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Improving crop immunity by exploitation of the ubiquitin system (UbS-plants)

Karolina Brzezinska[1] and Beatriz Orosa[1]

[1] *University of Edinburgh, Institute of Molecular Plant Sciences, EH9 3BF, Edinburgh*

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1. Abstract

One of the biggest challenges of our time is to feed the growing global population. World arable land is practically at its maximum capacity, and will decrease in area in coming years due to climate change and urban development. Therefore, the productivity of our food system must be increased to tackle world hunger and increase food security. One exceptional strategy to achieve this goal is to reduce losses by pests. Barley (*Hordeum vulgare*) is the fourth most important crop worldwide and second in the UK. Diseases, including those caused by the brown rust pathogen *Puccinia hordei*, represent the largest threat to barley, causing yield losses of up to 40%. On the AHDB Recommended Lists (RL), no spring barley variety has a resistance rating above 5 (on a 1 to 9 scale, where 9 represents high resistance), indicating only moderate resistance to *P. hordei*. Efforts to reduce disease-related losses are mainly focused on development of resistant varieties and new chemicals to prevent and control pathogens. Improving the plant's own natural defences can alleviate disease-related losses and is a cost-effective and environmentally sustainable alternative to chemical treatments. However, pathogens tend to mutate rapidly, overcoming these strategies and causing the rise of new virulent strains.

Understanding host-pathogen interactions is key to obtaining durable plant resistance to pathogens, and, in this regard, ubiquitination (a post-translational modification of proteins) plays a fundamental role. Ubiquitination of regulatory immune proteins is a crucial mechanism for appropriate immune activation, regulating pathogen perception and defense responses. Its central regulatory roles makes ubiquitination a potential mechanism to enhance crop resistance to pests.

Here, the immune-induced substrates in barley upon activation of the immune response by the plant hormone salycilic acid (SA) and jasmonic acid (JA) was studied. By using SA and JA, the immune activation of biotrophic and necrotrophic pathogens was mimicked and isolated without the interference of the pathogen and their effectors. In addition, the ubiquitin-dependent immune activation on winter and spring barley cultivars infected with the fungal pathogen *P. hordei* in field conditions was studied, correlating ubiquitination and the level of resistance of each cultivar to decipher the key ubiquitin regulated immune targets associated with resistance to puccinia.

Results showed a correlation between the level of accumulation of ubiquitin targets and the expression of immune genes in response to treatment with SA and JA under control conditions, finding a positive feedback between both pathways. However, this correlation was not observed under field trial conditions. Instead, the analysis of the barley cultivars infected with puccinia in a field trial showed a better correlation between the accumulation of the ubiquitinated proteins and their resistance level.

Results suggest that i) in response to SA and JA, there is a clear connection between ubiquitination and induction of immune genes. Nevertheless, environmental conditions affect transcriptional activation of immune genes, and therefore ii) the overall level of accumulation of ubiquitination is a better marker to determine the level of resistance of commercial cultivars than the expression of specific genes.

Characterising the activation of the ubiquitin pathway will provide a potential fast test to predict the level of resistance of new cultivars to rust, but also for assessing the prevalence of the immune response during breeding programs, where yield is the main selective character, in many cases jeopardising pathogen resistance.

2. Introduction

Eradication of hunger is one of the key sustainable development goals of the UN. The human population continues to increase and is predicted to reach 9.73 billion by 2050; therefore, to feed the world we will have to produce ~50% more food. Cereals are by far the most important source of total food intake for human consumption, providing 56% of total calories, and are also indirectly critical for animal production: 44% of total crop production is destined for animal feeds [1]. Unfortunately, increasing global crop production is not straightforward since suitable agricultural land continues to decrease due to sprawling urban development and climate change [2]. In this context, preventing crop yield losses caused by biotic stresses is a vital priority for the agri-industry sector [3]. Despite the large commitment and investment to reduce pathogen-associated losses, these are still a major threat to agriculture, and in fact, pathogens alone are estimated to cause yield losses of 21% in crops annually [3]. One of the main obstacles to obtain tolerant plants is that the interplay between plants and pathogens is dynamic, representing an ecological and epidemiological interaction with strong selective pressures. Highly virulent pathogens increase the selective pressure on the plant host to modify and improve its defence response, which in parallel increases the selective pressure on the pathogen to overcome these defence mechanisms [4]. Plant-pathogen interactions are constantly evolving, and pathogens frequently develop escape mutations, thereby avoiding existing host resistance or protection strategies.

Barley (*Hordeum vulgare*) is the fourth most important crop worldwide and second in UK; and diseases, including those caused by the **brown rust pathogen *Puccinia hordei***, represent the largest threat to barley production, causing yield losses of up to 40% [9]. Due to climatic change, warmer and more humid springs are increasing the incidence of *P. hordei* epidemics, which is becoming a major problem in UK due to the absence of resistant commercial cultivars [8]. In this context, **understanding the interaction between *P. hordei* and barley and improving plant immune responses against *P. hordei* is of vital importance to guarantee food security**.

Host-pathogen interactions

Plants are sessile organisms, and therefore depend heavily on efficient regulatory mechanisms to cope with external stressors, including pathogens. Plant immune responses are initiated by the recognition of conserved pathogen-associated molecular patterns (PAMPs) by plasma membrane receptors, which activate the first layer of defence termed pattern-triggered immunity (PTI). PTI initiates a cascade of events that results in the induction of a battery of immune responses that is sufficient to fend off most pathogens [5-8]. However, some pathogens are able to suppress PTI through the release of effector proteins into plant cells. The presence of these effector proteins can be sensed by intracellular receptors, which activate effector-triggered immunity (ETI). ETI is characterised by the rapid onset of programmed cell death at the site of infection, which is thought to isolate and prevent the spread of invading pathogens [12-14]. The regulation of this chain of events reflects the dynamic nature of host-pathogen interactions and requires a high degree of proteomic

plasticity from the host [15]. This plasticity is largely dependent on post-translational modifications where the contribution of ubiquitin is crucial [6, 15-18].

Ubiquitination as a key regulator of plant immune responses

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. Modifications by ubiquitin and ubiquitin-like proteins provide dynamic modulation of protein function in response to abiotic stress and pathogen attack [2, 13, 14]. In fact, ubiquitination of regulatory immune proteins is a crucial mechanism for appropriate immune activation [14, 17-19], regulating the stability of plasma membrane receptors [1-3] or of proteins involved in transcriptional reprogramming [15,16]. In agreement, preliminary findings in the model plant *Arabidopsis* showed that in response to infection by the bacterial leaf pathogen *Pseudomonas syringae* pv. *maculicola* (Psm), an accumulation of ubiquitin targets is observed **(Figure 1A)**, suggesting wide-spread substrate ubiquitination is an important aspect of immunity.

Attachment of ubiquitin to substrate proteins occurs through the sequential activity of the activating (E1), conjugating (E2), and ligating (E3) enzymes. Plasticity of ubiquitination is achieved mainly through E3 ligases, which provide substrate and linkage specificity in a temporal and spatial regulated manner [20]. Over 1,000 plant genes are estimated to encode E3 ligases, a number that suggests extreme specialisation in their cell-signalling roles [20]. E3 ligases are classified in three families based on their E2-binding domains: HECT, U-box and RING, each generating different ubiquitin linkages depending on the E2 with which they are paired [22]. Some E3 ligases can directly bind to substrates, while others require adaptor proteins that provide substrate specificity. Although E3 ligases are key regulators of ubiquitination, relatively little is understood about how their selectivity and activities orchestrate plant immunity. Importantly, this lack of knowledge impedes the development of novel targeted approaches to improve immunity of crops.

Objectives

The aim of UbS-plants was to provide new ground-breaking insight into the mechanisms underpinning pathogen immunity in plants, extending this knowledge from the model plant species *Arabidopsis thaliana* to crops. Barley (*Hordeum vulgare*) was used as a suitable model to deliver frontier knowledge on how ubiquitin signalling is at the core of this process. Barley has a diploid genome and abundant genomic resources [23], which make it the ideal crop model species. Additionally, barley is the fourth most important crop worldwide [5], and diseases currently represent the largest threat to sustainable production, causing yield losses of up to 40% [24].

Therefore this project provided new ground-breaking insight into the mechanisms underpinning pathogen immunity in crops, delivering frontier knowledge on how ubiquitin signalling is at the core of this process (Objective 1 and 2), and comprehensive insights into the ubiquitin system that will

enable the fine modulation of immune responses to enhance immunity in Barley (Objective 3 and 4). Furthermore, this project provided a potential fast test to predict the level of resistance of new cultivars to rust, but also for assessing the prevalence of the immune response during breeding programs, where yield is the main selective character, in most cases jeopardising pathogen resistance. And finally, this project identified new targets involved in Barley immune responses, which can be used to improve resistance to diseases

3. Materials and methods

3.1. Plant growth and treatment

Barley seeds were germinated on plates lined with filter paper and supplied with 20 ml of sterile water for 3 days at 4°C in the dark, followed by incubation for 3 days at 21°C with access to light. Barley seedlings were then transferred to soil and grown in a growth room at 21°C with access to the natural light supplemented by Valoya RX400 Ns12 daylight spectrum lights (16h light, 8h dark) for 3 weeks.

Plants were treated with immune elicitors by infiltration with 0.5 mM salicylic acid and 0.1 mM jasmonic acid. Six biological replicates for both elicitor treatment and negative control treatment were obtained. Samples were collected at 6h post-infiltration, frozen in liquid nitrogen and stored at -80°C until ready for processing.

3.2. Obtaining barley samples from the field trial

Dr Neil Havis and Dr Francois Dussart from SRUC provided barley samples of 24 commercial barley cultivars infected with *P. hordei* in the field trial. A pull of 3 biological replicates from 24 Puccinia-infected and 2 non-infected barley samples of winter and spring barley cultivars were collected in June and July 2021 respectively, frozen in liquid nitrogen and stored at -80°C until ready for processing.

3.3. Measuring levels of protein ubiquitin targets

Leaf samples were ground on dry ice using mortar and pestle. 1/3 of the ground leaf tissue was treated with 3x lysis buffer. The remaining tissue was transferred into Eppendorf tubes and stored at -80°C. Protein concentration in the protein extract was quantified using standard Bradford assay(Bio Rad). Ubiquitination levels in each sample were assessed by performing an immunoblot with ubiquitin antibody (P4D1). Actin was used as loading controls.

3.4. Real-time quantitative PCR

1/3 of the remaining ground leaf tissue was used for RNA extraction using the Phenol:chloroform method. RNA concentration and purity was measured using NanoDrop. cDNA library was prepared using 1.5 µg of total RNA, according to the First-strand Superscript II protocol. qPCR was performed using the SYBRGreen reagent for PR (pathogenesis-related), LOX2 and WRKY28 genes using primers listed in **Table 1.**

Table 1. Primers for QPCR

Gene name	GenBank ID	Forward primer (5' → 3')	Reverse primer (5' → 3')	Amplicon length
BPR1-1	Z48728.1	GTAGTCTTGCAATGTTCGCAC	GTTCTGTGCGAACCTGGCC	163 bp
TIFY11B	KAE8802733.1	GTGCTCAGCCAGTACGTCAAG	GTGCTCAGCCAGTACGTCAAG	188 bp
WRKY28	DQ863112.1	CATGTGTTTCAACCCGTTCCAG	GAAGGCAGAAATGTCGAAGTTGG	187 bp
Lox2	U56406.1	CGCACCAGTGTCTCCAAG	GAGGAAATCACGAACCCCGG	204 bp
Actin	AY145451.1	GCCGTGCTTTCCCTCTATG	GAAGGAGTAACCTCTCTCGG	189 bp

3.5. Proteomics

A total of ninety-six samples: Eighteen samples from three independent experiments of SA treated and not treated plant material (6 conditions x 3 experiments) and seventy-eight samples from 24 Puccinia-infected and 2 non-infected barley (26 conditions x 3 replicates) were then subjected to purification of ubiquitin conjugates with tandem ubiquitin binding domains (HALO-tagqed recombinant ubiquilin protein) that recognise ubiquitin substrates, followed by a trypsin digestion to reveal unique di-Gly remnants at sites that were ubiquitinated.

Last part of the ground leaf tissue was subjected to purification of ubiquitin conjugates with tandem ubiquitin binding domains using HALO magnetic beads and HALO-tagqed recombinant ubiquilin protein. This was followed by a trypsin digestion and protein-identification by mass spectrometry at the Roslin Institute Proteomics and Metabolomics Facility and Institute of Genetics and Cancer (University of Edinburgh) facilities for field-trial samples and elicitor-treated samples respectively. Comparison between infected and non-infected samples was carried out using Cytoscape software.

3.6. RNA-sequencing

The transcriptome of infected and non-infected barley leaves was analysed using RNA sequencing. Ninety-six mRNA sequencing libraries (treated vs not treated and infected vs control samples) were prepared and sequenced as 50 bp paired-end reads in an Illumina NovaSeq in the facilities of Novogene (https://en.novogene.com/). Raw data underwent basic filtering and trimming using Trimmomatic, gene expression was estimated using Kallisto ([44], with the barley transcriptome as a reference), and differential expression was carried out with the R/Bioconductor package DESeq2. Transcriptomic differences between control and treated/infected plants provided the contextual

transcriptional response for the ubiquitome isolated in objective 1 and 2, informing on the extent of transcriptional reprogramming underlying barley immune response to *P. hordei.*

3.7. Integrated Transcriptomic-Proteomic analysis

The differentially expressed transcripts obtained in Objective 3 were evaluated in conjunction with the linkage-specific ubiquitome obtained in Objectives 1 and 2. The integration of ubiquitination and expression highlighted key ubiquitinated transcription factors regulating downstream pathways associated with defence responses in barley. Functional protein associated networks generated by GENEmania and STRING were used to obtain the network of interactions, which were visualised using Graphia [28] to identify ubiquitinated proteins at the centre of the immune regulatory networks. The biological role of these ubiquitinated proteins was inferred through gene ontology (GO) analysis of its gene cluster via AGRIGO and REVIGO. Comparative analysis of data in Arabidopsis and Barley enabled the identification of proteins with conserved regulatory roles at the centre of immune pathway activation, providing robust candidates to alter plant immune responses and create potential resistant varieties in different plant species.

4. Results

Objective 1. Immune ubiquitome profiling of barley upon pharmacological activation of immunity.

Ubiquitin is a central regulator of immunity in plants. Upon activation of immunity, key immune targets exhibit changes in their ubiquitination status as part of the activation of the defense response. Preliminary findings in the model plant *Arabidopsis* showed that in response to infection by the bacterial leaf pathogen *Pseudomonas syringae* pv. *maculicola* (Psm), an accumulation of ubiquitin targets was observed **(Figure 1A)**, suggesting wide-spread substrate ubiquitination is an important aspect of immunity. Using a new ubiquitin proteomics workflow that has been recently designed, 1,294 proteins that changed their ubiquitination status upon infection, including key immune targets such as PAD3, MC2 metacaspase 2 or eIF-5A 1 (negatively regulators of cell death) and transcription factors such as AP2/EREBP family members, MYB26 or ERF53 (unpublished results) were identified. The majority of proteins experienced an increase in ubiquitination. Clustering analyses by gene ontology revealed that ubiquitin controls distinct cellular processes **(Figure 1B)**. For instance chromatin remodelling and DNA repair, calcium signalling, phosphorylation cascades and cell death, and regulation of gene expression as well as salicylic acid signalling are regulated by the ubiquitin pathway **(Figure 1B)**.

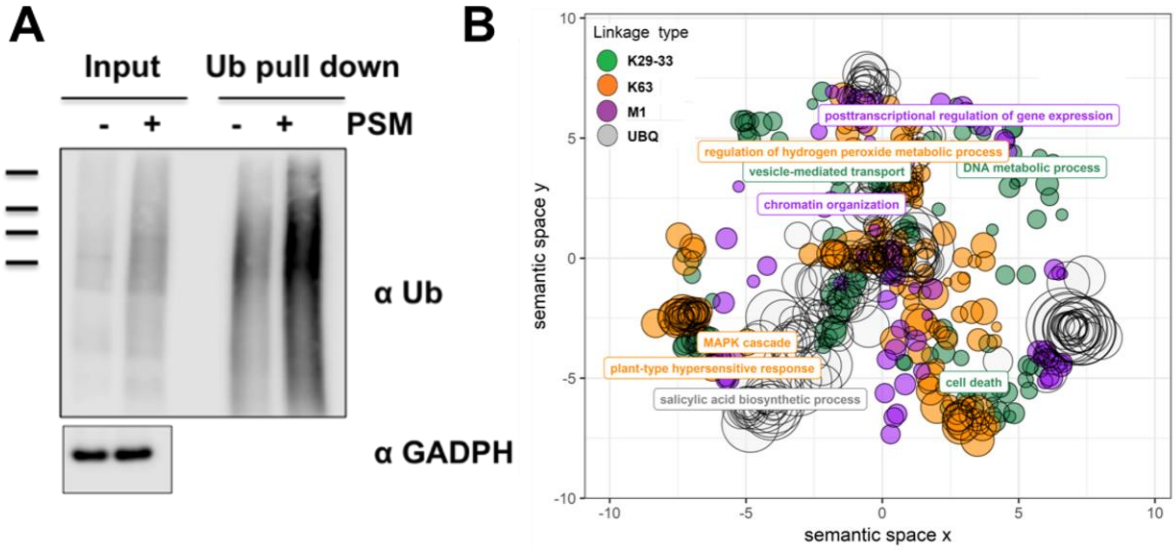

Figure 1. *(A) Pseudomonas (Psm) increased the levels of ubiquitin targets in* Arabidopsis thaliana*. Total proteins were subjected to pull-down with Ubiquilin recombinant protein (binds ubiquitinated proteins) followed by immunoblot analysis with anti-ubiquitin antibodies (α Ub) to detect ubiquitinated targets. GADPH protein has been used as housekeeping control (B) Scatter-plots showing the enrichment of biological process gene ontology (GO) terms by ubiquitin targets.*

Furthermore, functional links between transcriptional reprogramming and the immune ubiquitome were found, where 30% of the ubiquitinated transcription factors were ubiquitinated in their DNA binding domains. Therefore, data suggest a fundamental regulation and coordination of pathogen-induced immune signalling by the ubiquitin pathway. **Thus, elucidating the roles and ubiquitin targets is necessary to fully understand and exploit the ubiquitin-mediated regulation of immunity to enhance plant resistance.**

While advances have been made in our understanding of the roles of ubiquitination in response to pathogens in the model plant Arabidopsis, whether ubiquitin plays similar roles in other plants, and specifically in those of commercial interest, is mostly unknown. When plants are infected by pathogens, two distinct responses can occur, the early being a local response in the infected area, and later a systemic response in non-infected tissues. Even though both responses are essential for a correct immune response, by enhancing a local response, plants will be able to stop the progression of the disease, induce a higher systemic response, and, consequently, a better performance against pests.

Therefore the activation of the ubiquitin pathway in response to the treatment with the key immune hormones Salicylic Acid (SA) and Jasmonic Acid (JA) was studied, looking for ubiquitin targets involved in the activation of the defense response. Although treatments with SA and JA are a recurrent mechanism to study immunity in model plants, only a few studies have been performed in crops. Therefore, first it was necessary to optimise the SA and JA concentration and the timing of the response in barley. Different concentrations have been used (0.5 mM,1mM,10mM for SA and 0.5mM,1mM of JA) at 6h and 24h.

Results showed increased ubiquitin conjugates in response to treatment with SA. The accumulation occurs at 6h post-treatment and disappears at 24h (**Figure 2 A**), implying that ubiquitin signalling plays a key role in the SA-dependent early immune response in barley, and the ubiquitin response is stronger and faster than transcriptional activation of immune marker genes “pathogenesis-related proteins PR1 (HvPR1)” and “lipoxygenase” LOX2 (**Figure 2B),** suggesting ubiquitination may precede gene expression of PR1 and LOX2**.** 0.5mM SA 6h post-treatment was selected to identify ubiquitinated proteins that could be used as an early marker for infection and potential targets to enhance resistance. Ubiquitinated proteins and ubiquitin-associated proteins (interactors) of non-treated and SA-treated barley plants (three biological replicates and two technical replicates) were identified by Mass Spectrometry. The volcano plots showed 2136 proteins changed their ubiquitinated status in response to SA (**Figure 3**). A total of 146 proteins were differentially accumulated after applying statistical analysis (p-value of 0.05 and fold change 2), including key immune targets such as Thioredoxin, Lipoxygenases or Peroxidases. The follow-up work will consist of investigating the selected proteins to decipher their role in the activation of the immune response, looking for marker proteins for early detection of infection but also for potential targets to enhance resistance.

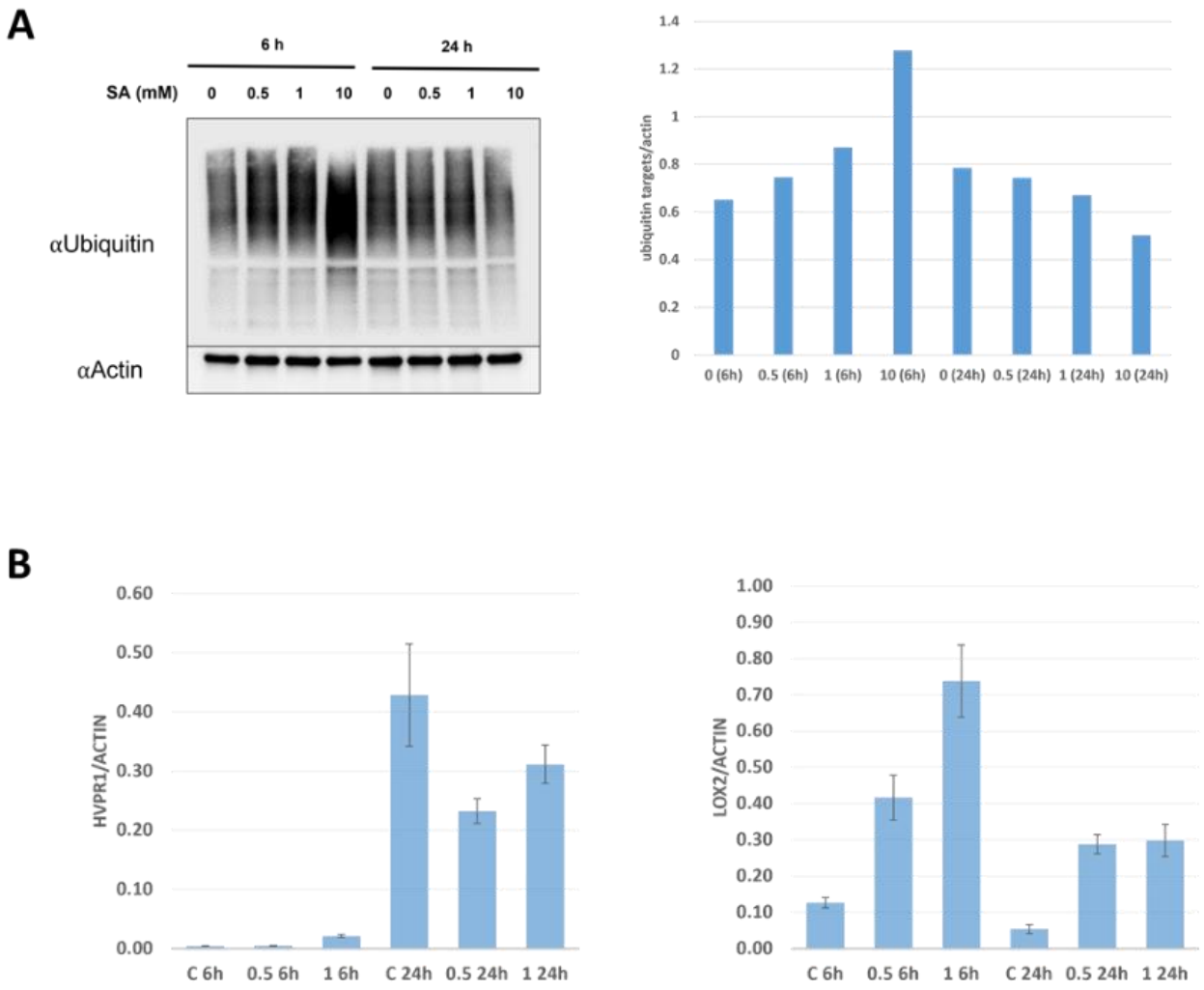

***Figure 2**. Salicylic acid (SA) increased the levels of ubiquitin targets in barley. **(A)** Treatment with SA increased the levels of ubiquitin targets in barley. Total proteins were subjected to pull-down with Ubiquilin recombinant protein (bind ubiquitinated proteins) followed by immunoblot analysis with anti-ubiquitin antibodies to detect ubiquitinated targets. **(B)** Gene expression of PR1 and LOX2 marker genes in response to SA treatment. The relative expression of the marker gene was normalised by the housekeeping gene Actin.*

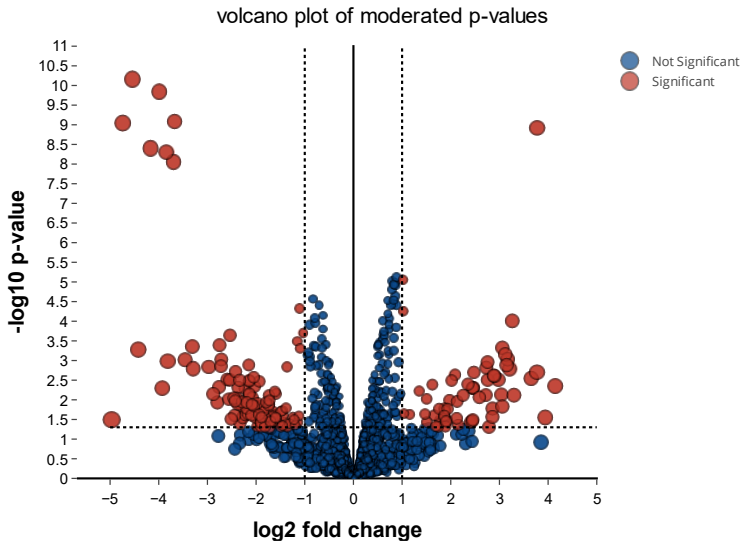

Figure 3. *Volcano plot showing ubiquitinated proteins and interactors in SA-activated barley plants. Volcano plots were depicted with the fold change of ubiquitinated proteins and interactors and the differences were calculated as p-value ≥0.05 and fold change ≥2. Red circles show proteins that have significant differences. Blue circles are proteins without any differences.*

Even though Puccinia is a biotrophic pathogen, and therefore it will activate the SA-dependent immune response, we were also interested in investigating the barley response to necrotrophic pathogens since under field conditions plants are often infected with more than one pathogen, and in order to select new candidates for enhancing resistance it is necessary to take into account the negative crosstalk between JA and SA.

Results showed increased ubiquitin conjugates in response to treatment with JA at 6h and 24h post-treatment (**Figure 4A**) that correlate with the transcriptional activation of immune marker LOX2 (**Figure 4B**). 0.1mM JA 6h post-treatment was selected to identify ubiquitinated proteins that could be used as an early marker for infection and potentially targets to enhance resistance against necrotrophic pathogens, and to compare with SA immune ubiquitinated proteins.

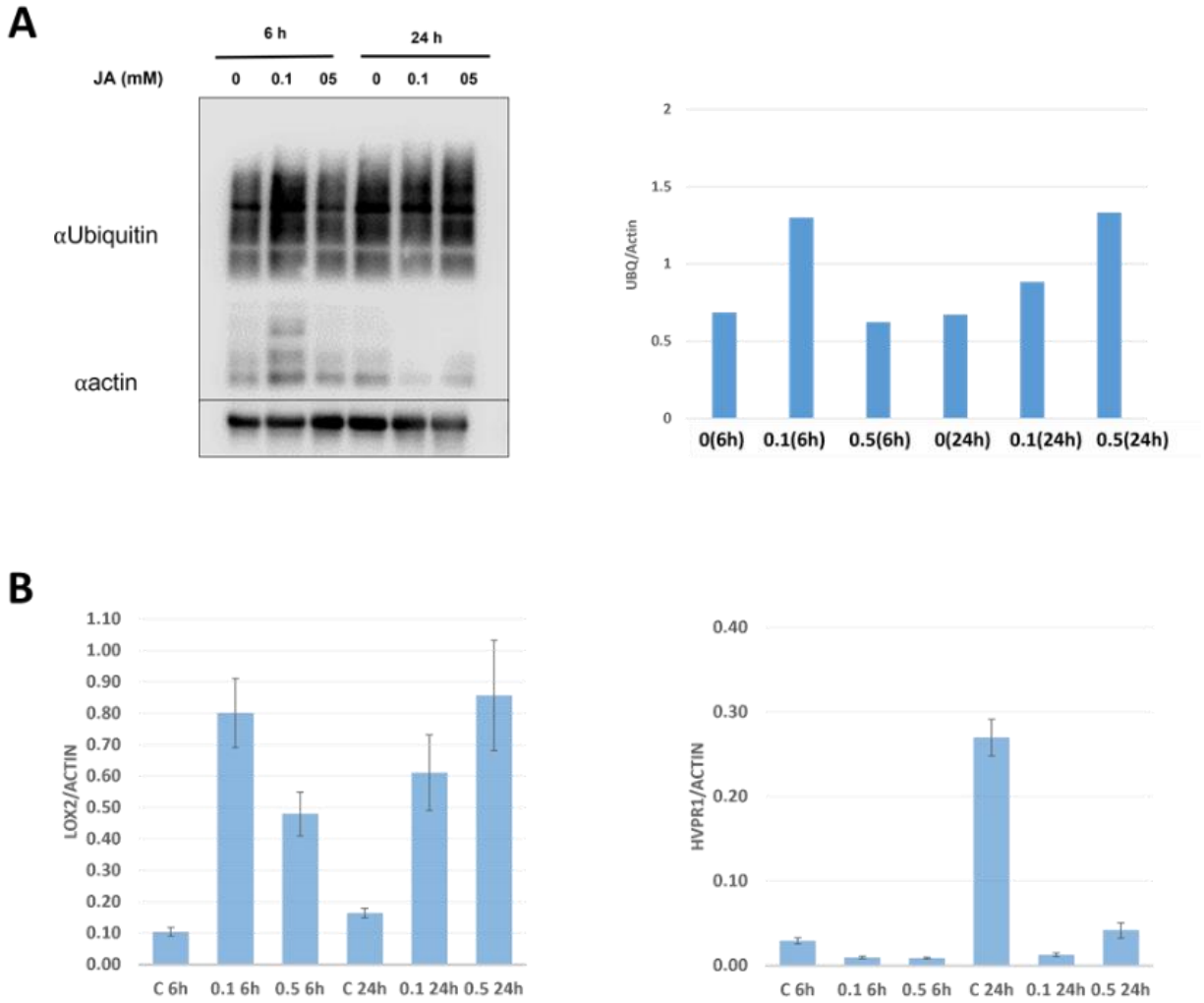

Figure 4. *Jasmonic Acid increased the levels of ubiquitin targets in barley.* ***(A)*** *Treatment with JA increased the levels of ubiquitin targets in barley. Total proteins were subjected to pull-down with Ubiquilin recombinant protein (bind ubiquitinated proteins) followed by immunoblot analysis with anti-ubiquitin antibodies to detect ubiquitinated targets.* ***(B)*** *Gene expression of PR1 and LOX2 marker genes in response to JA treatment. The relative expression of the marker gene was normalised by the housekeeping gene Actin.*

Ubiquitinated proteins and ubiquitin-associated proteins (interactors) of non-treated and JA-treated barley plants (three biological replicates and two technical replicates) were identified by Mass Spectrometry. The volcano plots showed 2136 proteins changed their accumulation status in response to JA; of those 67 proteins were differentially accumulated (**Figure 5**), containing immune proteins such as Thioredoxin, Lipoxygenase or Lipase 3.

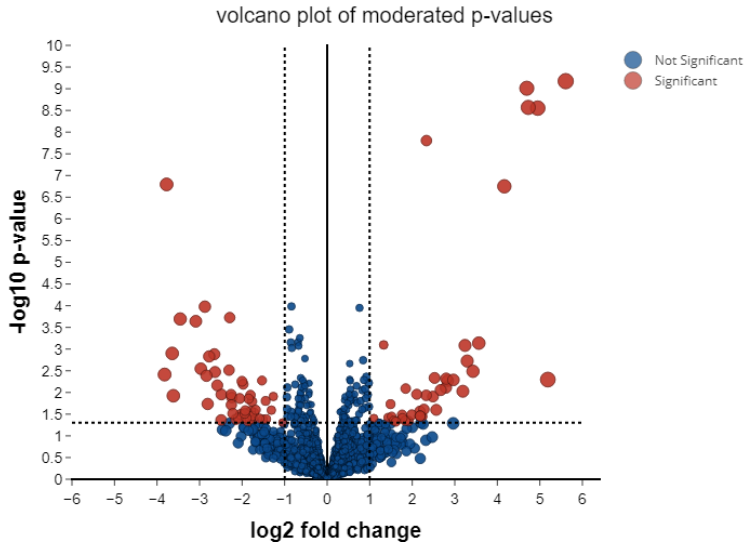

Figure 5. *Volcano plot showing ubiquitinated proteins and interactors in JA-activated barley plants. Volcano plots were depicted with the fold change of ubiquitinated proteins and interactors and the differences were calculated as p-value ≥0.05 and fold change ≥2. Red circles show proteins that have significant differences. Blue circles are proteins without any differences.*

In order to select potential targets for resistance against biotrophic and necrotrophic pathogens, differentially ubiquitin-associated proteins in response to SA and JA were compared (**Figure 6A _6B).** The results showed an overlap of 20 proteins associated with both treatments and therefore promising targets to enhance resistance against an ample spectrum of pathogens. Two of these proteins are A0A287JJR9 and M0Y1R9, which code for Lipoxygenase (LOX). The LOX pathway is a key regulator for lipid peroxidation, which is crucial for plant senescence and defense pathways. LOX genes are promising targets, and therefore, further experiments are needed to validate LOX genes as potential targets to enhance resistance.

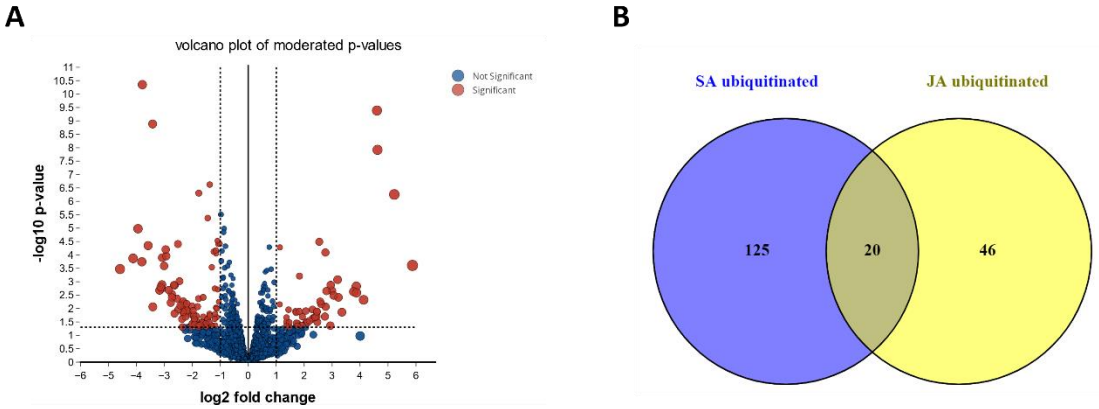

Figure 6. *Comparison between SA and JA activated barley plants.* ***(A)*** *Volcano plots were depicted with the fold change of ubiquitinated proteins and interactors and the differences were calculated as p-value ≥0.05 and fold change ≥2. Red circles show proteins that have significant differences. Blue circles are proteins without any differences.* ***(B)*** *Venn diagram showing the overlap between SA and JA treatments.*

Objective 2. Immune ubiquitome profiling of Puccinia-infected barley in a field trial

Environmental stress conditions can have a huge impact on plant performance against pests. Field conditions involve the simultaneous exposure of plants to more than one stressor, and abiotic stresses are frequently combined with pathogen infection or even co-infection with different pathogens. These conditions add more pressure to the performance of the plant defense response to pests, and this can have variable effects on each cultivar. The ubiquitome of *Puccinia*-infected barley samples was analysed, for winter and spring barley cultivars. This ubiquitome is a unique resource to identify signalling pathways orchestrated by ubiquitin that will explain the different levels of resistance between cultivars.

To optimise the methodology, winter barley cultivars infected in a field trial with Puccinia were used. Results showed different cultivars had a different accumulation of ubiquitinated proteins (**Figure 7A),** and increased infection correlates with a higher level of ubiquitination and expression of PR genes into the same cultivar (cultivar 16: KWS Orwell and cultivar 27: LGBU16-6889). Differences in activation of ubiquitin pathway and expression of marker immune genes were observed between cultivars (**Figure 7A-7B**). For example, cultivar 61 (LGBU16-6889) showed a lower accumulation of ubiquitin targets but higher expression of PR1 and PR2 than cultivar 32 (SY Thunderbolt). PR1 was selected as the representative gene since it was by far the most expressed PR gene in response to infection (**Figure 7B**). Although most barley cultivars are resistant to stripe rust of wheat, caused by *Puccinia striiformis* f. sp. *tritici* (yellow rust), some co-infection with yellow rust was observed in the field trial (Dr Neil Havis helped with the identification of the disease). A representative sample of the infected barley plant was analysed to determine if there was an activation of immunity against this pathogen, and an accumulation of ubiquitin proteins and high expression of PR3 was found. Further experiments are needed to validate the recognition of yellow rust by the barley immune response.

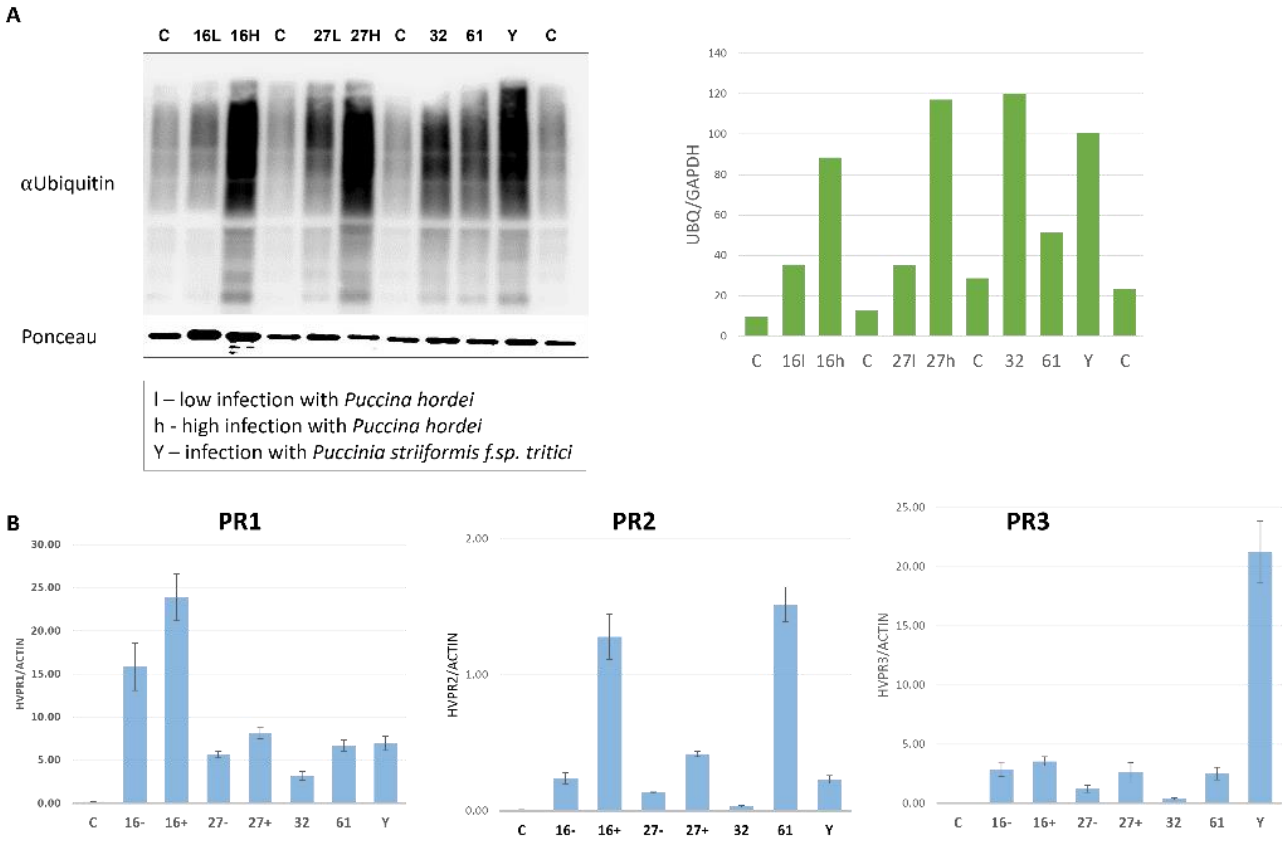

***Figure 7**. **(A)** Accumulation of ubiquitin targets after infection with* Puccinia hordei*. Infected (samples 16, 27, 32, 61, Y) and non-infected (c-) winter barley cultivars on a field trial with* P. hordei*. Total proteins were subjected to pull-down with Ubiquilin recombinant protein (bind ubiquitinated proteins) followed by immunoblot analysis with anti-ubiquitin antibodies to detect ubiquitinated targets. **(B)** Gene expression of the PRs marker genes in response to infection with brown rust. The relative expression of the marker gene was normalised by the housekeeping gene Actin.*

In order to determine if there was a correlation between performance in the field and molecular markers, the ratio of infection in the field (the % Brown rust score) was compared with the accumulation of ubiquitinated proteins and PR1 gene expression (**Figure 8**). The combined analysis with the three parameters suggests that the level of ubiquitination could explain the performance of most cultivars under field conditions, showing a correlation between infection and ubiquitination level and PR1 expression. In fact, plants with different levels of infection from the same cultivar (L vs H in Figure 8) showed a different level of ubiquitination, where increased ubiquitination correlated with higher infection (% brown rust scale represents the level of infection for the full cultivar on the field trial). An exception was cultivar 32 (SY Thunderbolt), where high ubiquitination levels did not correlate with rust resistance. Therefore, it could be possible that novel ubiquitinated proteins in SY Thunderbolt enhanced resistance to brown rust.

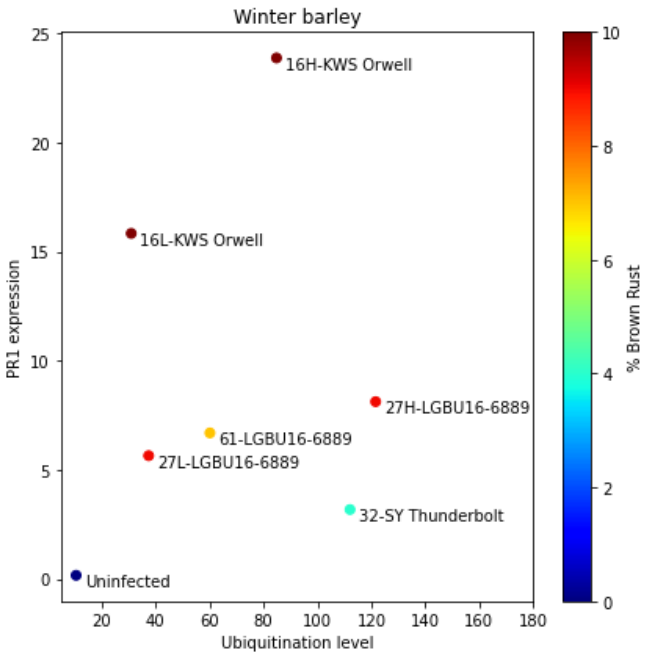

Figure 8. *Correlation between level of resistance / susceptibility to pathogens in the field with the activation of the ubiquitin pathway in winter barley cultivars. 2D graph comparing ubiquitination level and PR1 expression. Colour represents the % of infection on the field trial (Data provided by Dr Neil Havis (Crop & Soils Systems, Scotland's Rural College (SRUC)).*

Since the results in winter barley showed a correlation between infection and activation of the ubiquitin pathway, Spring barley cultivars were analysed. About half of the barley in the whole of the UK is of the spring variety and almost all the barley in the north and in Scotland is planted in the spring (https://www.gov.uk/government/statistics/farming-statistics-2021-uk-wheat-and-barley-production-first-estimate). First, non-infected/infected barley plants were selected across the field trial to establish the negative and positive controls of the study (**Figure 9A**). Once it had been validated that infected plants accumulate ubiquitinated proteins as part of their immune response, the different spring barley cultivars were analysed (**Figure 9B**).

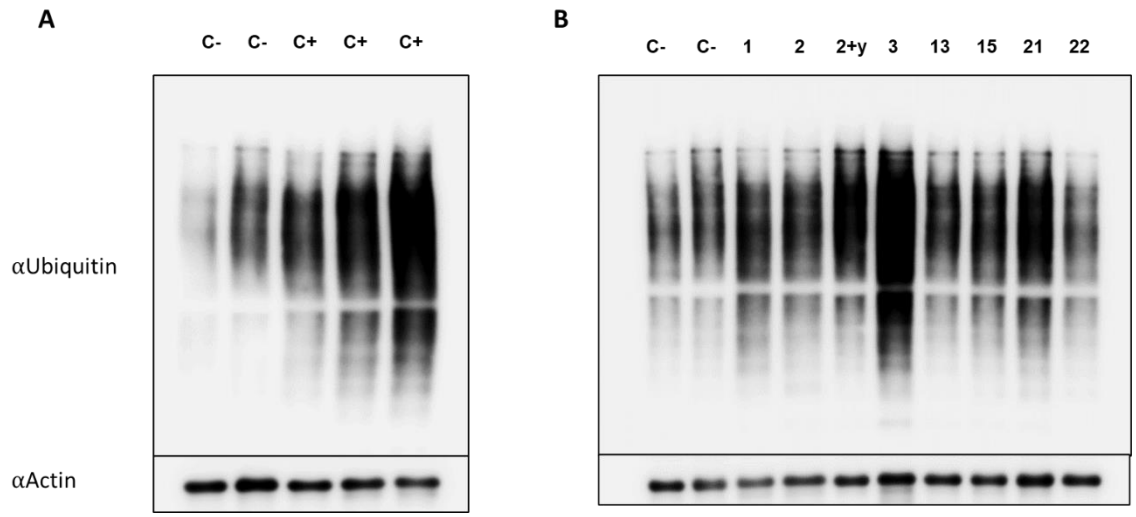

Figure 9. *Accumulation of ubiquitin targets after infection with* Puccinia hordei*. Total proteins were subjected to pull-down with Ubiquilin recombinant protein (bind ubiquitinated proteins) followed by immunoblot analysis with anti-ubiquitin antibodies to detect ubiquitinated targets. (A) Highly infected barley plants used as positive control and non-infected (c-). (B) Infected (samples 1,2,3,13,15,21,22) and non-infected (c-) Spring barley cultivars on a field trial with* P. hordei*.*

As was observed previously in winter barley, different cultivars had a different level of ubiquitination, and the combined infection of brown and yellow rust increased ubiquitination levels (cultivar 2: KWS Sassy). Analyses included their performance in the field(the % Brown rust score in the field), ubiquitin accumulation and PR1 expression (**Figure 10**). The results suggested thatPuccinia had a greatly impact on the immune response by activating the ubiquitin pathway and PR1 expression. Analysis of PR1 expression, ubiquitin accumulation and rust score showsed a correlation between performance against Puccinia and activation of the ubiquitin pathway, where high Rust score was associated with an important accumulation of ubiquitin targets in some cultivars but not in others. For example, Cadiz presented a high brown rust score and accumulation of ubiquitin targets but lower expression of the PR1 gene. However, SY Bronte had a high rust score, and PR1 expression but a low accumulation of ubiquitin targets. The differences between cultivars, activation of ubiquitin targets, and their performance in the field were key in this project since it could decipher novel targets and activated pathways in resistant cultivars that could be transferred to more susceptible cultivars.

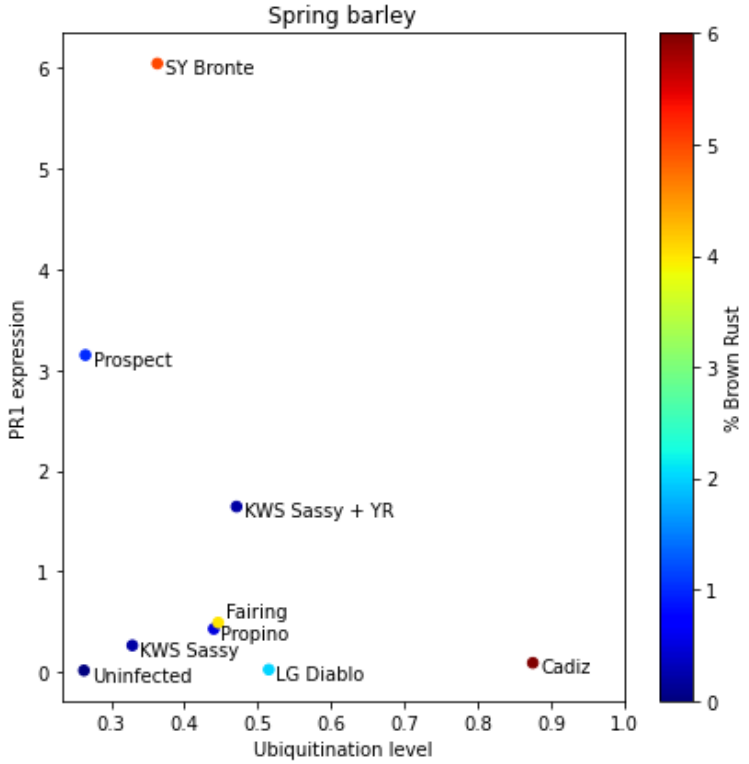

Figure 10. *Correlation between level of resistance / susceptibility to pathogens on the field with the activation of the ubiquitin pathway in Spring barley cultivars. 2D graph comparing ubiquitination level and PR1 expression. Colour represents the % of infection on the field trial (Data provided by Dr Neil Havis (Crop & Soils Systems, Scotland's Rural College (SRUC)).*

Data suggested that a unique parameter was not able to predict the performance of a cultivar in the field in response to Puccinia, however, a combined transcriptomic and ubiquitomic study could provide clues on the performance of the different cultivars. Different cultivars have different genetic backgrounds that can determine their performance in the field, therefore an RNASeq study was performed to compare the immune response against Puccinia for the different cultivars. To assess the data a PCA was performed where samples were distributed in 2D, with the two first principal components explaining 60.57% and 13.11% of the variation between samples (Figure 11).

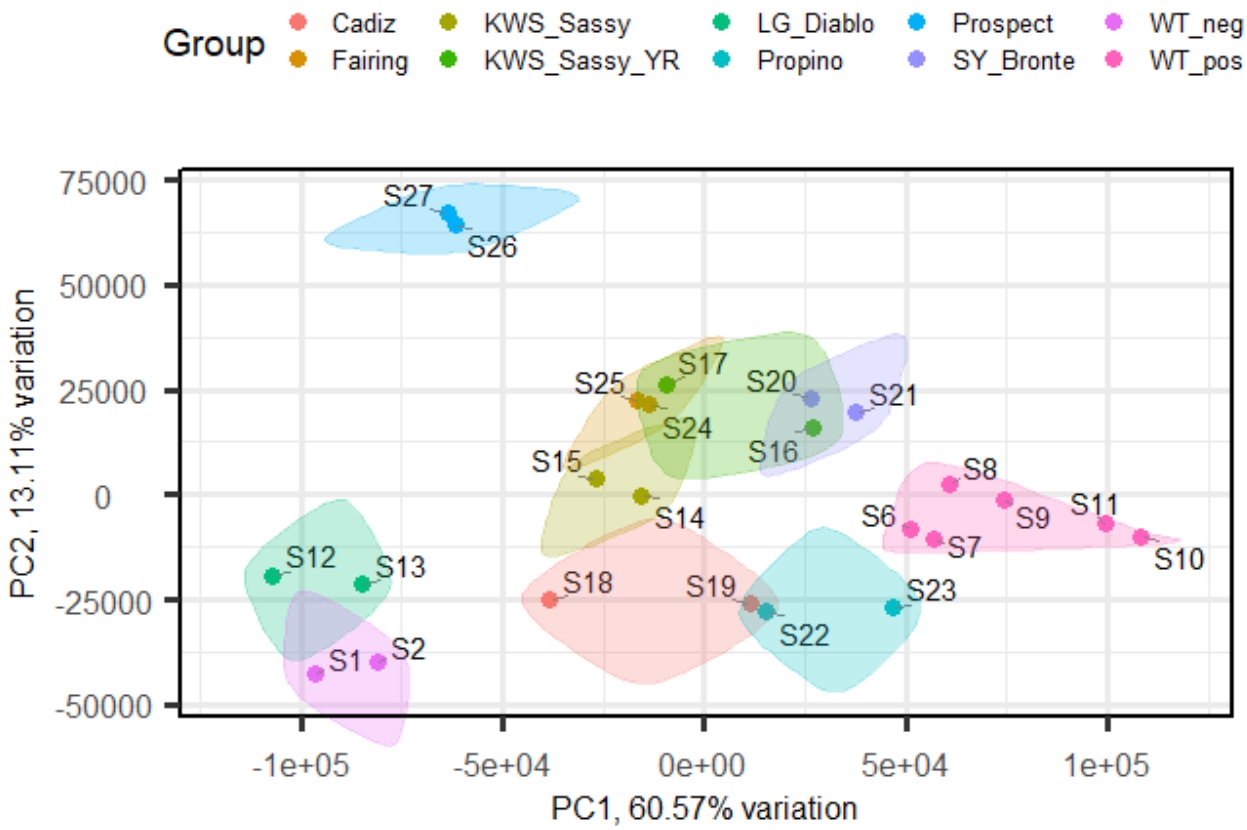

Figure 11: PCA graph showing the distribution of the different cultivars in 2D. The distributions was explained by the two main principal components which explain 73% of the variation between samples.

In the PCA, the different cultivars are located between negative / non-infected plants (S1, S2) and positive controls / plants used as spreaders (S6 to S11). Considering that some cultivars showed a higher level of resistance / susceptibility than others, each cultivar was compared with the positive and negative control. As expected, the differential gene expression of the cultivars reflected the level of resistance in the field. For example, KWS-Sassy showed a low brown rust score, low accumulation of ubiquitin targets and PR1 expression (Figure 10), and when the KWS-Sassy transcriptome was compared with that of non-infected barley it was found that only a small group of genes were differentially expressed. On the contrary, the differences with the positive control were high, suggesting KWS-Sassy did not have a full transcriptomic reprogramming. On the other side, Bronte and Cadiz presented a high similarity with the positive control, markedly different to that of uninfected barley (Figure 12).

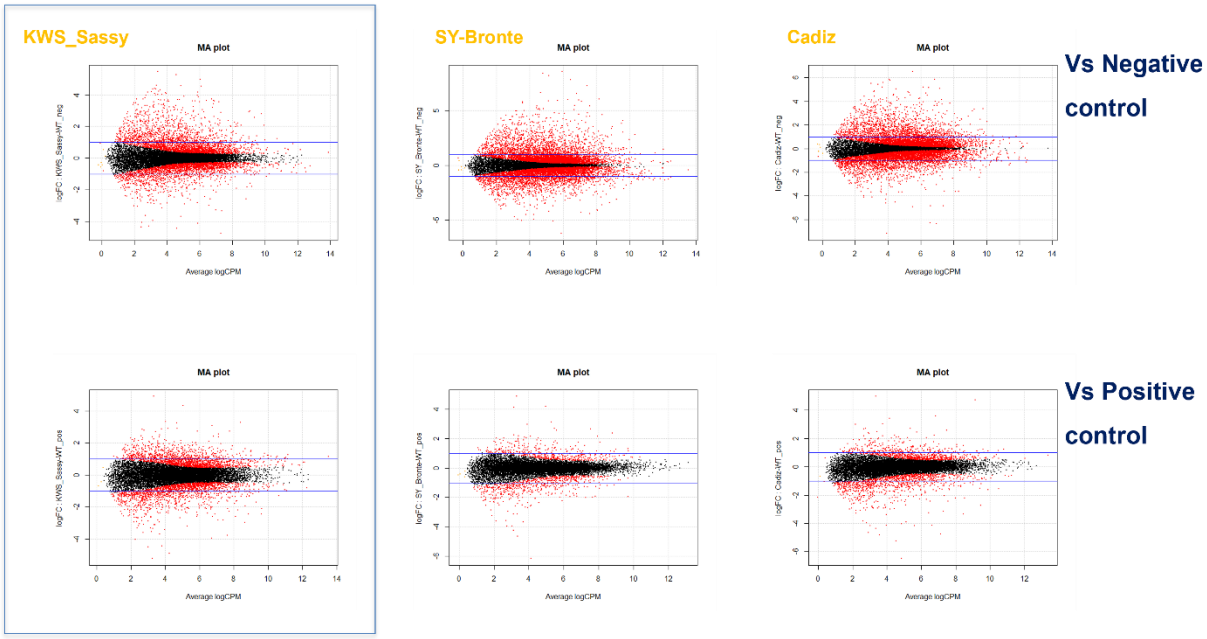

Figure 12: Differentially expressed genes of the different cultivars. The transcriptome of the different cultivars was compared with that of non-infected barley cultivar Laureate (top line) or the positive control / spreaders (bottom line). Black dots are non differentially expressed genes and red dots are differentially expressed genes. The blueline defines the gene expression difference threshold Fold Change ≥ ±2.

After assessing the transcriptomic differences between cultivars, the next step was to decipher the ubiquitome of the different spring barley cultivars infected with Puccinia. Ubiquitinated proteins, and proteins associated with them, were purified using ubiquitin binding domains (UBD: Ubiquilin) and the proteins were identified by Mass spectrometry.

The ubiquitome of the different cultivars showed a completely different pattern to that observed in the transcriptome. Proteins present across the cultivars and their level of accumulation are presented in Figure 13. Similar to the transcriptomic analysis, negative (NC) and positive (PC) controls showed different ubiquitome profiles, and the differences in ubiquitination were correlated with the level of performance in the field. Interestingly, the cultivar Cadiz presented a completely different ubiquitome, only partially overlaping with the positive control. If the overlap at the transcriptomic level was taken into consideration, it can be hypothesised that the differences in Cadiz were mostly at the proteomic level, where novel ubiquitinated and associated proteins seemed to define its performance in the field. The identity of the differentially accumulated proteins and their role during the immune response could provide the answers to why this cultivar shows a bad score on the field trial. Another surprising finding was the change in the ubiquitome for KWS-Sassy when it was co-infected with yellow rust (YR). Previously, it was described that the co-infection between Brown rust and Yellow rust primed the expression of PR1 gene and accumulation of ubiquitinated proteins (Figure 10). The proteomic results show that KWS-Sassy presented minor changes of the ubiquitome in response to the infection with Brown rust, however, the co-infection with yellow rust changed the profile, which

showed some overlap of certain ubiquitinated proteins with the positive control and some others with Cadiz, while a few were unique of this cultivar and co-infection. Further experiments are needed to be able to understand the implication of the yellow rust infection in barley plants, but results here suggest the co-infection with Brown rust aggravates the performance of the cultivar on the field and, therefore, could constitute a potential threat to barley.

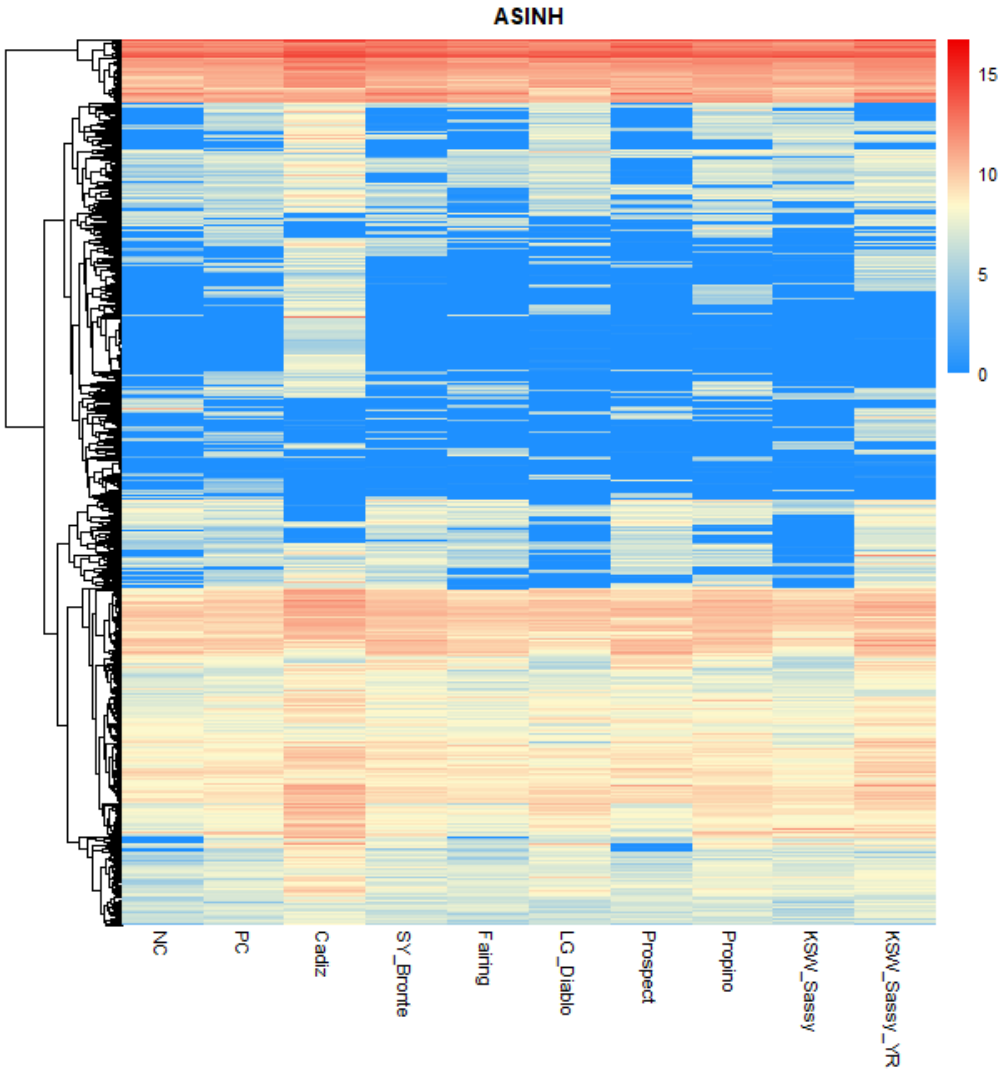

*Figure 13: Heatmap represents the differentially accumulated ubiquitinated and associated proteins across the cultivars. NC was the average of two independent samples and PC of three independent samples. Data represent the ASINH (inverse hyperbolic sine function**)** of the raw area of each protein.*

Ubiquitinated proteins are by far the more interesting targets to enhance resistance. Studying how ubiquitin regulates the activity of these target proteins and their role in immune response will allow us to use them as targets to induce resistance in susceptible cultivars but also a desirable trait to consider during breeding programmes. Therefore differentiated ubiquitinated proteins were selected across the different cultivars as potential targets for resistance (Figure 14). Future work will use Virus-induced gene silencing (VIGS) and transient overexpression to validate the relevance of these target genes in defence response against *Puccinia*.

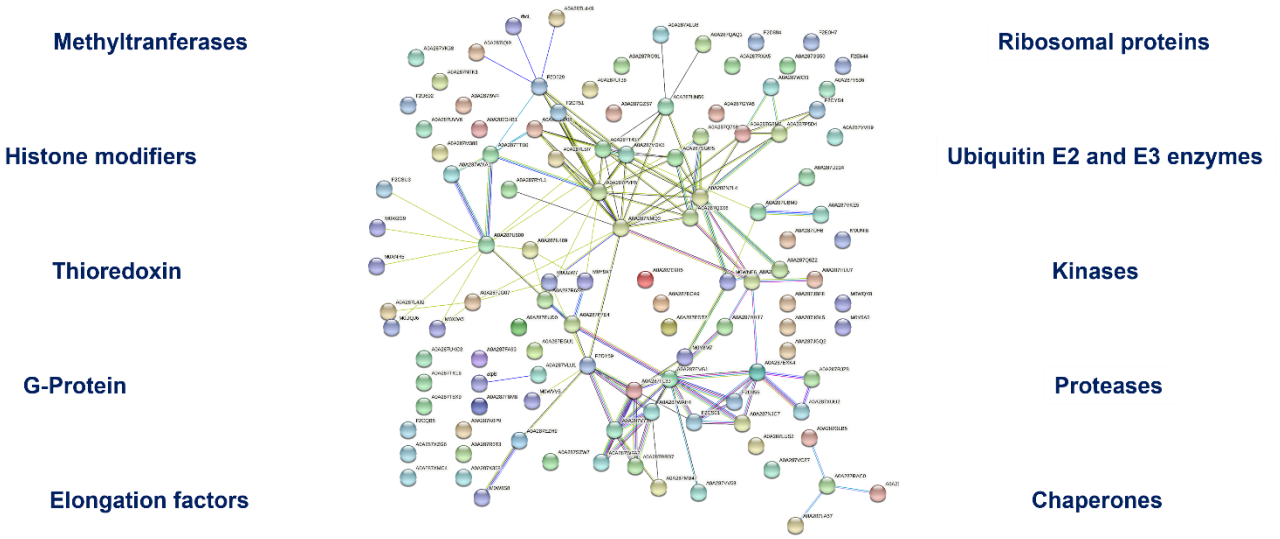

Figure 14: Network showing the differentially ubiquitinated proteins between cultivars. The network shows the predicted interaction between ubiquitinated proteins. The most interesting proteins have been highlighted in both sides. Node colours were used as a visual aid.

5. Discussion

The dynamic nature of host-pathogen interactions requires a high degree of immune plasticity from the host. This plasticity is largely dependent on post-translational modifications of immune proteins where the contribution of ubiquitin is crucial [4-7]. 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. Modifications by ubiquitin and ubiquitin-like proteins provide dynamic modulation of protein function in response to abiotic stress and pathogen attack [8, 6, 9]. In fact, ubiquitination of regulatory immune proteins is a crucial mechanism for appropriate immune activation [5, 10-12], regulating the stability of plasma membrane receptors [8] or of proteins involved in transcriptional reprogramming [6,7].

In agreement with previous results on the model plant Arabidopsis, in response to the treatment with the key immune hormones Salicylic Acid (SA) and Jasmonic Acid (JA), here an accumulation of ubiquitinated proteins in barley plants was observed. 146 differentially SA-dependent and 67 JA-dependent ubiquitinated proteins have been identified by mass spectrometry. Key immune markers were part of the immune ubiquitome, such as peroxidases, thioredoxins, phenylalanine ammonia lyase (PAL) or lipoxygenases. The next step is to study how ubiquitination regulates the activity of these key immune players, looking for targets to enhance plant resistance to diseases. Furthermore, these results in barley cultivars on a field trial with *P. hordei* showed a general ubiquitin-mediated immune activation of all infected barley cultivars, implying that ubiquitin regulation of the immune system is a conserved mechanism across cultivars.

Furthermore, the accumulation of ubiquitin targets correlated with the performance in the field trial, suggesting wide-spread substrate ubiquitination was a key aspect of the immune response. Being able to detect early pathogen infection is key, since during this period, called the latent period, the pathogen grows in the leaves without visible symptoms, acquiring sufficient biomass to establish infection. Fungicide use is much more effective in the early phases of the latent period, but without visible symptoms farmers are not able to apply fungicide treatments with precision. The results of this project suggest that ubiquitination could be used as a marker for accurate quantification of early pathogen infection. To follow up on this exciting result, a synthetic method to detect early pathogen infection by measuring the accumulation of ubiquitin in plants will be designed, consisting of a split-GFP based sensor that will measure the accumulation of ubiquitinated proteins during infection.

In parallel, comparing the immune proteome of the different spring barley cultivars will highlight ubiquitin immune targets present in resistant cultivars. Key ubiquitin targets involved in the regulation of immune pathways or transcriptional reprogramming will be selected. The differences between the resistant and susceptible cultivars will highlight key immune targets explaining the different phenotypes observed in the field trial. Furthermore, the further comparison with the ubiquitome obtained from SA/JA treated barley will display the potential spectrum of molecular responses associated with the selected targets. In summary, this project has identified ubiquitinated regulated immune targets which will allow us to obtain a holistic view of ubiquitin-driven regulation of plan immunity and reliable targets for improving the defense response of crops against pathogens.

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