).

Given that differences in the genetic background resulting from breeding programs may influence the ubiquitination profile and the level of immune response activation, I decided to combine ubiquitome profiling with transcriptome analysis. I performed RNA sequencing and compared transcription profiles of each of the cultivars. Principal Component Analysis (PCA) revealed a wide distribution of expression profiles, with 62.7 % of the variation in expressed genes between cultivars explained by the first principal component, with uninfected control and infected control lying on the opposite ends of the axis. 12.9 % of the variation was associated with the second principal component, with Prospect placed significantly higher on the axis than others (Figure 4). The expression of genes in comparison to the uninfected control, shows a similar profile for all cultivars, with the presence of cultivar-specific clusters (Figure 4.7B). I also did not observe clustering between susceptible and resistant cultivars; therefore, cultivar's transcriptional profile is unable to predict a resistance phenotype.

To gain more insight into the regulatory network of ubiquitin-mediated signalling, I compared transcriptomics and proteomics data from the cultivar data. Overall, 653 proteins were found to be differentially expressed and regulated by ubiquitin for both datasets (Figure 4.7C) which makes up 33% of the field ubiquitome and 14% of the

field transcriptome. These contain 305 with upregulated gene and enriched protein levels, 165 downregulated gene and enriched protein levels, 135 with downregulated gene and depleted protein levels and 43 with upregulated gene expression but depleted protein levels. The positive association between gene expression and ubiquitin association suggests that fates other than proteolysis must be involved in regulation at the protein level. Proteins found in this group were found to be involved in amino acid metabolism, carbohydrate transmembrane transport and glutathione metabolism, which are all important during pathogen infection (Figure 4.7D). Interestingly, many of the proteins with this behaviour were known immune proteins (Figure 4.7E). Proteins that were enriched after infection, but experienced downregulation at the gene level, were second most abundant and found to be involved in translation, response to light and temperature, photosynthesis and plastid organisation, suggesting a role of ubiquitin in reorganisation and signal transduction.

Overall, these results show, that ubiquitin regulation can work independently of transcriptional regulation and highlights the diversity of ubiquitin signalling, dependent on the cultivars response to the environmental cues.

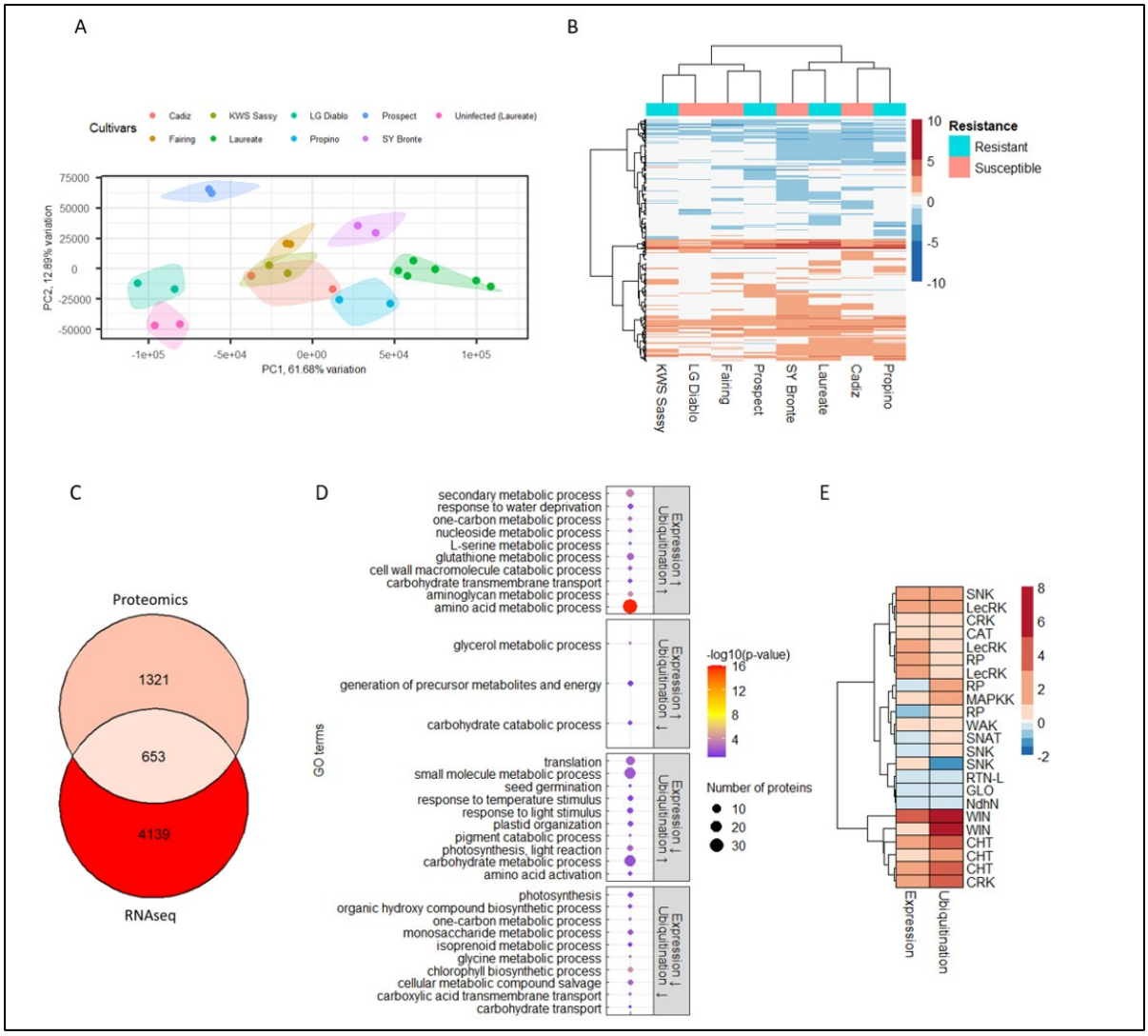

Figure 4.7 The genetic background affects transcriptional and ubiquitin regulation in the field. (A) A Principal Component Analysis (PCA) performed on expressed genes for each cultivar. (B) Differentially expressed genes (DEGs) for each cultivar (infected cultivar/uninfected control – Laureate cultivar; Log2FC>2, FDR<0.05, logCPM >2). Colour scale represents Log2FC. (C) Overlap between significant proteins and DEGs for all cultivars. (D) GO analysis shows BP terms associated with proteins exhibiting different transcriptional and posttranslational behaviour (average Log2FC for RNA ± 0.065 and protein ± 0.0625; GO analysis based on g:Profiler g:SCS multiple testing correction method applying significance threshold of 0.05, term size >2). (E) Expression and ubiquitination FC of selected immune proteins; names based on protein class: SNK - serine/threonine protein kinase, WIP - wound induced protein, CAT – catalase, RTN-L - reticulon like, CRK - cysteine rich protein kinase, WAK - wall associated protein kinase, CHT – chitinase, SNAT - Serotonin N-acetyltransferase,

NdhN - NAD(P)H-quinone oxidoreductase subunit N, GLO - Glycolate oxidase, RP - resistance protein, MAPKK - mitogen-activated protein kinase kinase

4.2.4 The interplay between hormone signalling, environmental factors and *P. hordei* infection

The plant–pathogen interaction is a highly dynamic and complex process. Since *P. hordei* secretes effectors that could manipulate both host immunity and the ubiquitin pathway, it is informative to compare the *P. hordei*-induced immune ubiquitome with that triggered by chemically activated immunity via salicylic acid (SA) and jasmonic acid (JA). This comparison provides insights into the overlap and specificity of ubiquitin-mediated immune regulation. As expected, the greatest protein overlap was observed between the *P. hordei*-induced ubiquitome and the SA-induced ubiquitome (Figure 4.8A). This is consistent with the knowledge that SA signalling is induced during infection with biotrophic fungi (Mengiste & Liao, 2025). The highest overlap was for Day 7, followed by Day 4 and finally Day 1. This difference in the number of proteins, however, could be due to the sizes of ubiquitomes themselves. Comparison of the ubiquitome profiles and protein enrichment patterns (Figure 4.8B) reveals that the SA-treated and Day 1 infection datasets exhibit the most similar profiles. This is consistent with the activation of hormone signalling pathways during the early stages of *P. hordei* infection. Interestingly, several proteins that are depleted following SA treatment are also depleted at day 7 post-infection. This suggests that SA-mediated regulation of the immune ubiquitome may operate not only during the early response but also at later stages of *P. hordei* infection. Such regulation could arise from long-term signalling feedback, heterogeneous infection states across the tissue, or systemic acquired resistance (SAR) in uninfected cells responding to the infection. Some of the JA enriched proteins show similar behaviour to Day 7, which suggests activation of JA immune pathway at the late stages of infection. This may reflect a shift in the host response as leaves become colonised, potentially triggering defence mechanisms more typically associated with responses to necrotrophic pathogens.

In the field, barley cultivars are exposed not only to *P. hordei*infection but also to a range of opportunistic pathogens and abiotic stressors. Therefore, it is important to interpret the *P. hordei*-induced immune ubiquitomes within the broader context of

additive field stressors. To identify processes regulated by ubiquitination independently of environmental conditions, I compared *P. hordei* infections under laboratory and field conditions. The majority of ubiquitinated proteins overlapped with the Day 7 laboratory dataset (Figure 4.8C), suggesting that most plants in the field had reached later stages of infection. A comparable number of proteins also overlapped with the Day 1 (90 proteins) and Day 4 (99 proteins) datasets. This distribution likely reflects the asynchronous progression of infection in the field, resulting in a heterogeneous activation of immune response both across plants and within individual leaves. When looking at the biological processes enriched among these proteins (Figure 4.8D), for the overlap with Day 1 dataset, the same processes are enriched as the ones from Day 1 Puccinia ubiquitome (Figure 4.4B). Day 4 showed enrichment in processes – response to temperature stimulus, circadian rhythm and carbohydrate catabolism – which were not enriched in Day 4 ubiquitome. This highlights the importance of processes associated with environmental responses at the colonisation stage of the pathogen infection. Day 7 overlap consisted of GO terms associated with Day 7 infection before - the phenylpropanoid pathway and lignin synthesis - which suggests the importance of this pathway in mediating immune response.

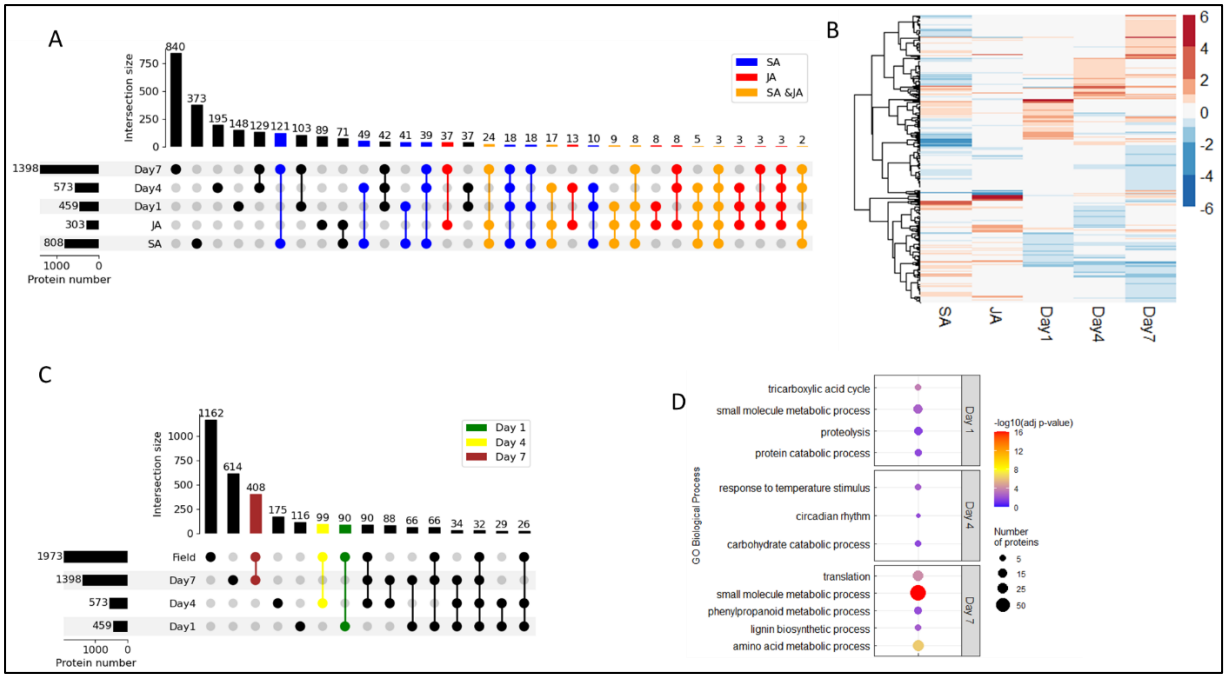

Figure 4.8 Ubiquitin-regulation of hormone signalling during *P. hordei* infection. (A) Protein overlap between *P. hordei*-infected time course and immune hormones datasets (B) Heatmap showing FC for proteins included in the selected overlap (colour-coded) from panel A. (C) Overlap between *P. hordei*-infected time course and Field datasets; highlighted are overlaps between each Day and Field proteins. (D) GO BP enriched in each of the highlighted sets from B (GO analysis based on g:Profiler g:SCS multiple testing correction method applying significance threshold of 0.05, term size >2).

4.2.5 Analysis of the ubiquitination sites reveals candidate immune proteins

Additional to the ubiquitome data, each dataset contained information about the ubiquitination sites. These were identified by the analysis software, based on a remnant glycine-glycine residue left at the ubiquitinated amino acid after the Trypsin Lyc-C digestion, and which corresponds to the C-terminal glycines of the ubiquitin protein attached to a lysine residue of the substrate. Interestingly, multiple unconventional ubiquitination sites on threonine, serine and cysteine residues were identified in the field data (Table S4). Although highly interesting, due to identification in only one dataset, I decided to focus on lysine ubiquitination in this chapter. 808 lysine ubiquitination sites on 542 proteins were identified among all datasets

combined. The majority of ubiquitinated peptides were dataset-specific (Figure 4.9A), a trend that was also reflected at the protein level (Figure S3). The most overlap was found between Day 1 and Day 4, (25 ubiquitination sites on 19 protein), which shows that some proteins are persistently ubiquitinated throughout *P. hordei* infection.

To understand, whether specific classes of proteins are more often ubiquitinated, I looked at the Protein Class annotation of ubiquitinated proteins from each dataset (Figure 4.9B). The most abundant class of ubiquitinated proteins were those involved in metabolite interconversion, which were present for all treatments. This is consistent with the enrichment of metabolic GO terms for ubiquitome datasets and highlights the importance of cellular reprogramming upon pathogen infection. In addition, I identified dataset-specific protein classes: scaffold proteins at Day 1, and RNA/DNA metabolism together with transcriptional regulators at Day 7. These findings align with the earlier results, supporting a regulatory role for ubiquitination in these processes during infection. As expected, proteins involved in folding (such as chaperones), protein-modifying enzymes, and transporters were ubiquitinated in response to all treatments. Additionally, cytoskeletal proteins were consistently identified across all *P. hordei*-infected samples.

Given the specificity of peptides found in each dataset, I performed a motif enrichment analysis to determine if this specificity is also reflected in the sequence of ubiquitinated peptides. Looking at the probability of amino acids appearing at ±7 position from the central lysine, I found that there is enrichment of alanine and lysine amino acids in Day 7 peptides, as well as neighbouring lysine residues (Figure 4.9C). In contrast, Day 4 peptides were enriched for polar residues such as glycine and serine downstream of the ubiquitination site. Across datasets, enriched motifs were identified (Table 4.1), with most enriched residues positioned 3–5 amino acids downstream or 1–7 amino acids upstream of the modified lysine.

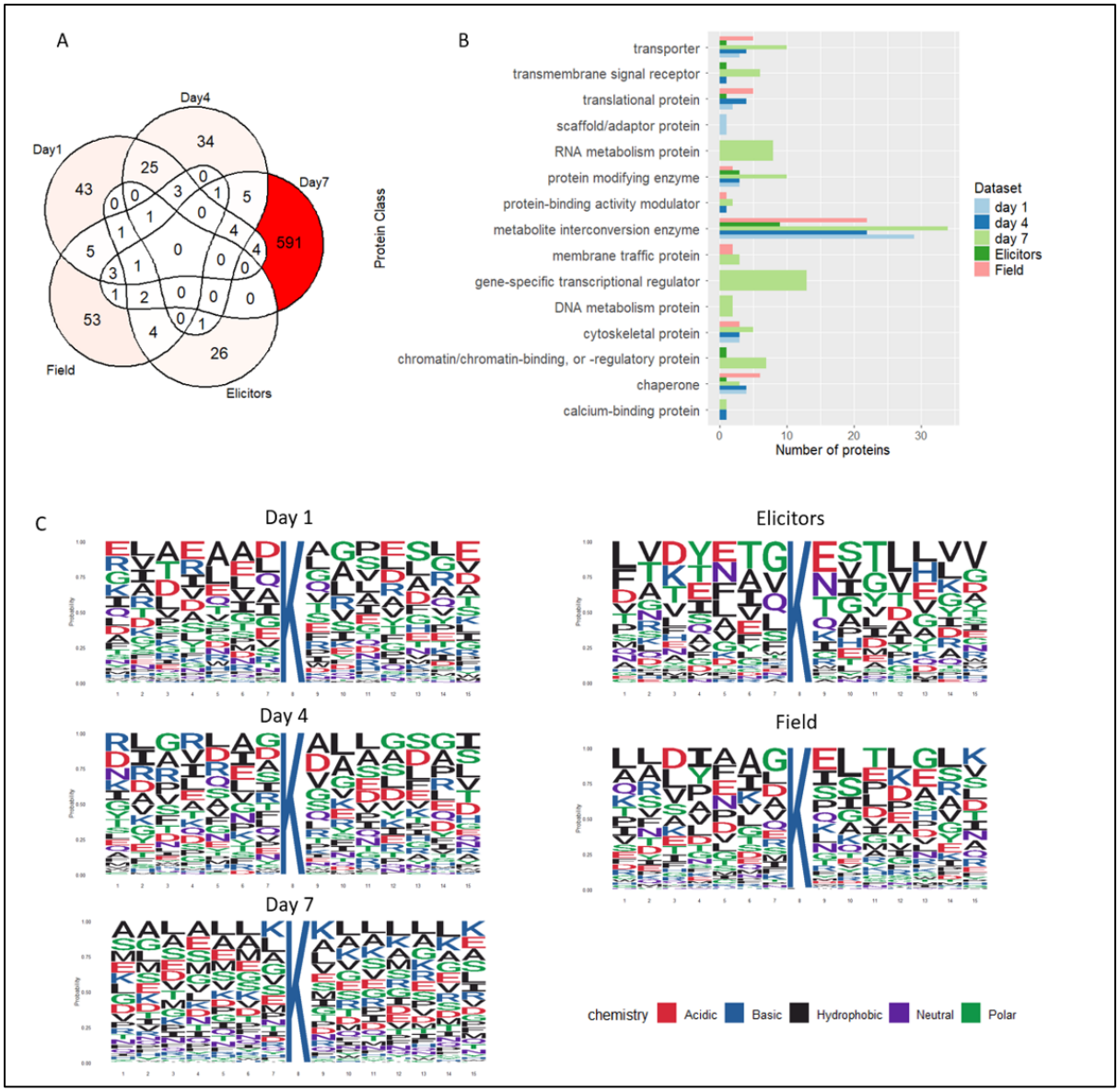

Figure 4.9 This proteomics studies identified novel ubiquitination sites in barley proteins. (A) Overlap between ubiquitination sites identified in target proteins in all datasets (B) Protein function of annotated proteins for which ubiquitination sites were identified (C) Ubiquitination motifs for each dataset (based on probability)

Table 4.1 Motifs enriched among the DiGly peptides for each dataset. Performed using MEME-SUITE MoDL algorithm (minimum occurrences = 5)

Dataset	Motif	Peptides matched	Adjusted p-value
Field	xxx[IY]xxx_K_xxxxxxx	15/73	2.2e-3
	xxxxxxx_K_xxxxxx[DK]	16/73	1.2e-3
Immune hormones	xxxxxxx_K_[EKNT]xxxxxx	21/32	8.0e-7
Puccinia Day 1	xx[DR]xxxx_K_xxxxxxx	16/89	3.8e-3
Puccinia Day 4	xxx[AHKLM]xxx_K_xxxxxxx	20/72	8.2e-4
Puccinia Day 7	xxxxxxx_K_xxxx[PR]xx	83/612	1.1e-6
	xxxx[MN]xx_K_xxxxxxx	78/612	1.6e-3

To understand how the ubiquitinated proteins fit into the protein interaction network of the immune ubiquitome, I overlapped the data from each ubiquitome and highlighted ubiquitinated proteins (Figure 4.10). I found that ubiquitinated proteins often occupy central nodes within the interaction network, potentially serving as key hubs that stabilise the network architecture. Others are located on the network periphery, where they may act as bridges linking the core network to less characterised pathways, interactors, or regulatory modules. I found enrichment of similar processes as for *P. hordei* ubiquitome (Figure 4.5) and, as expected, proteins from different immune ubiquitome datasets were found to form interaction networks with each other. Altogether, these findings reveal the interconnected nature of the ubiquitin-regulated network during immune responses, underscoring the complexity and multi-layered regulation of ubiquitin-mediated interactions.

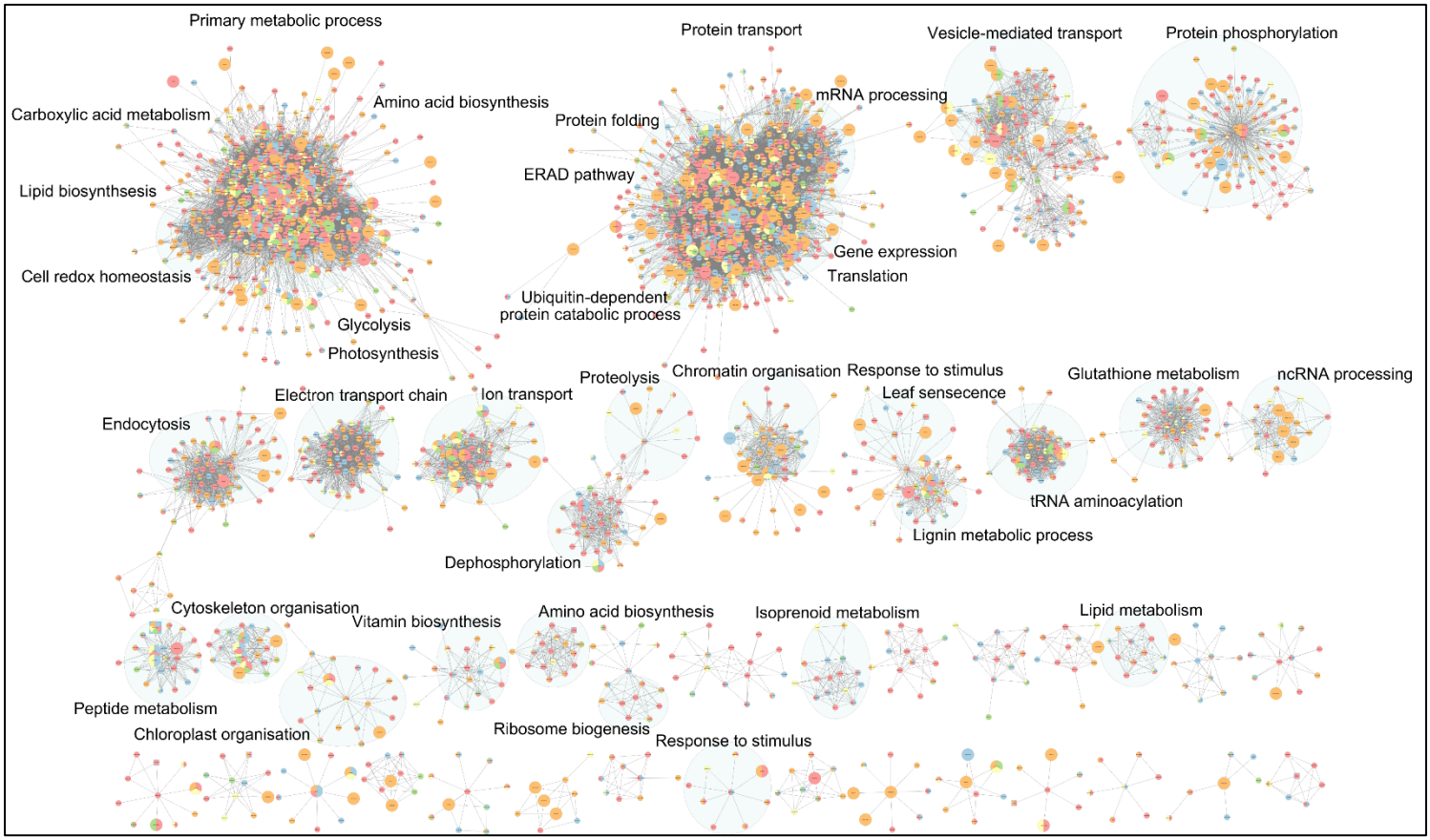

Figure 4.10 Ubiquitinated proteins form central nodes of the barley immune ubiquitome. Ubiquitinated and ubiquitin regulated proteins form a protein interaction network using (STRING v12, based on >90% sequence overlap); nodes represent proteins, coloured based on appearance (Day 1 – green, Day 4 – yellow, Day 7 – orange, immune hormones treatment – blue, field – red), while edges represent STRING full interaction score (edge cut-off >0.5). MCL clustering based on the interaction scores (inflation value =2.5, number of proteins in a node >6) was used to extract functional nodes and GO enrichment analysis was performed on each node and the top BP annotated to each node; nodes without annotation did not have enriched GO BP terms.

The analysis of barley immune ubiquitomes highlighted the importance of several pathways based on (i) the extent of ubiquitin regulation; (ii) significance at different stages of immune activation; (iii) presence of ubiquitinated targets. These include: translation, phenylpropanoid biosynthesis, protein processing and transport and the ubiquitination pathway (Figure 4.11).

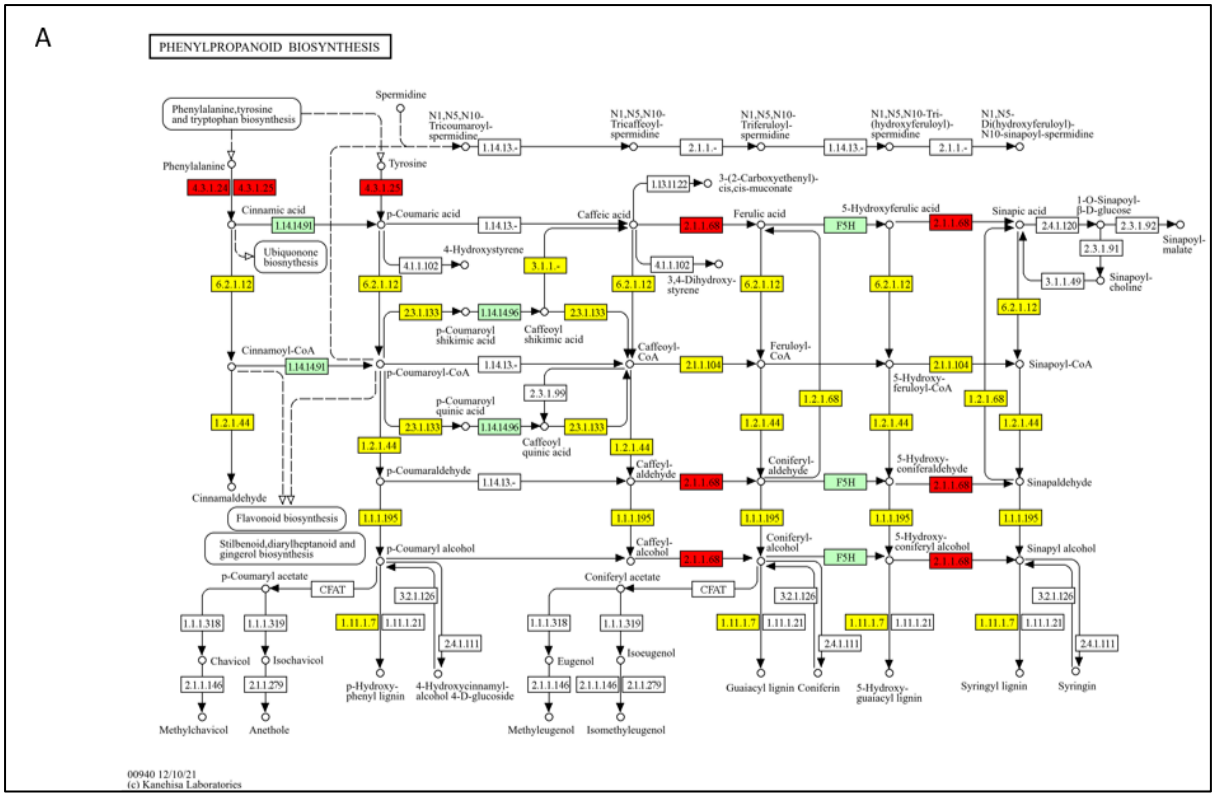

Figure 4.11 Proteins identified in barley ubiquitome are involved in essential pathways. (A) Phenylpropanoid biosynthesis pathway. (B) Protein processing in endoplasmic reticulum pathway. Graphics extracted from KEGG. Annotation based *Oryza sativa subsp. japonica* KEGG pathway (red nodes – ubiquitinated proteins;

yellow nodes – ubiquitin-associated proteins, green nodes – not found in the barley ubiquitome, white nodes – not annotated in *Oryza sativa subsp. japonica.*

4.3 Discussion

The aim of this study was to elucidate the biological processes and pathways regulated by ubiquitination during infection with *Puccinia hordei* and to identify ubiquitinated proteins that may serve as potential targets for enhancing barley immune responses against this pathogen. In order to gain an overview of the barley immune ubiquitome, I used a two-step protein enrichment protocol, coupled with LC-MS/MS analysis, to capture ubiquitinated and ubiquitin-associated proteins. This approach allowed us to establish complex networks of ubiquitin regulation, with each ubiquitome showing a unique profile and specific pathways commonly regulated during activation of the immune response.

As little is known about the role of ubiquitin signalling in mediating barley immunity, this study provides valuable insights into this area. I showed that both in response to immune hormones (Figure 4.1) and the pathogen (Figure 4.3), ubiquitination levels increase prior to transcriptional changes, as seen in the expression of immune marker genes. I also observed that ubiquitination response is more sensitive to lower doses of the immune hormones. This underscores the importance of ubiquitination in orchestrating early immune responses and aligns with previous studies highlighting its central role in regulating immune signalling immediately following pathogen perception (Furlan et al., 2012). Interestingly, I show that the ubiquitination status could predict cultivar's infection load better than its transcriptional profile. Cultivars with similar *P. hordei* susceptibility showed similar ubiquitome profile, while no correlation was observed when comparing transcriptional profiles (Figure 4.6 & 4.7). This suggests that the plant ubiquitin proteome may be shaped by the activity of a limited number of key immune regulators. However, it is essential to note that RNA-seq is more sensitive and may capture transcriptional changes associated with the genetic background that are not reflected at the protein level. Furthermore, I were surprised by the low overlap between plant transcriptome and ubiquitome, with only 14% of differentially expressed genes being present in this ubiquitome data. This likely does not reflect the full extent of ubiquitin-mediated regulation, as the proteomics workflow is inherently limited by the depth of protein capture and analysis, compounded by the incomplete annotation

of barley proteins and transcripts. Nonetheless, recent studies report a ~20% overlap between the proteome and ubiquitome (Song et al., 2024; Jiang et al., 2022). Furthermore, given that only ~50% of expressed transcripts can typically be confirmed at the protein level (Álvarez-Urdiola et al., 2025), the observed overlap is consistent with findings from other studies.

Hormone signalling is essential for the activation of effective immune responses. These results demonstrate that ubiquitin signalling contributes to hormone-specific responses in barley, both by directly regulating hormone-dependent pathways and by modulating broader biological processes. Interestingly, although jasmonic acid (JA) treatment triggered stronger ubiquitin accumulation and more pronounced marker gene expression than salicylic acid (SA), the JA ubiquitome contained significantly fewer ubiquitin-regulated proteins than the SA ubiquitome. This suggests that JA elicits a narrower spectrum of proteomic changes compared to SA, consistent with the observation of stronger magnitude enrichment of JA-induced ubiquitin targets (Figure 4.B). Moreover, this data indicate that ubiquitin contributes to the cross-talk between SA and JA in barley, with evidence of mutual negative regulation and overlapping effects on metabolic processes (Figure 4.2D–E).

To the best of my knowledge, this is the first comprehensive profiling of the barley immune ubiquitome across three major stages of *P. hordei* infection. I identified day 4 as a critical stage, associated with pathogen colonisation, elevated ubiquitination, and a marked reduction in PR gene expression. Strikingly, however, the profile of total ubiquitination (Figure 4.3A) did not correlate with the number of ubiquitin-regulated proteins identified (Figure 4.4A). While protein numbers were similar at day 1 and day 4, more than twice as many were identified at day 7. One explanation could be that only a small number of proteins undergo heavy ubiquitination. However, this is unlikely, as peptide distribution showed a similar ratio across datasets (data not shown), and no strongly enriched individual proteins were evident (Figure 4.4C). Another possible explanation is that the increased ubiquitination observed at day 4 involves pathogen-derived proteins, which were not detected in the host-focused proteomics workflow, or results from *P. hordei* effectors directly targeting host ubiquitin pathways, such as the proteasome. Such ubiquitination of pathogen effectors or other pathogen-derived proteins could serve as a plant's defence mechanism, by most likely targeting them for degradation (Duplan and Rivas, 2014). A direct search of the

ubiquitome dataset against the *P. hordei* proteome would therefore provide valuable insight into the ubiquitin-mediated plant–pathogen interface.

Gene ontology enrichment analysis revealed robust differences between immune treatments in the biological processes regulated by ubiquitin. I found that although SA treatment induced ubiquitination of proteins associated with early activation of immune responses, like translation, protein processing and transport, it triggered a highly different ubiquitome to the early *P. hordei* infection (Figure 4.8). Additionally, these results show signs of pathogen-mediated regulation of the plant immune system, with oscillating immune gene expression (Figure 4.3B), suppression of specific immune processes at the early stages of infection (Figure 4.4B) and absence of immune-related proteins at crucial stages of the infection (Figure 4.4C). It is known that the pathogen can target plant ubiquitin system to evade the immune responses (Dielen et al., 2009) and this ubiquitome data can be used to dissect these interactions during *P. hordei* infection.

We also identified ubiquitination sites in over 500 barley proteins with potential roles during the immune response. Interestingly, we identified unconventional ubiquitination sites that are not as well understood as those involving lysine ubiquitination. Even though there are few reports of unconventional ubiquitination in plants (Gilkerson et al., 2015), there have been reports that connect it to immunity, especially in the context of pathogens' activity (McDowell and Philpott, 2013). Taking all this into consideration, the area of unconventional ubiquitination in plants is an interesting yet unexplored area of research.

Altogether, I established a comprehensive immune ubiquitome, which can serve as a mining database for identifying immune proteins in barley, dissecting the effect of the genome on the plant ubiquitome, and understanding how ubiquitin regulates proteins during immune response in barley.

Chapter 5

Characterisation of ubiquitin-regulated barley immune proteins

5.1 Introduction

Plant pathogens contribute significantly to food insecurity by causing up to 40% of yield losses (Fisher et al., 2012). To meet the growing demand for food production, we need to develop new strategies to mitigate food loss, one of which is improving crop resistance to reduce the disease burden. Conventional breading practices rely on natural variation and require a lot of time and resources (Dong and Ronald, 2019). Genetic engineering, however, offers several advantages over conventional breeding and can provide long-lasting resistance (Dong and Ronald, 2019). Developing pathogen resistance through genetic engineering requires, however, a very good understanding of the molecular mechanisms underlying the plant-pathogen interactions. While the barley ubiquitome provides clues as to which proteins could serve as targets for improving disease resistance, molecular studies are required to understand the mechanistic roles of these proteins.

Ubiquitination is important in regulating plant immune responses, and it has been used previously in developing plant resistance. For example, Arabidopsis mutants *pub22/pub23/pub24* showed increased resistance against bacterial and oomycete pathogens (Trujillo et al., 2008). In rice, knockout of a different PUB E3 ligase OsPUB41 was found to stabilise OsPAL1 protein, which led to increased lignin accumulation and strengthened cell walls, thereby enhancing resistance against *Magnaporthe oryzae* (Xu et al., 2025). This shows not only that by altering ubiquitination we can alter plant immune responses, but also that the E3 ubiquitin ligases are the key players in this pathway. This is not surprising, as the E3 ligases are the most diverse group of ubiquitination enzymes, with over 1 000 genes belonging to three families encoded in Arabidopsis (Vierstra, 2012). They are known to be involved in all stages of the immune response, from pathogen perception, regulation of signalling responses, to targeting transcription factors (Duplan & Rivas, 2014). Moreover, as they are often target-specific, they can be used to control the activity of downstream proteins. To this day, we know little about barley E3 ligases involved in the regulation of the immune response, with only one functionally validated (Table 5.1). Characterising barley E3 ligases can therefore be beneficial for identifying methods of inducing *P. hordei* resistance.

Table 5.1 Identified immune E3 ligases in barley

E3 ligase	Result	Pathogen	Reference
MIR1	Promotes degradation of MLA NLR receptors	*Blumeria graminis f. sp. hordei* (Powdery Mildew)	Wang et al., 2016
PUBs (24, 42, 23, 27, 55)	Induced expression during infection	*Blumeria graminis f.sp. hordei* (Powdery Mildew)	Ryu et al., 2019
RAR1/SGT1*	Induces R gene-mediated resistance against a variety of pathogens	Powdery Mildew	Azevedo et al., 2002

* SGT1 is part of a SCF complex SKP1

Chapter 3 of this thesis provided an insight into how infection with *P. hordei* modulates barley transcriptome, while Chapter 4 aimed to decode how the ubiquitin pathway regulates barley immune responses. These two chapters together provide a ground for identifying novel barley immune proteins, whose functionality is regulated or dependent on ubiquitin. As little is known about the role of ubiquitination in regulating barley immune responses, molecular characterisation studies are required to decipher these interactions.

Characterising the role of ubiquitination in regulating immune activity and identifying specific targets presents several challenges—particularly due to the limited availability of molecular tools for functional validation in barley. For example, transient expression in barley shows low efficiency, limiting the ability to test hypotheses effectively. To overcome this, we can use *Arabidopsis thaliana* and *Nicotiana benthamiana* as platforms for molecular validation and use synthetic biology approaches to decode functions of barley proteins.

In this context, Arabidopsis and *N. benthamiana* offer an exceptional model system to validate the function of ubiquitinated immune targets. Nevertheless, I am fully aware that differences between monocots and dicots may influence the results and must be

carefully considered when interpreting findings from Arabidopsis in the context of cereal crop immunity.

In this study, I aimed to validate selected immune proteins found to be ubiquitinated during *P. hordei* infection, along with several candidate immune-related E3 ligases. My results showed, that EF1a, BD1, CAT2 and CAM5 function as positive regulators of immunity in Arabidopsis. Furthermore, my proteomic screen enabled the identification of immune-associated E3 ligases that either co-purify with their targets during ubiquitin pull-down or undergo autoubiquitination during immune activation. E3 ligases are particularly interesting targets for modulating immunity, as they regulate immune responses by ubiquitinating key immune proteins. Therefore, identifying their targets is a critical step in characterising their function. However, identifying E3 substrate interactions is inherently challenging, due to the transient and labile nature of these interactions. Here, I present a synthetic approach that allows for the identification of HvRGLG2 targets and characterise it as a novel immune E3 ligase in barley. I further demonstrate that two known immune regulators, HvHSP70-2 and HvPAL1, are direct targets of HvRGLG2. These findings contribute new candidates to the list of barley immune proteins that can be manipulated to enhance resistance against *Puccinia hordei*.

5.2 Results

5.2.1 Identified ubiquitination targets are important during immune response

We used the barley immune ubiquitome dataset to construct protein interaction networks that highlight pathways regulated by ubiquitination during *Puccinia hordei* infection (sections 4.2.1 – 4.2.4). In parallel, by identifying the di-glycine (DiGly) remnant on peptides from pull-down proteins, I mapped specific ubiquitination sites on proteins involved in these immune-related processes (section 4.2.5). From these proteins, several candidates were selected for functional validation based on their putative roles in immunity. Selection criteria included enrichment in biological processes active at different stages of infection and the presence of closely related Arabidopsis orthologues, facilitating comparative functional analysis. Ichose proteins involved in translation (EF1a), gene expression (GRF7, BD1, WIP4), protein folding (HSP70-1, HSP70-2, BIP2), response to stress (CAM5, CAT2), hormone synthesis (PAL1, PAL3b) and ubiquitination (PUB11, PUB17, FBL12) (Table 5.2).

Table 5.2 Identified ubiquitinated targets chosen for validation.

Name	Dataset	UniProt protein ID	Ubiquitination site	Arabidopsis orthologue UniProt protein ID
EF1a	Field	M0Y9X7	K301	Q8GTY0/Q0WL56/ Q8W4H7/P0DH99
GRF7	Day 1	F2CRF1	K75	Q96300
HSP70-1	Day1	A0A8I6YLW7	K564	P22953
HSP70-2	Day 1, Field	F2D884	K513, T69	P22954
PAL3b	Day 1, Day 4	F2DQ23	K111, K111	P35510
CAT2	SA, Day 1, Day 4	F2CVM1	K5, (K72, K464, K233), K233	P25819
BIP2	Day 4	A0A8I6WXH3	K356	Q39043
CAM5	Day 4	A0A8I6WU73	K95	F4IVN6
BD1	Day 7	A0A8I6X799	K236, K241, K242	F4K639
FBL12	Day 7	M0V6F2	K259, K260	Q9SRR1
PAL1	Day 7	A0A8I6XFK0	K598	P35510
PUB11	Day 7	F2E3L3	K596	Q9C9A6
PUB17	Day 7	F2DPH2	K544	Q9C7R6
WIP4	Day 7	A0A8I7B8E3	K273	O82615

To determine whether the selected proteins act as immune regulators, I used the *Arabidopsis* model system to characterise the immune phenotypes associated with loss-of-function mutations in their respective orthologues. I infected Arabidopsis knock-out lines with a bacterial pathogen *Pseudomonas syringae pv. maculicola* (*Psm*) and assessed the bacterial growth after 4 days. I found that lines mutant for *EF1A (triple knockout), BD1, CAT2* and *CAM5* were significantly more susceptible to *Psm* than the Wild Type (WT) (Figure 5.1). Among the tested lines, only the *ef1a(x3)*

plants displayed susceptibility levels comparable to *npr1* mutants. This phenotype reflects a strong impairment in salicylic acid (SA)-mediated defence, as *npr1* mutants are unable to mount an effective immune response and are highly susceptible to pathogen infection due to loss of NPR1 function. The similarity suggests that EF1A plays a critical role in plant immunity. In summary, I identified three proteins, for which a single gene knockout results in compromised immune response in Arabidopsis, which suggests that they might have a conserved immune role in monocots and dicots. Further work into their roles in barley, as well as the role of ubiquitination sites in mediating immune role is required to fully dissect their function during *P. hordei* infection.

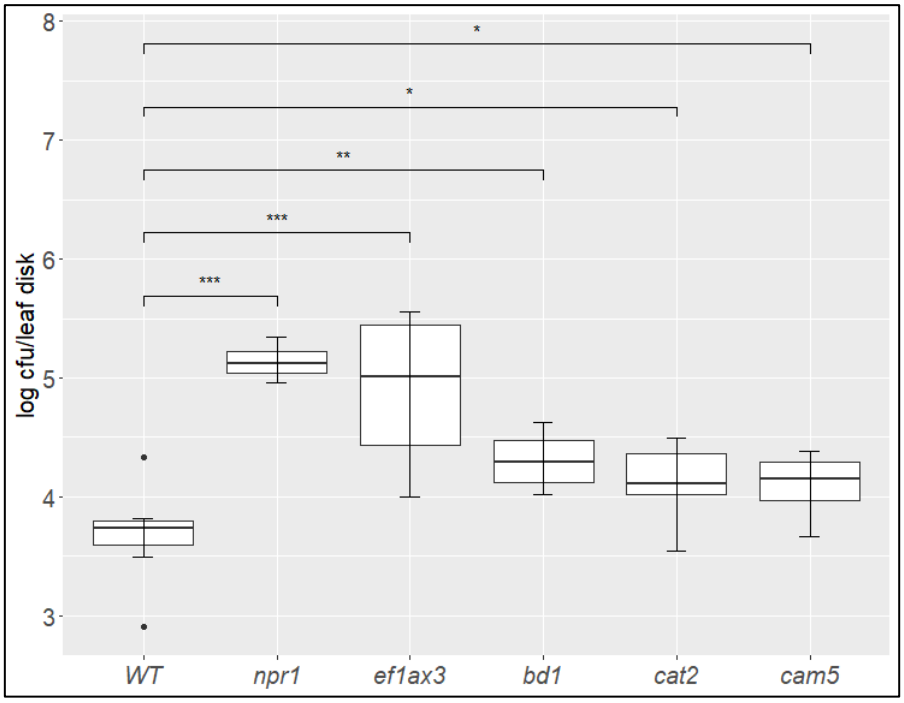

Figure 5.1 Identified ubiquitinated targets have immune roles in Arabidopsis. Arabidopsis plants containing knockout mutations for identified ubiquitinated targets EF1A(x3), BD1, CAT2 and CAM5 were infiltrated with a bacterial suspension (5 x 108 cfu/ml) and bacterial growth was assessed at 4 dpi. *npr1* genotype acts as a positive control (t-test, n=8).

5.2.2 Enriched E3 ligases control ubiquitination during *P. hordei* infection

Additionally, I identified 16 E3 ligase proteins, that were enriched in the ubiquitome data during *P. hordei* infection (Table 5.3). Five proteins were enriched at Day 1, six at Day 4 and eight at Day 7. Most of the proteins belonged to the RING family and F-box family. Three proteins were found enriched at various stages of the infection: Cullin 1 (CUL1) and Ubiquitin-protein ligase 1 (UPL1) were both positively enriched at Day 1 and Day 4, while BBR-like was enriched at Day 1 and 7, but depleted at Day 4.

Table 5.3 E3 ligases significantly differentially enriched during *Puccinia hordei* infection. ($p < 0.05$; $Log_2FC \geq \pm 0.5$). Protein names are based on the closest Arabidopsis orthologues when the corresponding barley gene name was unavailable.

Name	Dataset	Fold change	Type	UniProt protein ID
CUL1	Day 1	0.32	Cullin	A0A8I6WBL2
UPL1	Day 1	1.82	HECT	A0A8I6Y6Y3
CRT1	Day 1	3.12	RING	A0A287SQW8
BBR-like	Day 1	1.55	RING	F2D1F4
PUB44	Day 1	1.35	U-box	F2CTE6
SKP1	Day 4	0.49	BTB-POZ	A0A8I7BG29
BBR-like	Day 4	-0.54	BTB-POZ	F2D1F4
CUL1	Day 4	0.48	Cullin	A0A8I6W4Q8
UPL1	Day 4	0.79	HECT	A0A8I6Y6Y3
BTL8	Day 4	0.32	RING-H2	A0A8I6X2E4
FBD	Day 7	4.86	F-box	A0A8I6Z890
GID2	Day 7	1.21	F-box	A0A8I6WNE6
KMD	Day 7	1.02	F-box	F2CSB7
PP2	Day 7	0.24	F-box	A0A8I7BHS6
UPL3	Day 7	0.36	HECT	A0A8I6XWA2
RGLG1	Day 7	2.39	RING	F2EJA0
BBR-like	Day 7	1.64	RING	F2D1F4
RGLG2	Day 7	1.40	RING	F2DMD1

We found two closely related proteins RGLG1 and RGLG2, to be highly enriched at Day 7 after infection. These proteins are closely related to AtRGLG1 and AtRGLG2. Earlier, I found RGLG2 to be upregulated in infected plants during *P. hordei* infection, while RGLG1 showed downregulation during infection (Figure 3.9). Additionally, Arabidopsis orthologues are known to be involved in K63-linked ubiquitination, which is associated with vesicular transport GO term highly enriched in the immune ubiquitome data (Figures 4.5 & 4.10). It was also demonstrated that Arabidopsis RGLG1/2 positively regulate immune signalling by preventing PUB25-mediated ubiquitination and subsequent proteasomal degradation of Botrytis-induced kinase 1 (BIK1) (Bai et al., 2023). Taking all of this into consideration, I selected the E3 ligase RGLG2 as a potential key regulator of immunity for further analysis. A target-trapping strategy identifies targets of HvRGLG2

Ubiquitin E3 ligases are key specificity factors in plant immune signalling, directing substrate ubiquitination to modulate protein activity, stability, and localisation. Therefore, defining their direct substrates, and integrating these with immune ubiquitinome data, will clarify how individual E3 ligases fit within the ubiquitin-regulated proteome and highlight the regulatory nodes they control. However, functions of many immune E3 ligases remain unclear because interactions with their substrates are transient and therefore difficult to capture, hindering substrate identification. To overcome this, I decided to design a synthetic E3 ligase construct that would enable us to “trap” targets of E3 ligases.

In this approach, I utilised tandem ubiquitin binding entities (TUBEs), to enable the capture of proteins ubiquitinated by HvRGLG2 (Watanabe et al., 2020). The designed construct contained four UBA domains from Ubiquilin1 acting as TUBEs (the same were used for ubiquilin immunoprecipitation in Chapter 4) in the N-terminus, internal FLAG-tag followed by the HvRGLG2 sequence. This synthetic HvRGLG2 (SynRGLG2) construct was cloned into pET-28a(+) vector with C-terminal HIS-tag for protein expression in *E.coli* (Figure S3). The approach involved expressing SynRGLG2 construct in *E.coli* and purifying the protein using a HIS-tag specific nickel-charged affinity resin, followed by protein immobilisation on anti-FLAG antibody-coupled magnetic beads. Immobilised SynRGLG2 could then be combined with plant extract and incubated to promote protein ubiquitination. As proteins are ubiquitinated by SynRGLG2 the growing ubiquitin chain binds to the TUBEs, and as a result the

target proteins are trapped bound to the SynRGLG2 and can be subsequently characterised using mass spectrometry (Figure 5.2A).

The first step was to confirm that SynRGLG2 retains E3 ligase activity after the addition of a long N-terminal tag. To do this, I expressed SynRGLG2 in *E. coli* and performed in vitro ubiquitination assays. My results indicate that SynRGLG2 is well expressed, remains active after heterologous expression, and exhibits E3 ligase activity, including the ability to auto-ubiquitinate in vitro (Figure 5.2B). RGLG2 is a RING-type E3 ligase. The “RING E3 zinc” designation refers to a class of E3 ligases that contain a RING (Really Interesting New Gene) finger domain, which coordinates two zinc ions to form a cross-braced structure essential for E3 ligase function. This structural motif enables the RING domain to fold and recruit an E2 ubiquitin-conjugating enzyme, which carries ubiquitin for transfer to the substrate. To generate a catalytically inactive control, EDTA was included in the reaction mixture. EDTA chelates zinc ions and thereby disrupts the structural integrity of the RING domain, leading to a loss of E3 ligase activity (Lorick et al., 1999). Consistently, the addition of EDTA to the reaction strongly impaired SynRGLG2 activity, confirming its zinc-dependent catalytic function and validating the use of EDTA treatment as an effective negative control (Figure 5.2B).

I then performed a semi–in vitro ubiquitination assay, in which purified SynRGLG2 was immobilised using FLAG-affinity immunoprecipitation. Protein extracts from healthy and *P. hordei*-infected barley leaf tissue were then added to the immobilised SynRGLG2. To promote ubiquitination and avoid limiting reaction components, exogenous ATP, E1, and E2 enzymes were added to the reaction mixture. Following the ubiquitination reaction, SynRGLG2-associated substrates were purified and analysed by western blot (Figure 5.3 C). A ubiquitination smear was observed in both healthy and infected samples which was reduced after the addition of EDTA. This suggested that SynRGLG2 successfully trapped ubiquitinated targets. The ubiquitination signal was slightly stronger in control than in the infected tissue, however, the autoubiquitination signal from SynRGLG2 was the same. The difference may stem from the number of substrates or the degree of ubiquitination.

These results showed that SynRGLG2 is active and can be used to pull-down substrates of HvRGLG2 from barley.

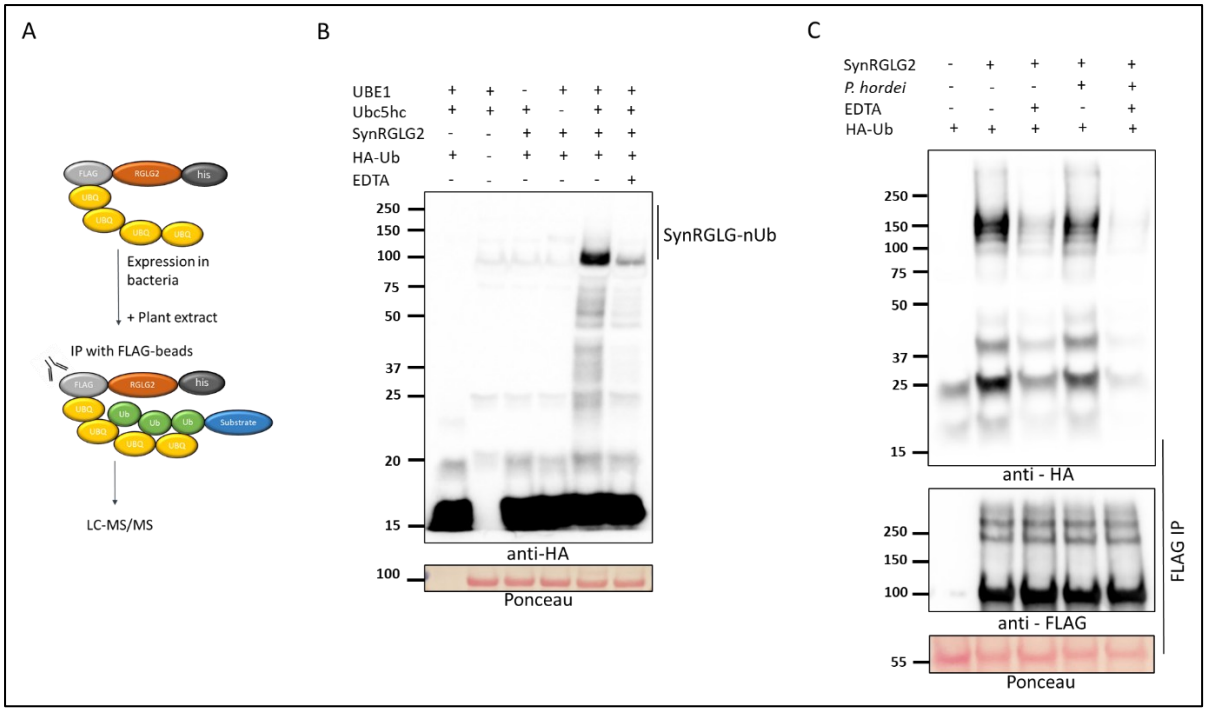

Figure 5.2 A synthetic HvRGLG2 possesses E3 ligase activity and can bind ubiquitinated substrates. (A) Substrate trapping method using a synthetic HvRGLG2 (SynRGLG2) construct; (B) SynRGLG2 shows ubiquitin ligase activity in vitro. Sample with EDTA was used as a negative control; (C) SynRGLG2 was used to pull ubiquitinated substrates from barley leaf extract from healthy plants and plants infected with *P. hordei*. Leaf tissue was collected 7 dpi. Sample with EDTA was used as a negative control.

I employed the substrate trapping approach as described above, and followed with a LC-MS/MS analysis to identify HvRGLG2 substrates. To specifically identify immune-related RGLG2 substrates, I excluded all proteins that bound in the non-infected samples or in the infected samples in the presence of EDTA. Ultimately, I identified 73 proteins, which were significantly enriched in infected sample (Figure 5.3 A). These represent proteins which are likely ubiquitinated by SynRGLG2 during infection, and therefore can be potential real targets of HvRGLG2. Among SynRGLG2 targets, I found many immune proteins, like pathogenesis related proteins PR1 and PR10, phenylalanine ammonia lyase 1 (PAL1), Receptor-like serine/threonine-protein kinase 1 (RLK1), peroxidases PER50 and PER52 and many others. 46 of these proteins were present in the barley immune ubiquitome data, for three of which we found ubiquitination sites (Glutathione transferase 17, PAL1 and PAL3) (Figure 5.4 B).

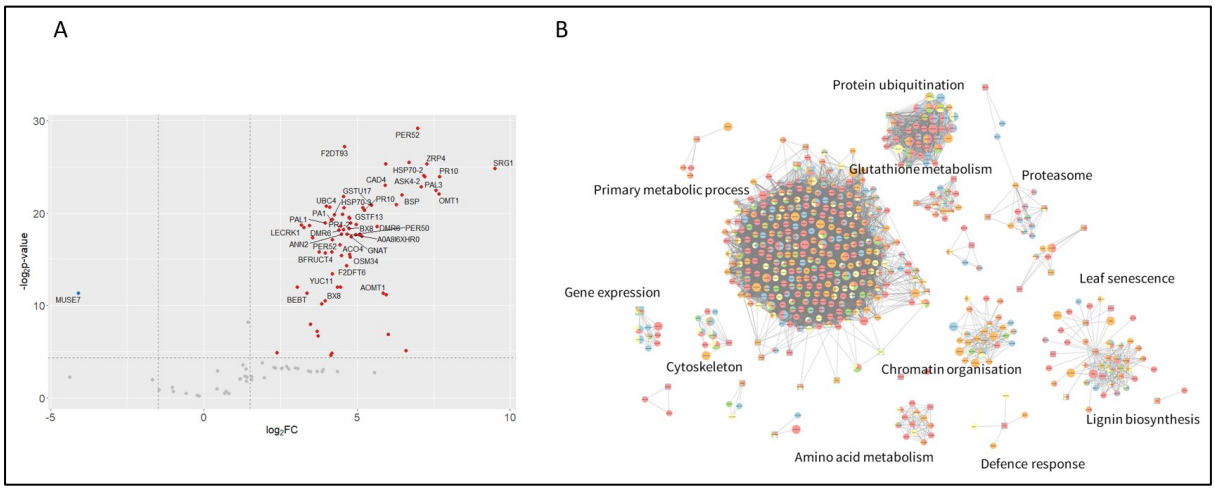

Figure 5.3 Target trapping method identified targets of SynRGLG2. (A) Targets of active SynRGLG identified using LC-MS/MS enriched after infection. (B) Overlap between proteins present in the barley immune ubiquitome (Figure 4.10) and targets of SynRGLG from (A) representing targets of SynRGLG (square nodes) and their first neighbours present in the immune ubiquitome (circular nodes). Nodes are coloured based on appearance (Day 1 – green, Day 4 – yellow, Day 7 – orange, immune hormones treatment – blue, field – red), while edges represent STRING full interaction score (edge cut-off >0.5). Large nodes represent proteins for which ubiquitination sites were identified. Top BP are annotated to each node. Nodes without annotation did not have enriched GO BP terms.

5.2.3 RGLG2 ubiquitinates barley immune proteins HSP70 and PAL1

To validate the mass spectrometry data, I selected two candidate SynRGLG2 targets, HvHSP70-2 and HvPAL1, that were also previously identified in the barley immune ubiquitome dataset. Both proteins were found to be ubiquitinated during *P. hordei* infection (Table 5.1) and are involved in biological processes that were significantly enriched at specific stages of infection (Figures 4.10 and 4.11). Although knockout lines for their Arabidopsis orthologues did not display a clear immune phenotype (Figure S5, this may be due to functional redundancy among gene family members. Wild-type and mutant variants of these proteins, carrying mutations in the identified ubiquitination sites (HvHSP70-2 withT70A and HvPAL1 withK238R mutations), were cloned and transiently expressed in *N. benthamiana*. They were then pulled-down and

incubated with Ubiquitin carboxyl-terminal hydrolase 2 (USP2), to remove any ubiquitination that might have occurred *in planta*. Deubiquitinated proteins were then incubated with SynRGLG2 in an *in vitro* ubiquitination assay. This data shows, that both HvHSP70-2 and HvPAL1 can be ubiquitinated by SynRGLG2 in vitro (Figure 5.4). The mutation in the ubiquitination site does not affect the ability of SynRGLG2 to ubiquitinate either of the proteins. HvHSP70 showed a faint smear of low molecular weight, which suggests that it might be modified with short chains. Additionally, the unmodified HvHSP70-2 protein appeared as two closely migrating bands, which may indicate post-translational modifications, such as phosphorylation, or partial cleavage (Figure 5.4A).

In contrast, HvPAL1 showed a pronounced ubiquitination pattern when incubated with SynRGLG2, including a high molecular weight smear extending ~50 kDa above the unmodified protein and an additional discrete band approximately 2.5 times the molecular weight of the native protein (Figure 5.4B). These bands were absent in control reactions lacking SynRGLG2, suggesting that they result from extensive ubiquitination, possibly via long polyubiquitin chains.

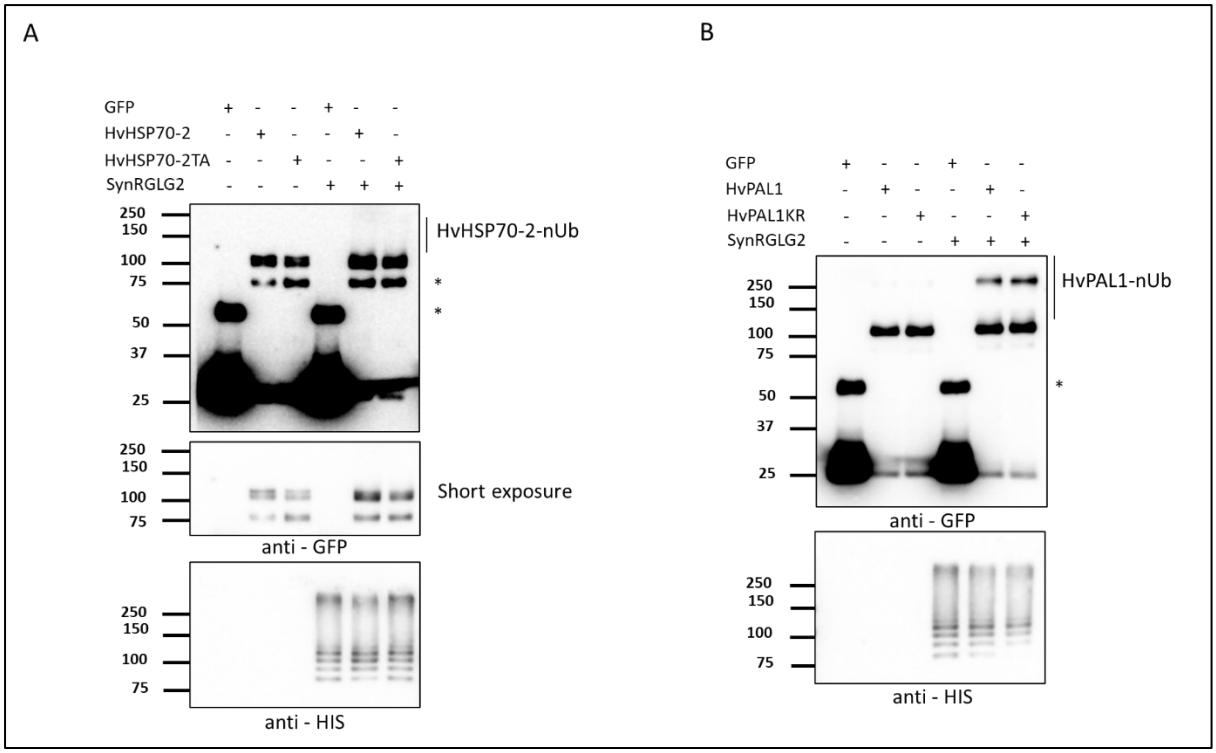

Figure 5.4 SynRGLG2 can ubiquitinate HvHSP70-2 and HvPAL1 in vitro. HvHSP70-2 (A) and HvPAL1 (B) transiently expressed in Tobacco and pulled-down using anti-GFP beads, followed by a deubiquitination treatment with USP2. In vitro ubiquitination reaction with SynRGLG was then performed for wild type and mutant proteins carrying a point mutation in the ubiquitination site identified earlier (n=2).

Altogether, I found that RGLG2 is an immune E3 ligase in barley and it is active during *P. hordei* infection. These results suggest that it can ubiquitinate HvHSP70-2 and HvPAL1 *in vitro*, which could have physiological implications in planta during the immune response.

5.3 Discussion

My results (Chapter 4) reveal the immune ubiquitome of barley plants infected with *P. hordei*, suggesting that ubiquitination plays a central role in regulating the immune response. To validate the findings from the mass spectrometry dataset, I selected a subset of immune-related proteins with established roles in plant defence and tested their function in the model plant *Arabidopsis thaliana* using mutant lines of the

homologous genes. Of the identified proteins, only four showed a measurable immune phenotype in Arabidopsis (Figure 5.1).

The most pronounced susceptibility was observed in the triple knockout of the EF1a gene, which encodes translation elongation factor 1A. Arabidopsis contains four EF1a paralogues, and single-gene mutants did not exhibit an immune phenotype (data not shown). While EF1a is best known for its role in translation elongation, it has also been reported to interact with the cytoskeleton and participate in signal transduction pathways (Liu et al., 2016). In addition, EF1a has been implicated in abiotic stress responses, including heat and salt tolerance in Arabidopsis and drought tolerance in rice (Li et al., 2024; Xu et al., 2023; Gu et al., 2023). Its role in mediating immune responses in plants is not confirmed, with only one paper suggesting its importance in suppressing PAMP-triggered immunity in rice, by sequestering a *M. oryzae*-derived PAMP protein Ebg1 (Liu et al., 2023). Interestingly, in mammals, EF1a has been shown to inhibit infection with a fish rhabdovirus by promoting the ubiquitin-proteasome degradation of a matrix protein, the activity relying on the C-terminus of EF1a (Meng et al., 2023). The identified ubiquitination site is located in a loop, within 150 bp from the C-terminus, and structurally close to the C-terminus of the protein (Figure S6). Moreover, another ubiquitination screen in Arabidopsis identified another three ubiquitination sites upstream (K438, K441, K449) (Saracco et al., 2009). This together suggests, that EF1a can be involved in immune-mediated ubiquitination.

Other identified proteins are also potential interesting immune regulators. BD1 is an uncharacterised bromodomain-containing protein, likely functioning as a reader of the histone lysine acetylation mark (Bardani et al., 2023). Next, CAT2 is a catalase enzyme involved in ROS detoxification (Mhamdi et al., 2010). Lastly, CAM5 is a calmodulin protein functioning as a calcium sensor and mediating calcium signalling (Ranty et al, 2006). Members of families of all of these proteins were previously associated with immunity and have been shown to be regulated by ubiquitination in different plant systems (Bardani et al., 2023; Saberi Riseh et al., 2024; You et al., 2022; Cheval et al., 2013; Zhang et al., 2014). My findings demonstrate that these targets are ubiquitinated and enriched in barley plants infected with *P. hordei*, and that Arabidopsis mutants of the corresponding homologous genes display altered immune phenotypes.

From the dataset, I also selected several E3 ubiquitin ligases. These enzymes are key regulators of ubiquitin-mediated processes, as they confer substrate specificity by selectively recognising and ubiquitinating target proteins. They can also themselves be ubiquitinated and their activity is highly controlled (de Bie & Ciechanover, 2011). It is not surprising therefore, that I found some of them ubiquitinated in the proteomics data (Table 5.2) and others highly enriched (Table 5.3). Previous studies have shown that members of the ATL and PUB E3 ligase families are specifically involved in mediating plant responses to both biotic and abiotic stresses in different plant species (Su et al., 2024). Interestingly, I did not detect any ATL family proteins as enriched in the dataset. This absence could be due to several technical or biological reasons: their protein levels may be below the detection threshold of mass spectrometry; their peptides may ionize poorly ("do not fly well"); they may not have been efficiently extracted due to limitations of the buffer composition, which lacked sufficient detergents and salts required for solubilising trans-membrane proteins; or they may simply not be involved in the immune response to *P. hordei* in barley. I did however identify PUB44, which was highly enriched in Day 1 dataset. Interestingly, Arabidopsis PUB44, also known as SAUL1 (Senescence-associated ligase 1) is a positive regulator of PAMP-triggered immunity and its homeostasis is regulated by an NLR receptor SOC3 (Tong et al., 2017).

In this study, I also identified CUL1 (and its adaptor protein SKP1) and multiple F-box proteins, that can form potential SCF complexes and are essential regulators of plant responses, including hormone signalling (Trujillo and Shiras, 2020; Marino et al., 2012). UPL1 was also found to be enriched throughout the infection, highlighting the role of proteasome-mediated degradation in mediating immune responses in barley (Furniss et al., 2018). In fact, UPL1 was indeed a particularly interesting E3 ligase candidate; however, I decided not to generate a synthetic version (SynUPL1) due to its large protein size. Although removing certain domains to reduce its size was considered, this approach risked compromising its function, regulatory properties, or interaction with target proteins.

E3 ligases that attracted my attention were RGLG1 and RGLG2 as they were two of the most highly enriched E3 ligases in my datasets. In Arabidopsis, there are five RGLG proteins, and four of them have been reported to affect hormone signalling. RGLG1 was shown to control abscisic acid signalling by controlling the turnover of

phosphatase PPCA (Wu et al., 2016), while the *rglg1/rglg2* double mutant exhibits misregulation in auxin and cytokinin signalling (Yin et al., 2007). Additionally, RGLG3 and RGLG4 were reported to be essential for jasmonate-mediated responses (Zhang et al., 2012). Moreover, RGLG2 has been identified as an E3 ligase with an ability to mediate K63-linked chain formation, by association with K63-specific MMZ2/UBC35 E2 conjugating enzyme (Yin et al., 2007). K63-linked ubiquitination is a non-degradative type of ubiquitination which has been shown to regulate DNA replication and repair, protein synthesis and trafficking, and immune signalling (Zhou and Zeng, 2017). This is highly interesting in the context of my barley immune ubiquitome study, which revealed enrichment of these processes during *P. hordei* infection (Figure 4.10). Previously, RGLG2 has been associated only with abiotic responses, by negatively regulating drought response, but not immune responses in Arabidopsis (Cheng et al., 2011). Recently however, RGLG1/2 was shown to positively regulate immune signalling by preventing PUB25-mediated ubiquitination and subsequent proteasomal degradation of BOTRYTIS-INDUCED KINASE 1 (BIK1) (Bai et al., 2023); another RGLG protein, rice OsRGLG5, has been shown to confer basal resistance against *M. oryzae*, by targeting the AvrPi9 effector for degradation (Liu et al., 2023). All this, makes RGLG2 an interesting protein with a likely role in mediating immune signalling.

As the interaction between the E3 ligase and its targets is transient, I designed a synthetic construct utilising tandem ubiquitin binding entities, to enable us to capture ubiquitinated proteins (Watanabe et al., 2020). I was concerned that this approach could lead to false positives, as UBIQUILIN may bind not only to direct substrates of SynRGLG2, but also to ubiquitinated proteins that are merely in close proximity, or part of the same complex. To address this, I included EDTA as a chelating agent to disrupt the activity of the RING domain of RGLG2, which depends on zinc ions for structural integrity and catalytic function. In the presence of EDTA, I observed a significant reduction in the number of trapped proteins, supporting the specificity of the substrate identification strategy (Figure 5.3B). Interestingly, I was able to trap proteins from both healthy and infected barley leaf extracts, suggesting broad functions of RGLG2 in regulating different responses (Figure 5.3C).

The proteomics analysis yielded 73 proteins which were enriched in the target trapping pulldown from infected barley tissue. Among the identified proteins, there were many associated with immune signalling, which supported myhypothesis that RGLG2 could

have an immune function in barley. Presence of multiple PR proteins, as well as heat shock proteins and PALs suggested roles in immune regulation, protein homeostasis and hormone signalling. Interestingly, I also identified UBC4, which suggests that RGLG2 could mediate K48-linked ubiquitination. Unfortunately, I did not identify MMZ2 or UBC35 to confirm its role in mediating K63-linked ubiquitination.

We found peptides for multiple members of the PAL and HSP70 families in my target trapping data, suggesting RGLG2 may ubiquitinate several members within the same family. The *in vitro* ubiquitination data confirmed that they can indeed be ubiquitinated by SynRGLG2. Results for HSP70-2 were less striking, with only a slight increase in the band intensity without a clear ubiquitination smear (Figure 5.5A). Interestingly, I observed two very faint bands in the control lanes, which could indicate phosphorylation. Additionally, the T70A mutant version of the protein showed a fainter upper band, suggesting lower phosphorylation. This would be consistent with the fact that threonine can also be phosphorylated, and brings a hypothesis that phosphorylation of HSP70-2 T70 could block the availability of the site for ubiquitination. HSP70 proteins can in fact be phosphorylated (Berka et al., 2022).

Ubiquitination of PAL1 showed a clearer result, with a stronger ubiquitination smear and an interesting high molecular weight band (Figure 5.5B). The explanation of this phenomenon is not straightforward and could be dependent on multiple factors. For example it could simply be a result of poly- or mono- ubiquitination on multiple residues, and performing a shorter assay could help identify if this was the case. As mutation in the ubiquitination site did not seem to affect the ability of SynRGLG2 to ubiquitinate PAL1 it is likely that the protein contains more ubiquitination sites.

Altogether, this study predicted and characterised multiple immune proteins that were not previously described as having immune roles in barley. Additionally, I propose that HvRGLG2 is an immune E3 ligase with a defined substrate specificity. These data can be used further to decipher the roles of noted proteins and the role ubiquitination plays in their regulation.

Chapter 6

General Discussion

Pathogens pose a significant threat to global food security, a challenge that is being further exacerbated by climate change and the demands of a rapidly growing world population. At the same time, there is increasing societal and industrial pressure to develop sustainable disease management strategies and to enhance pathogen resistance in economically important crops. Barley is Scotland's most valuable crop and one of the world's most important cereals. Its production is threatened by a wide range of pathogens, among which the emerging biotrophic fungus *Puccinia hordei* remains one of the least studied in the UK. This study was therefore designed to investigate strategies to enhance barley resistance to *P. hordei*, with relevance for both organic and conventional farming systems. In particular, I focused on two complementary approaches: leveraging symbiotic associations to influence host immunity and dissecting the role of the plant ubiquitin system in regulating immune responses.

Accordingly, in Chapter 3 I demonstrated that association with arbuscular mycorrhizal fungi (AMF) can prime barley immune responses against *P. hordei*, although this priming was not sufficient to significantly reduce disease burden. Intriguingly, my findings also indicated that the plant ubiquitin system plays a role in mediating both symbiosis and immune responses, as evidenced by changes in the transcriptional activity of ubiquitination-related enzymes and global ubiquitination levels. Building on this, in Chapter 4 I investigated the barley immune ubiquitome under immune elicitation and pathogen challenge, identifying ubiquitin-regulated pathways involved in immune activation. Finally, in Chapter 5 I characterised several immune-related proteins and E3 ligases uncovered in the ubiquitome dataset, highlighting their potential roles in mediating barley immune responses and their promise as targets for improving resistance.

6.1 Using omics approaches to dissect plant-microbe interactions

Plants adapt to environmental changes by modulating gene expression and post-translational modifications, which together fine-tune metabolic pathways and the accumulation of secondary metabolites. These regulatory layers are particularly complex in plant–microbe interactions, where both host and pathogen manipulate cellular processes to their advantage. Dissecting such multilayered responses requires integrative approaches. Recent advances in omics technologies have

provided the tools necessary to capture these dynamics at scale and resolution. In this study, I combined transcriptomics and proteomics to explore these processes in the context of barley immunity.

In Chapter 3, together with collaborators we designed a tripartite experiment to investigate the interaction between AMF and barley in the presence and absence of a foliar fungal pathogen, applying a transcriptomic approach. While transcriptomics has previously been used to study AMF symbiosis—such as characterising its developmental stages (Serrano et al., 2024) or uncovering the molecular mechanisms of AMF interactions in diverse plant species (Ma et al., 2024; Velásquez et al., 2025), our study is, to my knowledge, the first to apply this method to barley–AMF interactions in the context of foliar pathogen challenge. I showed that AMF alone induces expression of defence response genes (Figure 3.5), specifically calcium oscillators and WRKY transcription factors (Figure 3.7), which likely regulate protein phosphorylation to prime immune-responsive genes. These findings are consistent with studies in other plant systems (Puccio et al., 2023), and highlight the extent of transcriptional changes induced by AMF root colonisation in barley leaves. Importantly, I demonstrated that AMF primes salicylic acid (SA)-responsive genes, enhancing the amplitude of their induction during *P. hordei* infection (Figure 3.3). AMF also induced expression of genes associated with SA and jasmonic acid (JA) metabolism and defence responses during infection (Figures 3.5B and 3.6B), while at the same time limiting the scale of transcriptional reprogramming compared with non-mycorrhizal plants (Figure 3.6A). Conversely, AMF downregulated genes linked to protein phosphorylation, cell surface receptor signalling, and broad responses to other organisms. These results support the hypothesis that AMF prime barley against *P. hordei* and provide a valuable resource for identifying the molecular mechanisms underlying AMF-induced transcriptional regulation. However, my analysis was limited to a single time point, providing only a snapshot of the AMF–barley–*P. hordei* transcriptome. This constraint makes it difficult to capture the dynamics of early immune responses, which could in part explain the absence of a clear immune phenotype at the whole-plant level. Future work should incorporate a time-course transcriptomic analysis spanning early and later stages of infection to capture the temporal dynamics of AMF-mediated immune priming and host responses to *P. hordei*. This could be complemented by tissue-specific sampling and phenotypic

measurements, such as fungal biomass and plant grain development, to better link transcriptional changes with local and systemic immune outcomes.

In Chapter 3 I identified that infection with *P. hordei* results in an increase in global ubiquitination and induces expression of many ubiquitination genes (Figure 3.9). Building on this, in Chapter 4 I applied a ubiquitin-enriched proteomics approach to identify the biological processes and specific proteins regulated by ubiquitination during *P. hordei* infection. This strategy enabled us to establish complex networks of ubiquitin regulation under different immune conditions. A comparison between the SA- and JA-induced ubiquitome revealed the specificity in ubiquitin regulation in terms of activation of the immune responses, and highlighted processes uniformly regulated by both hormones (Figure 4.2). This comparison contributed to a broader understanding of plant immune systems by showing that ubiquitin-mediated regulation forms part of both hormone-specific and shared defence networks, thereby supporting a more nuanced view of SA–JA crosstalk in plants, including the less strictly antagonistic interactions often observed in monocots. Interestingly, by integrating proteomic and transcriptomic approaches, I found that only a small portion of ubiquitin-mediated regulation is reflected in the transcriptome, and vice versa, and the protein-gene behaviour often follows the same trend (Figure 4.7). This is perhaps not unexpected, given that ubiquitination primarily acts at the post-translational level by altering protein stability, turnover, localisation, or activity, rather than directly affecting transcript abundance. Conversely, many transcriptional changes were not mirrored at the protein level, likely reflecting the additional influence of translational regulation and other proteostatic mechanisms. Nevertheless, in cases where both transcript and protein changes were observed, these frequently followed the same directional trend, suggesting coordinated regulation of specific pathways at multiple molecular levels. Collectively, this highlights the importance of combining transcriptomic and proteomic approaches to obtain a more complete view of regulatory processes.

In Chapter 4, I performed ubiquitome profiling at three distinct infection stages, which enabled us to identify biological processes critical to different phases of *P. hordei* infection. Interestingly, unlike the transcriptome data from Chapter 3, where protein phosphorylation emerged as a major process associated with infection-induced genes, the *P. hordei* ubiquitome did not show enrichment for phosphorylation-related processes (Figure 4.4). These processes were only detected in the higher-resolution

immune ubiquitome dataset (Figure 4.10). Incorporating phosphoproteomics in future studies would therefore provide deeper insights into the regulatory cross-talk between ubiquitination and phosphorylation and their roles in shaping immune responses in barley. This would be particularly interesting in the context of non-canonical ubiquitination, such as the regulation of quality control proteins as HvHSP70-2, and my hypothesis that ubiquitination may compete with phosphorylation as a regulatory mechanism.

Finally, I showed that a proteomics approach can be used to identify ubiquitination sites on proteins with diverse functions and abundances. By establishing an immune ubiquitome in barley across different immune elicitation regimes and stages of pathogen infection, this work also demonstrates the feasibility and value of applying mass spectrometry-based ubiquitin proteomics to non-model species. In doing so, it expands the scope of PTM-focused proteomics beyond well-characterised model systems and provides a framework for investigating dynamic immune regulation in crops with more limited molecular resources. Here, we must highlight the importance of designing an appropriate LC-MS/MS data acquisition workflow to maximise the discovery rate. Given that ubiquitinated peptides are often low in abundance and technically challenging to detect, methodological choices can strongly affect site coverage, reproducibility, and confidence of identification. Even with the same sample preparation technique, the results I obtained varied significantly between datasets, according to the processing methodologies. Therefore, the ubiquitinated peptide data were not interpreted quantitatively in terms of protein abundance, nor were direct comparisons made between treatments and datasets. Rather, they were used as a resource for the identification of ubiquitination sites in barley.

6.2 Ubiquitination regulates barley immune responses

Ubiquitination is one of the most conserved and ubiquitous mechanisms regulating biological processes in eukaryotic cells. Consisting of thousands of proteins, it controls plant's health, reproduction, growth and responses to the changing environment. All of these processes can impact agronomic traits (Linden and Callis, 2020, Theodoulou et al., 2022). By understanding the molecular basis of ubiquitin-mediated regulation, we can develop crops with improved resilience to biotic stresses. In this study, I demonstrated that ubiquitination plays a central role in regulating barley responses to

both symbiotic and pathogenic fungi. In Chapter 3, I showed that while AMF colonisation reduces global ubiquitination levels, *Puccinia hordei* infection induces them, likely reflecting reduced versus heightened cellular stress, respectively (Figure 3.9A).

Immune-related E3 ligases provide critical insight into the dynamics of the immune-associated ubiquitome. They are themselves embedded within the immune-associated ubiquitome, where they may act as dynamic regulatory nodes that help fine-tune the strength, timing, and duration of defence responses. This is why these enzymes are frequently subject to auto- or self-ubiquitination, and were therefore co-purified along with their substrates in the ubiquitin proteomics pipeline. Among them, HvRGLG2 stood out as one of the E3 ligases strongly induced during *P. hordei* infection and was highly enriched in Day 7 ubiquitome dataset (Table 5.2). Functional analyses demonstrated that HvRGLG2 acts as an immune E3 ligase in barley, directly ubiquitinating two key immune proteins, HvHSP70-2 and HvPAL1 (Figure 5.5). This places HvRGLG2 at the intersection of two major immune pathways—the protein quality control pathway and the phenylpropanoid pathway (Figure 4.11). Supported by previous reports implicating RGLG ligases in stress responses, these findings establish HvRGLG2 as a promising molecular target for improving resistance in barley. Future work should focus on further characterisation of the direct substrates of HvRGLG2 and defining its broader role within immune signalling pathways, followed by targeted genetic manipulation in barley to assess its potential for enhancing disease resistance while minimising associated trade-offs with growth. Ubiquitin is directly involved in regulating immune responses by controlling the turnover of immune receptors or mediating the hormone signalling crosstalk (Gao et al., 2022). More generally, however, it is also involved in the transcriptional regulation, protein quality control and protein transport within the cell – processes important during the transition from growth to defence state (Gao et al., 2022). The barley ubiquitome profiling provided insight into how these processes are regulated by ubiquitin under different immune triggers. My data suggested that SA triggers ubiquitin regulation of a wider range of biological processes than JA (Figure 4. 2). This could be explained by the nature of these hormones in protecting against types of pathogens with very different lifestyles (Mengiste & Liao, 2025). I also showed that the plant ubiquitome is dynamically remodelled throughout *P. hordei* infection (Figure 4.4). It therefore

highlights the ubiquitome as an integral and temporally responsive layer of immune regulation, likely involved in coordinating stage-specific defence processes as infection progresses.

Altogether, ubiquitin was found to highly regulate not only immune-specific pathways, but also processes associated with primary metabolism, protein processing and transport, and gene expression (Figure 4.10). It was surprising to see that even during pathogen infection, the most prominently ubiquitinated proteins are metabolite interconversion enzymes (Figure 4.9). This could be due to the bias of the MS machine for the most abundant proteins, and using a data independent approach (DIA) could improve this. In this study, I opted for a data-dependent approach (DDA), for the identification of ubiquitination sites, due to the lack of advanced PTM detection tools for DIA analysis in barley. However, studies suggest that activity of primary metabolite enzymes modulates signalling transduction cascades and actively promotes defence responses (Rojas et al., 2014).

6.3 Identifying ways of improving barley resistance against *Puccinia hordei*

Puccinia hordei is one of the main pathogens of barley, and an emerging pathogen in the UK. Considering that most commonly grown barley cultivars are highly susceptible and the lack of known resistance genes conferring long-term protection against this pathogen, we need to identify ways to reduce brown rust burden on barley crops.

In Chapter 3, I hypothesised that association with AMF can protect barley against *P. hordei* infection. Even though AMF has been used successfully in the past to induce resistance against a variety of pathogens, we observed no decrease in the disease burden in plants experiencing symbiosis with *Rhizophagus irregularis* (Figure 3.2B). This outcome, although unfavourable, is not definitive, as many factors such as nutrient availability, AMF species, plant genotype or infection timeline can affect successful colonisation. For example, a study in wheat showed that all the above affect the efficacy of mycorrhizal inoculation against a different biotrophic pathogen, wheat powdery mildew, and an optimised combination can result in resistance (Mustafa et al., 2016). Additionally, as I saw promising activation of MIR in inoculated plants, further studies exploring different AMF strains and barley cultivars are needed to explore this approach.

Our barley ubiquitome data provided a useful mining platform for pathways and proteins that function during barley immune response. This helps to expand current knowledge of immune-associated ubiquitin regulation beyond model species, showing that complex ubiquitin-mediated defence processes are conserved and biologically significant in crop plants. Additionally, I identified immune-related ubiquitinated proteins which might be important in mediating immune responses. Targeting these pathways for resistance breeding could prove beneficial for improving barley defence responses. I also identified day 4 as crucial for the transition between early and late stages of *P. hordei* infection, with strong evidence of pathogen-induced immune suppression at this stage, which also targets the plant ubiquitin system. Identifying ways to improve barley defence mechanisms prior to this stage could be significant in inducing *P. hordei* resistance in barley.

I showed that three of the ubiquitinated proteins found in the barley ubiquitome, EF1a, CAM5 and CBD1, function as positive regulators of immunity in Arabidopsis (Figure 5.1) and identified HvRGLG2 as an E3 ligase that targets multiple immune substrates (Figure 5.4). This is very exciting, as these proteins were not previously associated with immunity in barley. Additionally, this study is the first to report HvRGLG2 as an immune E3 ligase and show that it can ubiquitinate both HvHSP70-2 and HvPAL1 (Figure 5.5). Supplementary *in planta* experiments, such as co-immunoprecipitation from barley plants, are needed to confirm these results and extend them to the other identified targets. This study can be taken further by investigating the role of ubiquitination sites in mediating protein activity and deciphering the molecular function of identified immune candidates. Ultimately, an approach utilising engineered E3 ligases can be developed, where modifying ubiquitination status of substrate proteins triggers an altered immune response in the plant.

6.4 Conclusions and impact

The work in this thesis establishes the barley immune ubiquitome, identifies ubiquitin-regulated immune proteins, and explores strategies for improving crop health. The data have shown that ubiquitination regulates various aspects of plant-microbe interactions. By investigating the effect of different immune contexts on ubiquitin-mediated regulation, I conclude that the plant ubiquitome is highly dynamic and adjustable. As ubiquitination is one of the most conserved post-translational

modifications, this work can likely be translated to other plant systems and provide a practical insight into improving agronomic traits in crops.

As demonstrated in this study, omics approaches hold great potential to revolutionise our understanding of plant–microbe interactions. Integrating multiple omics layers provides powerful insights into transcriptional and translational regulation, yet it remains technically challenging. In particular, this work demonstrates that mass spectrometry-based proteomics can be successfully applied in a non-model crop species to interrogate dynamic post-translational regulatory networks, despite the additional challenges posed by more limited genomic and proteomic resources. By establishing these approaches in barley, this study helps expand the scope of mass spectrometry beyond model systems and provides a framework for applying PTM-focused proteomics to other non-model organisms. Continued development of multi-omics workflows, data integration strategies, and higher-resolution datasets will be critical to advance these approaches as platforms for protein function discovery.

Although this thesis does not propose definitive strategies for improving barley resistance to *P. hordei*, it identifies several promising avenues toward this goal and establishes a valuable resource for the broader research community. I hope that these findings will contribute to the development of effective and sustainable disease management strategies, ultimately strengthening crop resilience and health.

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Appendix

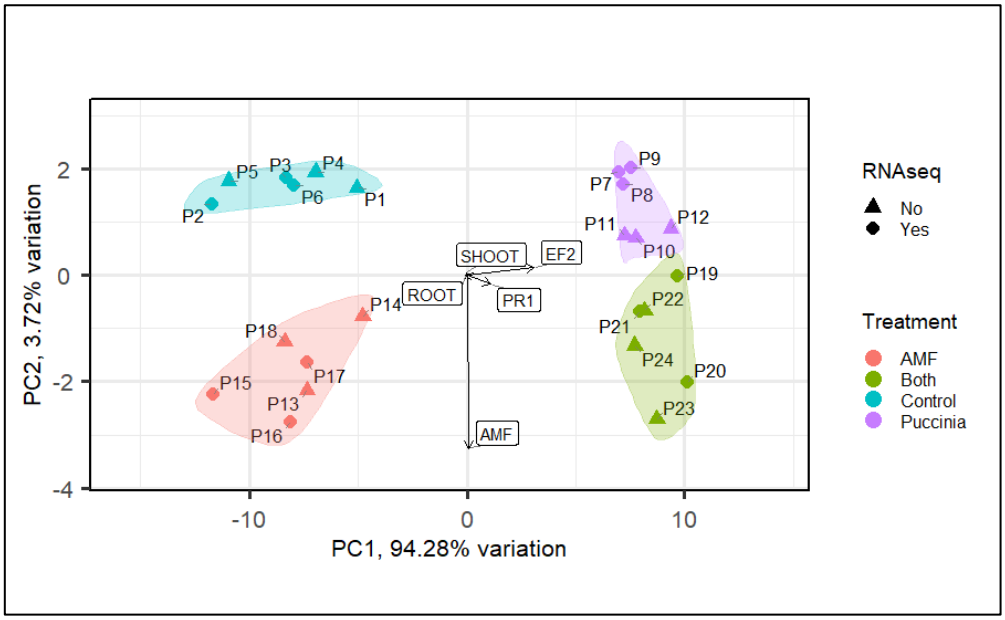

Figure S1. PCA plot showing sample clustering for each treatment based on measured characteristics: root and shoot dry mass, PR1 expression, AMF copy number, *P. hordei* load (EF2 expression).

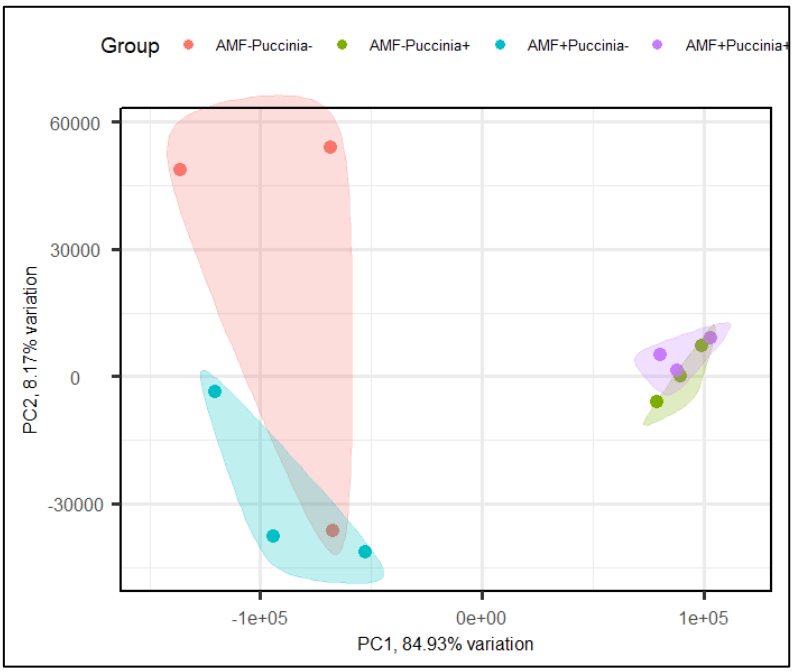

Figure S2: PCA plot showing sample clustering for each treatment based on the transcriptome profile.

Table S1: Annotation of WRKY transcription factors

Protein name	Barley gene ID	Arabidopsis orthologue	Rice orthologue	Role	Reference
HvWRKY1/38	HORVU.MOREX.r3.6HG0568570	WRKY40/60/18	WRKY71	Biotic	Xu et al., 2006
HvWRKY10	HORVU.MOREX.r3.2HG0158690	WRKY17/11	WRKY42	Biotic	Han et al., 2014
HvWRKY105	HORVU.MOREX.r3.2HG0111890	WRKY54/70	WRKY47	Biotic	Chen et al., 2021; Wei et al., 2013
HvWRKY106	HORVU.MOREX.r3.2HG0111920	WRKY54/70	WRKY47	Biotic	Chen et al., 2021; Wei et al., 2013
HvWRKY12	HORVU.MOREX.r3.2HG0096750	WRKY45	WRKY72	Biotic	Zhu et al., 2025
HvWRKY15	HORVU.MOREX.r3.5HG0511790	WRKY57	WRKY3	Biotic	Jiang et al., 2016; Liu et al., 2005
HvWRKY17	HORVU.MOREX.r3.1HG0029360	WRKY51	WRKY10	Biotic	Gao et al., 2011; Wang et al., 2023
HvWRKY19	HORVU.MOREX.r3.1HG0080920	WRKY51	WRKY7	Biotic	Gao et al., 2011; Son et al., 2024
HvWRKY2	HORVU.MOREX.r3.7HG0743270	WRKY40/60/18	WRKY28	Biotic	Xu et al., 2006
HvWRKY20	HORVU.MOREX.r3.1HG0080940	WRKY51	WRKY7	Biotic	Gao et al., 2011; Son et al., 2024
HvWRKY21	HORVU.MOREX.r3.3HG0276810	WRKY46	WRKY61	Biotic	Berri et al., 2009
HvWRKY22	HORVU.MOREX.r3.5HG0474120	NA	NA	NA	
HvWRKY23	HORVU.MOREX.r3.7HG0743280	WRKY40/60/18	WRKY28	Biotic	Xu et al., 2006
HvWRKY26	HORVU.MOREX.r3.1HG0090520	WRKY46	WRKY61	Abiotic	Wang et al., 2024; Ding et al., 2014
HvWRKY27	HORVU.MOREX.r3.1HG0090460	WRKY46	WRKY61	Abiotic	Wang et al., 2024; Ding et al., 2014

HvWRKY3	HORVU.MOREX.r3.5HG0484070	WRKY40/60/18	WRKY76	Biotic	Xu et al., 2006
HvWRKY30	HORVU.MOREX.r3.2HG0123480	WRKY46	WRKY69	Abiotic	Ding et al., 2014
HvWRKY31	HORVU.MOREX.r3.1HG0070890	NA	NA	NA	
HvWRKY32	HORVU.MOREX.r3.2HG0200560	WRKY54/70	WRKY45	Biotic	Chen et al., 2021; Huangfu et al., 2016
HvWRKY33	HORVU.MOREX.r3.4HG0380330	NA	WRKY55	Abiotic	Huang et al., 2021
HvWRKY37	HORVU.MOREX.r3.3HG0237360	WRKY61/36/72	WRKY107	Biotic	Gao et al., 2016; Bhattarai et al., 2010
HvWRKY4	HORVU.MOREX.r3.2HG0111920	WRKY54/70	WRKY47	Biotic	Chen et al., 2021; Wei et al., 2013
HvWRKY42	HORVU.MOREX.r3.5HG0489860	WRKY34/2	WRKY90	Biotic	Dangol et al., 2021
HvWRKY43	HORVU.MOREX.r3.1HG0071500	WRKY25/26	WRKY70	Biotic	Li et al., 2024
HvWRKY45	HORVU.MOREX.r3.3HG0286660	WRKY65	WRKY14	NA	
HvWRKY47	HORVU.MOREX.r3.3HG0251900	WRKY6	WRKY1	Abiotic	Chen et al., 2009
HvWRKY48	HORVU.MOREX.r3.4HG0333220	NA	WRKY60	NA	
HvWRKY49/72	HORVU.MOREX.r3.3HG0297530	NA	WRKY108	Abiotic	Wang et al., 2021
HvWRKY50	HORVU.MOREX.r3.3HG0304380	WRKY25/26	WRKY24	Biotic	Yokotani et al., 2018
HvWRKY51	HORVU.MOREX.r3.3HG0278140	WRKY71/28	WRKY16	Biotic	van Verk et al., 2011
HvWRKY52	HORVU.MOREX.r3.5HG0484060	WRKY40/60/18	WRKY76	Biotic	Xu et al., 2006
HvWRKY55/87	HORVU.MOREX.r3.1HG0090460	WRKY46	WRKY61	Abiotic	Wang et al., 2024; Ding et al., 2014

HvWRKY56	HORVU.MOREX.r3.1HG0092420	NA	WRKY8	NA	
HvWRKY58	HORVU.MOREX.r3.4HG0340900	WRKY21/39/74	NA	NA	
HvWRKY60	HORVU.MOREX.r3.7HG0650260	WRKY61/36/72	WRKY73	NA	
HvWRKY63	HORVU.MOREX.r3.3HG0237200	WRKY51	WRKY10	Biotic	Gao et al., 2011; Wang et al., 2023
HvWRKY64	HORVU.MOREX.r3.3HG0273500	WRKY27	WRKY12	Biotic	Mukhtar et al., 2008
HvWRKY69	HORVU.MOREX.r3.1HG0088760	WRKY71/28	WRKY16	Biotic	van Verk et al., 2011
HvWRKY7	HORVU.MOREX.r3.2HG0192860	NA	WRKY68	Biotic	Shuo et al., 2016
HvWRKY92	HORVU.MOREX.r3.6HG0551140	WRKY55	WRKY75	NA	
HvWRKY96	HORVU.MOREX.r3.2HG0192860	NA	WRKY68	Biotic	Shuo et al., 2016
HvWRKY97	HORVU.MOREX.r3.2HG0192860	NA	WRKY69	NA	
HvWRKY98	HORVU.MOREX.r3.2HG0192860	NA	WRKY70	NA	
HvWRKY99	HORVU.MOREX.r3.4HG0378680	WRKY54/70	WRKY79	Biotic	Chen et al., 2021; Berri et al., 2009

Table S2: Annotation of calcium dependent protein kinase (CDPK) and CDPK-like genes based on the closest Arabidopsis homologue.

Protein name	Barley gene ID
CPK1	HORVU.MOREX.r3.5HG0450250
CPK1-2	HORVU.MOREX.r3.5HG0517140
CPK10	HORVU.MOREX.r3.4HG0334900
CPK13	HORVU.MOREX.r3.1HG0072410
CPK13-2	HORVU.MOREX.r3.3HG0303750
CPK28	HORVU.MOREX.r3.6HG0551960
CPK29	HORVU.MOREX.r3.2HG0188200
CPK3	HORVU.MOREX.r3.1HG0092880
CPK4	HORVU.MOREX.r3.4HG0349000
CPK5	HORVU.MOREX.r3.2HG0196120
CPK5-2	HORVU.MOREX.r3.6HG0604810
CPK6	HORVU.MOREX.r3.4HG0410700
CPK7	HORVU.MOREX.r3.2HG0133550
CPK7-2	HORVU.MOREX.r3.5HG0523860
CRK3-2a	HORVU.MOREX.r3.1HG0049840
CRK3-2b	HORVU.MOREX.r3.7HG0721290
CRK5-2	HORVU.MOREX.r3.2HG0124380

Table S3: Annotation of ubiquitination genes based on the closest Arabidopsis homologue.

Protein name	Barley gene ID
AIP2	HORVU.MOREX.r3.5HG0481290
ARI2	HORVU.MOREX.r3.4HG0341330
ATG3	HORVU.MOREX.r3.1HG0058170
ATG7	HORVU.MOREX.r3.3HG0271550
ATL-1	HORVU.MOREX.r3.7HG0664700
ATL-2	HORVU.MOREX.r3.3HG0233040
ATL-3	HORVU.MOREX.r3.3HG0245590
ATL-4	HORVU.MOREX.r3.5HG0487980
ATL-5	HORVU.MOREX.r3.6HG0560520
ATL-6	HORVU.MOREX.r3.2HG0198510
ATL-7	HORVU.MOREX.r3.4HG0352590
ATL2	HORVU.MOREX.r3.6HG0547990
ATL3	HORVU.MOREX.r3.5HG0511930
ATL31	HORVU.MOREX.r3.6HG0616830
ATL32	HORVU.MOREX.r3.7HG0709440
ATL4	HORVU.MOREX.r3.6HG0605120
ATL43	HORVU.MOREX.r3.1HG0052980
ATL44	HORVU.MOREX.r3.5HG0504280
ATL5	HORVU.MOREX.r3.3HG0233030

ATL52	HORVU.MOREX.r3.7HG0736710
ATL54	HORVU.MOREX.r3.2HG0172180
ATL57	HORVU.MOREX.r3.3HG0245610
ATL6-1	HORVU.MOREX.r3.1HG0057210
ATL6-2	HORVU.MOREX.r3.7HG0660720
ATL6-3	HORVU.MOREX.r3.3HG0303880
ATL60	HORVU.MOREX.r3.7HG0719250
ATL66	HORVU.MOREX.r3.1HG0071970
ATL74	HORVU.MOREX.r3.3HG0297680
ATL8	HORVU.MOREX.r3.2HG0196250
ATL9	HORVU.MOREX.r3.7HG0709620
BB	HORVU.MOREX.r3.2HG0187130
RBR-1	HORVU.MOREX.r3.2HG0211990
RBR-2	HORVU.MOREX.r3.5HG0481120
BRIZ1	HORVU.MOREX.r3.2HG0204960
RDUF2	HORVU.MOREX.r3.1HG0004730
ELC	HORVU.MOREX.r3.5HG0504530
ELCL	HORVU.MOREX.r3.5HG0523850
MBR2	HORVU.MOREX.r3.3HG0235900
MPSR1	HORVU.MOREX.r3.2HG0122190
MUF9	HORVU.MOREX.r3.2HG0100390
RGLG1	HORVU.MOREX.r3.7HG0720740

RGLG2	HORVU.MOREX.r3.3HG0308290
RHA1B	HORVU.MOREX.r3.7HG0709350
RHC1A	HORVU.MOREX.r3.3HG0312030
RHC2A	HORVU.MOREX.r3.4HG0388680
RHF2A	HORVU.MOREX.r3.2HG0150530
RMA	HORVU.MOREX.r3.2HG0183830
RMA3	HORVU.MOREX.r3.5HG0430240
UBA1	HORVU.MOREX.r3.5HG0465550
UBC26	HORVU.MOREX.r3.3HG0279940
UBC28	HORVU.MOREX.r3.7HG0710360

Table S4: Annotation of ubiquitination genes

A0A287EDT2	RK*DT*MDEMYYGVHEQLLYARK
A0A287EVG1	KLT*IIEEQRK*RD
A0A287EZH9	KMETDVT*DVNMQEPKG
A0A287F8M8	RAK*WAGTPLLLEAS*ELLSK*E
A0A287F8M8	RAKWAGT*PLLLEAS*ELLS*KE
A0A287FA93	RRS*RGIAVDPGAQS*ARA
A0A287FL70	RT*PASVAAQPSPLPRR
A0A287FL70	RTPAS*VAAQPSPLPRR
A0A287G8M8	KFVAS*AVNKAGLAKL
A0A287GLB5; A0A287GLA0; A0A287LA57; A0A287RAC0	KIYHPNIDK*LGRI
A0A287GYA5	RDFSSFVRT*T*DRS
A0A287HLU7	RIS*CFGSHDTS*PPRG

A0A287I9V4	RLGVS*CIDLYYQHRV
A0A287IJH6	KVRSGHC*LGAAGGLEAIATIKS
A0A287J1P2	RGLS*TIVILK*PSPARS
A0A287J1P2	RGLS*TIVILKPS*PARS
A0A287J1P2	RGLST*IVILK*PSPARS
A0A287JBF8	KDYDGSS*S*SYRN
A0A287JBF8	KDYDGSS*SS*YRN
A0A287JBF8	KDYDGSSS*S*YRN
A0A287JGL5	RLAVILNQIFS*GKN
A0A287JGQ2	KVQQLLQDFFDGKT*LCKS
A0A287L4J2	REMVS*VVHSC*HS*MGVFHRDLKPENFLFLNNKEDSPLKA
A0A287L4J2	REMVSVVHS*C*HS*MGVFHRDLKPENFLFLNNKEDSPLKA
A0A287L4K9	KGHYLNATVGTC*EEMIKR
A0A287L4K9	KGHYLNATVGTC*EEMIKRV
A0A287L5I7	KKVQT*EC*ASMPFDDQCTVLEKEAVNVSLQNLLTYPFVKE
A0A287L5I7	RVCPS*VTLGLEPGEAFTIRN
A0A287LT36	KQEALASIT*YSYKH
A0A287MB47	KAPQAAGAIHT*DFERG
A0A287NJC7; F2D355	KKT*AVAVAYCKPGRG
A0A287NMQ3	KGK*MCC*LFINDLDAGAGRM
A0A287NMQ3	KK*GKMC*CLFINDLDAGAGRM
A0A287NMQ3	KK*GKMCC*LFINDLDAGAGRM
A0A287NMQ3	KKGK*MC*CLFINDLDAGAGRM
A0A287NMQ3	KS*FQCELVFAKM
A0A287NMQ3	KYDFDNT*MGGFYIAPAFMDKL
A0A287NTK1	KS*KYIIGDGNNYYDFTYVENVAYGHVCADKT
A0A287P2H6	RDSRLIT*SPLK*FPGKL
A0A287P5D4	RALDEINAGVCDGMT*YDEVKKN
A0A287PVR5	KGADIPTYVVGVNEGDYDHDVANIIS*NASC*TTNCLAPFAKV
A0A287PVR5	KGADIPTYVVGVNEGDYDHDVANIIS*NASCTTNC*LAPFAKV
A0A287PVR5	KGADIPTYVVGVNEGDYDHDVANIISNAS*C*TTNCLAPFAKV
A0A287PVR5	KGADIPTYVVGVNEGDYDHDVANIISNAS*CTTNC*LAPFAKV
A0A287PVR5	KGADIPTYVVGVNEGDYDHDVANIISNASC*T*TNCLAPFAKV
A0A287PVR5	KGADIPTYVVGVNEGDYDHDVANIISNASC*TT*NCLAPFAKV

A0A287PVR5	KGADIPTYVVGVNEGDYDHDVANIISNASCT*TNC*LAPFAKV
A0A287PVR5	KGKLNGIALRVPTPNVSVVDLVINT*VKT
A0A287Q768	RGDAGIVEC*S*YIAS*QVT*ELPFFASKV
A0A287QAQ1	RGKAPITQQLPGESDAEYAEFS*SKVLHLK*G
A0A287R6S4	KNMITGTSQADC*VVLIIDSTTGGFEAGISKD
A0A287R6S4	KNMITGTSQADC*VVLIIDSTTGGFEAGISKDGQTRE
A0A287RG91	RNDMAC*GSTIGPILASGVGIRT
A0A287RJZ8; A0A287XUU2; A0A287XMC4; A0A287ECA9; A0A287EXK4; A0A287RRT7	RTLADYNIQKES*TLHLVLRLRG
A0A287RJZ8; A0A287XUU2; A0A287XMC4; A0A287ECA9; A0A287EXK4; A0A287RRT7	RTLADYNIQKEST*LHLVLRLRG
A0A287RJZ8; A0A287XUU2; A0A287XMC4; A0A287ECA9; A0A287EXK4; A0A287VVH9	KT*LTGKTITLEVESSDTIDNVKA
A0A287RJZ8; A0A287XUU2; A0A287XMC4;	KTLT*GKTITLEVESSDTIDNVKA

A0A287ECA9; A0A287EXK4; A0A287VVH9	
A0A287RJZ8; A0A287XUU2; A0A287XMC4; A0A287ECA9; A0A287EXK4; A0A287VVH9	KTLTGK*TITLEVESSDTIDNVKA
A0A287RJZ8; A0A287XUU2; A0A287XMC4; A0A287ECA9; A0A287EXK4; A0A287VVH9	KTLTGKT*ITLEVESSDTIDNVKA
A0A287RRB7; A0A287WFA2	RVVDSPC*CLVT*GEYGWTANMERI
A0A287RXA5	KADIDEIVLVGGS*TRI
A0A287RYL1	KQSKTSFS*IASPKV
A0A287SGM5	RS*IC*EGVAKSCPNAIVNLISNPVNSTVPIAAEVFKRA
A0A287SGM5	RSIC*EGVAK*SCPNAIVNLISNPVNSTVPIAAEVFKRA
A0A287SS50; M0W8S8; F2D884; M0WQY8	KNQVAMNPINT*VFDAKR
A0A287SVA7	RCASRLLGSAAAAPPAS*C*AS*T*EPPRF
A0A287SZW7	KIIHEMYPEFPVPDKC*ADDAPFATIYQVSKD
A0A287T437; A0A287VDK5	KDPNATIIMLAT*GT*GIAPFRS
A0A287T8X9	KFHWRPT*C*GVSCLMDDEATLVGGKN
A0A287T8X9	KFHWRPT*CGVSC*LMDDEATLVGGKN

A0A287T8X9	KFHWRPTC*GVS*CLMDDEATLVGGKN
A0A287T8X9	KFHWRPTCGVS*C*LMDDEATLVGGKN
A0A287TK16	RK*T*VALVGESGSGKS
A0A287U224	KK*AES*LNIS*RDVSGEGVQQALLKM
A0A287U800	KEYKSDIDIVSNASC*T*TNCLAPLAKV
A0A287U800	KEYKSDIDIVSNASC*TT*NCLAPLAKV
A0A287U800	KEYKSDIDIVSNASCTT*NC*LAPLAKV
A0A287U800	KSDIDIVSNAS*C*TTNCLAPLAKV
A0A287U800	KSDIDIVSNAS*CTTNC*LAPLAKV
A0A287U800	KSDIDIVSNASC*T*TNCLAPLAKV
A0A287U800	KSDIDIVSNASC*TT*NCLAPLAKV
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A0A287U800	KSDIDIVSNASCTT*NC*LAPLAKV
A0A287UBM0	RCPVNT*IC*IGQAASMGSLLLAAGARG
A0A287UKC3	RQNS*FS*FT*GSVSEVRHGDDAKY
A0A287UVV6	KIVMYSPAFYAAC*TAGGIAS*CGLTHMAVTPLDLVKC
A0A287V536	RT*PS*RFPAVAQPAPGPGARL
A0A287VDK5	KGVCS*NFLC*DLKPGADVNITGPVGKE
A0A287VKE5	KAQT*RLGSSESSC*PNVNARF
A0A287VKE5	RISMMPVGT*PRV
A0A287VV58	KS*GKLGFEGTQEVQHRI
A0A287WCI1	KLT*ISDITK*QIC*DAVQARA
A0A287WXA1	KDAPMFVVGVNEDKYTSDVNIVSNAS*CTTNC*LAPLAKV
A0A287XUU2 ; A0A287XMC4 ; A0A287ECA9	MQIFVKT*LTGKT
A0A287XZG6	RLT*LNYDGNLRL
F2CS01	KNVISVYET*ET*FSLIEK*K*S
F2D629; A0A287H208; F2CT51	KLPMFGC*TDATQVLNEVEEVKKEYPDAYVRI
F2D629; A0A287H208; F2CT51;	KLPMFGC*TDATQVLNEVEEVKKE

F2D629; A0A287H208; F2CT51; F2D629; A0A287H208; F2CT51	
F2DY59	RSGTEEAETNVNDSSEAVVT*EVVEPSEVRTENENDPWRD
F2E644	KFMVLS*LGTGSAKVEEKF
M0UZW7	RAALAEAGT*VK*Y
M0WNE6; A0A287Q8Z2	RKS*EHAFYLDWAVHSFRI
M0WVV5	KVVVRT*VVALPEKE
M0X0A5	RLDLAGRDLT*DNLMKI
M0XNR5	RVGNPSNYVT*NPGQTYGNRN
M0Y9X7	RGYVASNSKDDPAKEAANFTAQVIIMNHPGQISNGYAPVLDC*HTSHIA VKF
M0Y9X7; A0A287R6S4	KYSC*TVIDAPGHRD
M0Y9X7; A0A287R6S4	KYSC*TVIDAPGHRDFIKN
Q7YMR6	KGHYLNATAGTC*EEMIK*R
Q7YMR6	RAC*YECLRGGLDFT*KDDENVNSQPFMRW
Q7YMR6	RACYEC*LRGGLDFT*KDDENVNSQPFMRW
Q7YMR6	RVSPQPGVPPEEAGAAVAAES*S*TGTWTTVWTDGLTSLDRY
Q7YMR6	RVSPQPGVPPEEAGAAVAAES*ST*GTWTTVWTDGLTSLDRY
Q7YMR6	RVSPQPGVPPEEAGAAVAAESST*GT*WTTVWTDGLTSLDRY
Q7YMR6	RVSPQPGVPPEEAGAAVAAESSTGTWT*T*VWTDGLTSLDRY
S4Z0F2	KGSITS*IQAVYVPADDLTDPAPATTFAHLDATTVLSRG

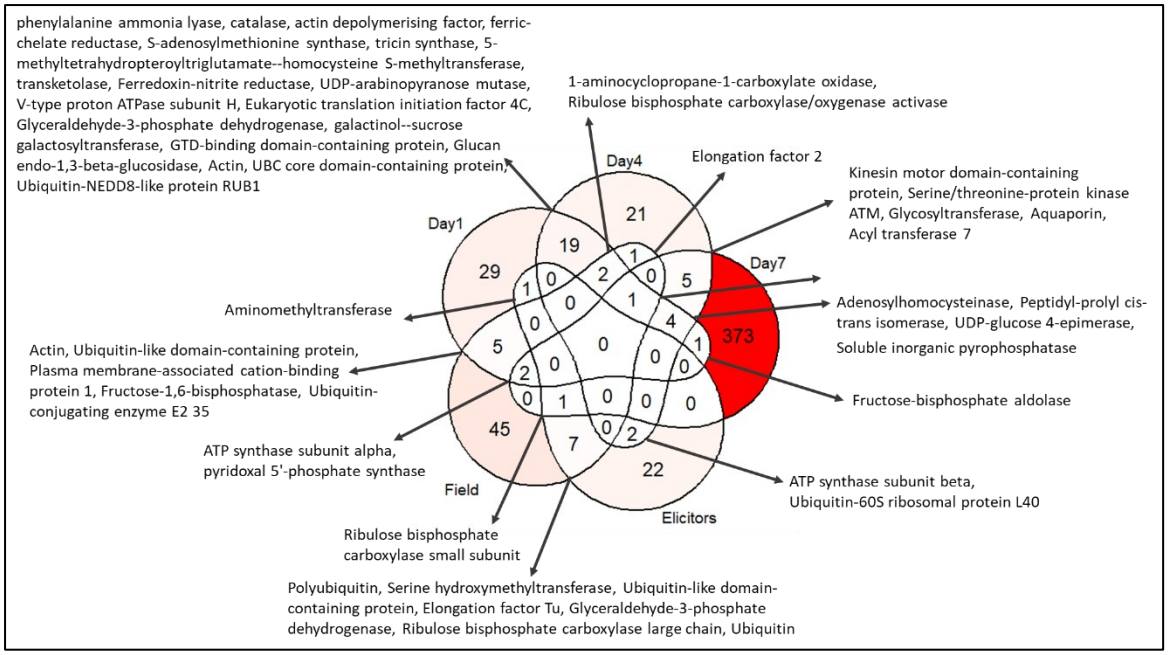


Figure S3. Overlap between proteins for which ubiquitination sites were identified.

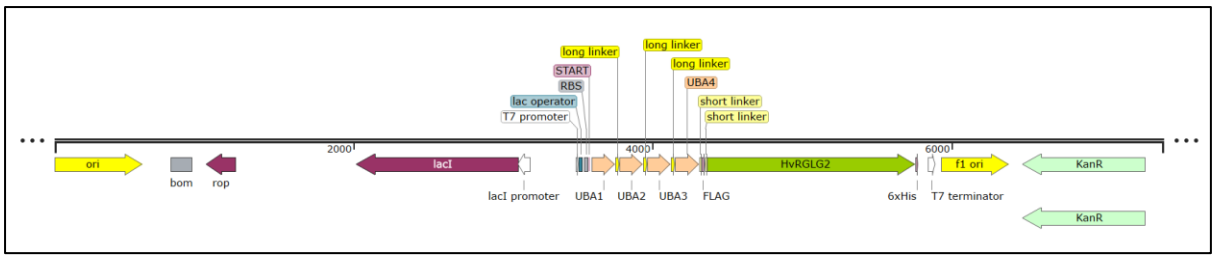


Figure S4. Vector map of the SynHVRGLG2 in PET28a construct.

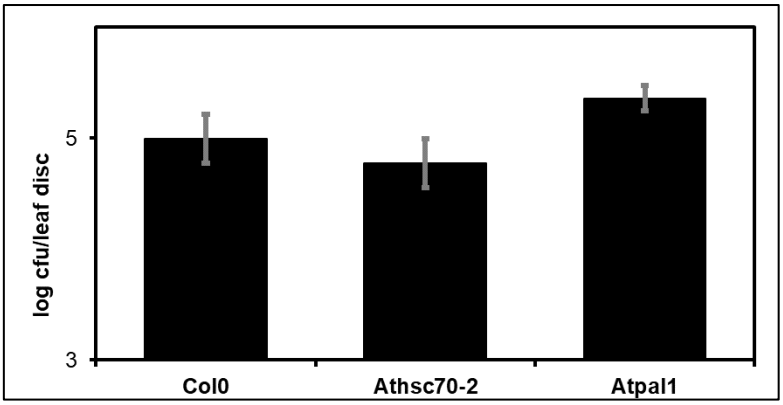
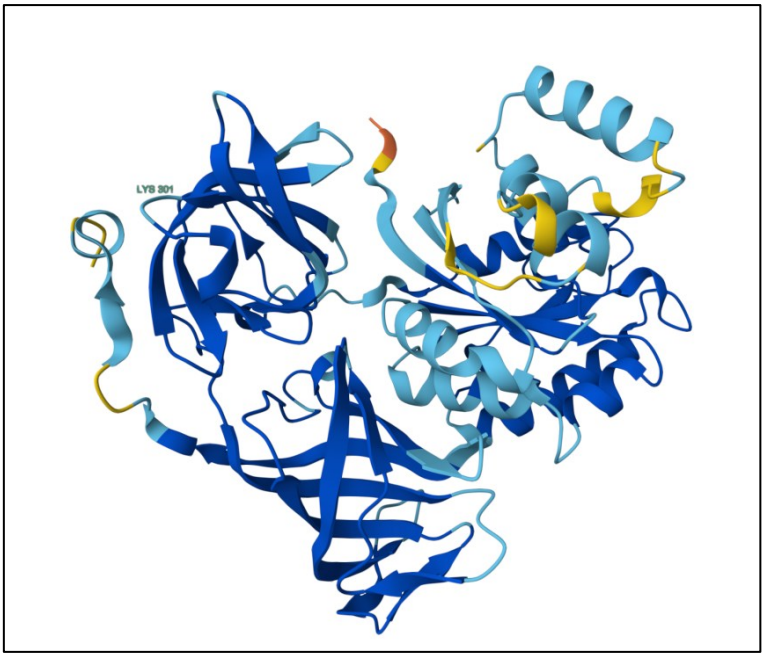


Figure S5. Disease assay for *Athsc70-2* and *Atpal1* mutants.



Supplementary Figure 6. Alphafold structure of HvELF1a with labelled lysine 301.

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