AMF primes immune genes against Puccinia hordei (Brown rust) in Hordeum vulgare but does not reduce pathogen burden

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Abstract

Barley (*Hordeum vulgare*) is susceptible to *Puccinia hordei* (leaf rust), a biotrophic foliar fungal pathogen contributing to yield losses globally. With projected increases in food demand and ongoing threats from plant diseases, sustainable approaches to crop protection are essential to achieve the UN Sustainable Development Goal 2: Zero Hunger. Arbuscular mycorrhizal fungi (AMF), including *Rhizophagus irregularis*, establish symbioses with barley roots and have been implicated in the modulation of host immunity via mycorrhiza-induced resistance (MIR). In this study, we investigated whether *R. irregularis* AMF colonisation primes barley immune responses to *P. hordei*. Although AMF did not alter disease severity or growth in infected plants, co-infected tissues exhibited enhanced expression of defence-associated genes, including *PR1, PR2*, *PR3* and *WRKY28*. AMF alone did not induce these genes, consistent with a priming mechanism. Transcriptome profiling revealed AMF-induced reprogramming of the leaf transcriptome, with enrichment of genes related to defence, protein modification, and metabolism. AMF also modulated host protein ubiquitination during infection. These findings demonstrate that *R. irregularis* primes barley immune responses through transcriptional and post-translational regulation, with potential applications in the development of sustainable disease management strategies.

Introduction

Food security faces mounting pressure as global demand is projected to rise by 35-56% by 2050 (van Dijk *et al.* 2021), yet pathogens already reduce yields of major crops by 20-40% (Ristaino *et al.* 2021). Barley (Hordeum vulgare), the world's fourth most cultivated cereal, is highly vulnerable to leaf rust (*Puccinia hordei), which* can reduce yield by up to 62% in susceptible cultivars (Cotterill *et al.* 1992). Epidemics are driven by mild winters, wet springs and warm summers; conditions which are predicted to become more frequent with climate change (West *et al.* 2012). Current management relies on partially resistant cultivars, removal of volunteer hosts and fungicide application, yet these strategies are constrained by limited genetic resistance in UK barley (maximum AHDB ratings of 6.2 for winter and 4.2 for spring varieties; AHDB 2025) and sustainability concerns. As the fourth most cultivated cereal, barley (*Hordeum vulgare*) is crucial for food security and industries such as brewing. With climate change expanding the geographical range and severity of disease outbreaks, new approaches are needed to enhance crop resilience, reduce reliance on chemical inputs, in line with UN Sustainable Development Goal 2 (Zero Hunger and promotion of sustainable agriculture).

Arbuscular mycorrhizal fungi (AMF) represent an important strategy for promoting sustainable agriculture and enhancing food security. AMF are symbiotic fungi (subphylum Glomeromycotina) that colonise the roots of most vascular plants, including cereals (Wang and Qiu 2006). They facilitate nutrient and water uptake in exchange for sugars/lipids, leading to improved crop growth and yield, particularly under resource-limited conditions. Meta-analyses show that AMF inoculation increases cereal grain yields by up to 23% under rainfed systems (Wu *et al.* 2022). Beyond nutrition, AMF also enhance tolerance to abiotic stressors such as drought and contribute to soil health via improved structure and microbial activity (Diagne *et al.* 2020). Crucially, AMF can reduce susceptibility to pests and pathogens by modulating host immunity in a phenomenon known as mycorrhiza-induced resistance (MIR) (Farhaoui *et al.* 2025). Consequently, utilising AMF symbioses is increasingly viewed

as a sustainable strategy to improve cereal crop productivity while reducing dependence on synthetic fertilisers and pesticides (Umer *et al.* 2025).

MIR provides systemic protection against a broad range of pathogens and shares similarities with both pathogen-triggered systemic acquired resistance and rhizobacteria-induced systemic resistance (Cameron et al. 2013). Unlike direct activation of immunity, MIR typically involves priming – a preparatory state in which immune responses are potentiated rather than constitutively expressed, minimising fitness costs (Jung *et al.* 2012). AMF colonisation remodels host immune signalling networks, enabling more rapid and robust defence activation upon pathogen challenge. Hormonal crosstalk plays a central role, with early models suggesting that AMF suppress salicylic acid (SA)-dependent defences while enhancing jasmonic acid (JA)-mediated responses, conferring resistance to necrotrophs and herbivores (Cameron *et al.* 2013). However, recent findings from tomatoes indicate that MIR can amplify both SA- and JA-responsive genes, showing a more integrated modulation of multiple defence pathways (Fujita *et al.* 2022).

The MIR phenotype has been reported across diverse plant taxa. In tomatoes (*Solanum lycopersicum*), *Funneliformis mosseae* colonisation enhanced resistance to *Alternaria* blight through JA-dependent signalling, and protection was lost in JA-pathway mutants (Song *et al.* 2015). In wheat (*Triticum aestivum*), AMF colonisation with *F. mosseae* reduced susceptibility to powdery mildew (*Blumeria graminis*) via enhanced accumulation of defence metabolites and upregulation of pathogenesis related (PR) and phenylpropanoid biosynthetic genes (Mustafa *et al.* 2017). Similarly, *F. mosseae* was found to prime wheat against *Zymoseptoria tritici* (Allario *et al.* 2025), and *Rhizophagus irregularis* improves rice (*Oryza sativa*) resistance to *Magnaporthe oryzae* which increased grain yield in field settings (Martín-Cardoso *et al.* 2025). These cases demonstrate the relevance of MIR to both monocot and dicot systems and highlight its potential to enhance disease resistance and yield concurrently, a central goal of SDG2.

Mechanistically, MIR involves transcriptional and post-transcriptional reprogramming of immune signalling networks. Mycorrhizal colonisation alters the expression of transcription factors, including WRKY proteins, which regulate both basal and inducible defences. In apple (*Malus domestica*), AMF-induced *WRKY40* enhanced resistance to *Fusarium* by activating β-1,3-glucanase expression (Wang *et al.* 2022). Priming also enhanced physical defences, such as callose deposition, which restricts pathogen entry. Blocking callose synthesis abolishes MIR protection in tomato (Sanmartín *et al.* 2021), indicating its functional importance.

Beyond transcriptional changes, MIR includes modulation of the ubiquitin-proteasome system (UPS), a major post-translational regulator of immunity. E3 ubiquitin ligases, which confer substrate specificity in UPS, target immune regulators for proteasomal degradation, balancing activation and repression of defences (de Vega, Newton and Sadanandom 2018). Several *PUB* and *Arabidopsis Toxicos en Levadura* (*ATL*)family ligases are upregulated in mycorrhizal roots and shoots (Wu *et al.* 2024), suggesting that AMF may fine-tune immune signalling by modifying proteostasis. This regulation may allow plants to remain immunologically alert without incurring the growth penalties of constitutive defence.

The magnitude and outcome of MIR can be highly context dependent. Factors including plant nutrient status, AMF genotype, pathogen lifestyle, and environmental conditions species can influence whether mycorrhiza-induced changes translate into effective resistance (Dejana *et al.* 2022; Weinberger *et al.* 2025). Understanding how these variables

interact in cereal systems is vital if MIR is to be harnessed as a reliable strategy for sustainable crop protection.

Despite compelling evidence from other pathosystems, the effectiveness and molecular basis of MIR in barley, particularly against biotrophic foliar pathogens like *P. hordei*, remains unstudied. Evidence from cereal-pathogen-symbiont tripartite systems is needed to validate MIR as a practical tool for disease management.

In this study, we hypothesised that colonisation of barley by *Rhizophagus irregularis* (a widely occurring AMF) primes the plant's immune system, which could attenuate *P. hordei* infection or disease development. We established a controlled tripartite system with barley (cv. Bowman), *R. irregularis*, and *P. hordei* to test: (1) whether AMF colonisation impacts barley growth or rust infection levels, (2) how AMF affects basal and infection-induced expression of key defence genes (PR proteins, WRKY transcription factors and ubiquitin enzymes), and (3) the broader systemic transcriptional changes and defence pathways modulated by AMF in leaves, including signalling elements like WRKYs and ubiquitin-related proteins. By integrating physiological measurements and transcriptomic analyses, we aimed to elucidate the mechanistic basis of MIR in this system. Our findings are discussed in the context of sustainable agriculture – how harnessing AMF symbioses could reduce reliance on chemical fungicides and improve crop resilience, contributing to food security under climate change.

Materials and Methods

Experimental design

A 2 x 2 factorial experiment was conducted to assess the impact of AMF colonisation and foliar pathogen infection on barley. Treatments comprised: (i) **Control** (no AMF, no pathogen), (ii) **AMF** only (*Rhizophagus irregularis*), (iii) ***Puccinia*** *hordei* only, and (iv) **both** AMF and *P. hordei*. Each treatment group included six biological replicates. Barley was grown for four weeks, then inoculated with *P. hordei* spores. The experiment was terminated one-week post-inoculation (Figure 1).

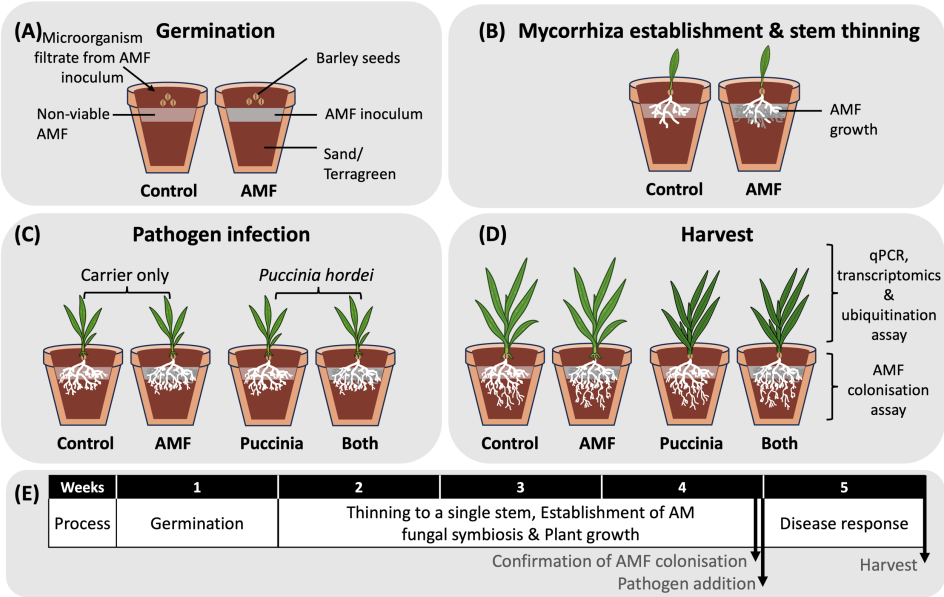

Figure 1 Overview of the experimental setup showing (A) treatment setup, (B) AMF colonisation phase when pots were thinned to a single stem, (C) rust inoculation, (D) final harvest and (E) experimental timeline.

132 **Plant growth conditions and AMF inoculation**

133 Seeds of *Hordeum vulgare* cv. Bowman, a rust-susceptible cultivar, were surface sterilised
134 and sown in a 1:1 mixture of autoclaved coarse silica sand and Terragreen® clay. Each 1 L
135 pot was supplemented with 0.5 g L^{-1} bonemeal and watered twice weekly with phosphate-
136 free Hoagland's solution to promote mycorrhization. Plants were grown in a controlled-
137 environment chamber at 21°C under a 16 h light/8 h dark photoperiod.

138 AMF treated plants received 20 g L^{-1} commercial *Rhizophagus irregularis* inoculum
139 (PlantWorks, UK); controls received autoclaved inoculum and microbial filtrate to standardise
140 non-AMF microbial communities.

141 **Pathogen inoculation**

142 *P. hordei* spores (~1.3×10^4 spores/plant) were applied using talcum powder as a carrier.
143 Control plants received talcum only. To promote infection, plants were enclosed at >95%
144 relative humidity for 48 hours post-infection.

145 **Confirmation of AMF colonisation**

146 AMF colonisation was verified by both microscopy and qPCR on root samples. For
147 microscopy, roots were stained using the ink and vinegar method (Vierheilig *et al.* 1998).
148 Roots were stored in 40% ethanol at 4°C, then cleared with 10% KOH at 70°C for 45
149 minutes. They were stained with 5% Brilliant Black ink (Pelikan) in 5% acetic acid, destained
150 in 1% acetic acid, and mounted on slides with glycerol.

151 For AMF qPCR, DNA was extracted from roots using the DNeasy Plant Mini Kit (Qiagen).
152 Each 10 µL qPCR reaction included Fast SYBR™ Green Master Mix (Thermo Fisher
153 Scientific), 350 nM of each primer, and ~10 ng of root DNA. AMF were quantified using the
154 18S rRNA gene with AMF-specific primers AMG1F and AM1 (Table S1; Helgason et al. 1998;
155 Hewins, Carrino-Kyker and Burke 2015), normalised to barley *Elongation Factor 1-α* using
156 primers HvEF1a-qF and HvEF1a-qR (Hua *et al.* 2015). Triplicate reactions were run on a
157 7500 Fast Real-Time PCR System. The program comprised an initial denaturation at 95°C
158 for 20 s, followed by 40 cycles at 95°C for 10 s and 60°C for 30 s, then a melt curve from 60-
159 95°C. Data were analysed with Applied Biosystems 7500 Software (v2.3) using the Pfaffl
160 method for relative quantification.

161 **Rust infection quantification**

162 Pathogen biomass in leaves was quantified by qPCR (see Gene expression analysis for
163 conditions). Leaf RNA was extracted and converted to cDNA (see below), and *P. hordei*-
164 specific primers targeting rust Elongation Factor 2 gene – *PhEF2* (***Error! Reference source***
165 ***not found.***) were used to measure pathogen cDNA relative to barley cDNA (using a barley
166 endogenous actin gene as reference – *HvACT*). This provided a relative infection index for
167 each plant.

168 **Barley resource allocation measurements**

169 Root and shoot dry weights were recorded at harvest. Plants were divided into above- and
170 below-ground fractions and dried at 60°C until a constant weight was reached. Total biomass
171 and root:shoot ratio were calculated.

172 **RNA extraction and cDNA synthesis**

173 Total RNA was extracted from barley leaf tissue using a phenol-chloroform-isoamyl alcohol
174 method with LiCl precipitation, adapted from established protocols (Verwoerd, Dekker and
175 Hoekema 1989). Briefly, frozen plant material was ground at -20 °C, extracted with warmed
176 phenol:chloroform:isoamyl alcohol (25:24:1), and subjected to sequential organic washes.
177 The aqueous phase was precipitated with 1/3 volume of 8 M LiCl overnight at 4 °C, and the
178 resulting pellet was washed with 70% ethanol. A secondary precipitation with sodium acetate
179 and 96% ethanol was performed to ensure purity, and RNA pellets were finally resuspended
180 in RNase-free water. cDNA was synthesised using the SuperScript™ III Reverse
181 Transcriptase (Invitrogen) according to the manufacturer's instructions.

182 **Gene expression analysis**

183 Quantitative RT-PCR was used to assess the expression of immune marker genes, *PR1b*,
184 *PR2*, *PR3*, and *WRKY28*. Reactions used SYBR™ Green Universal Master Mix (Applied
185 Biosystems, Thermo Fisher Scientific). Reactions were conducted in technical triplicates,
186 each containing 2 µL of cDNA and 200nM gene-specific primers (**Error! Reference source**
187 **not found.**). The reactions were run on a QuantStudio 5 system (Applied Biosystems,
188 Thermo Fisher Scientific). The cycling conditions were hold stage at 50°C for 2 minutes and
189 95°C for 10 minutes; 40 cycles of 95°C for 15 seconds, 60°C for 1 minute; and a melt curve
190 at 95°C for 15 seconds, 60°C for 1 minute, and 95°C for 15 seconds. Relative expression
191 was calculated by the ΔΔCt method and normalised to barley actin.

192 **RNA sequencing and transcriptomics**

193 RNA sequencing was conducted on a subset of samples (n=3 per treatment). RNA
194 sequencing was performed by Novogene Company Limited using Illumina NovaSeq Platform
195 (150bp PE). Reads were quality assessed using FASTQC (Andrews 2010). Adapter
196 sequences were trimmed using Trimmomatic (Bolger, Lohse and Usadel 2014). After adapter
197 and quality trimming, we obtained on average 43.69, 42.83, 32.90, and 44.11 Mb clean
198 reads from the Control, AMF, Puccinia, and AMF + Puccinia treatments, respectively (Table
199 S2), with a similar GC content across treatments (53.34%).

200 Reads were aligned and annotated against barley genome (MorexV3) using Histat2 (Kim *et*
201 *al.* 2019). Gene count data was quantified and standardised using HTseq2 (Putri *et al.* 2022).
202 Differential analysis was performed using edgeR (Chen *et al.* 2025) in R version 4.3.0 (R
203 Core Team 2024) by filtering out CPM < 1 and normalising using a TMM method (Robinson
204 and Oshlack 2010). differentially expressed genes (DEGs) were defined as: FDR < 0.05, FC
205 > 2, logCPM > 2. Gene Ontology (GO) enrichment analysis of DEGs was done using
206 g:Profiler with FDR correction. Hierarchical clustering (Ward's method) of DEGs was
207 employed to identify groups of genes with similar expression patterns across the four
208 conditions. RNA-sequencing data generated in this study have been deposited in the NCBI
209 Sequence Read Archive (SRA) under accession number PRJNA1328502.

210 **Ubiquitination assay**

211 Given the enrichment of ubiquitin-related genes in AMF-treated plants, global protein
212 ubiquitination was assessed by Western blot. Total protein was extracted from frozen barley
213 leaves using a RIPA-like buffer adapted from established plant protocols (Chico *et al.* 2020).
214 Briefly, tissue was ground under liquid nitrogen and homogenised in extraction buffer (20
215 mM Tris pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.5% NP40, 0.5% SDS, 0.5% sodium

216 deoxycholate) supplemented with protease inhibitors, 50 µM MG132, and 10 mM N-
217 ethylmaleimide (NEM). Extracts were vortexed, incubated on ice for 10 min, and centrifuged
218 at 10,000 rpm for 15 min at 4 °C. The supernatant was collected for downstream analysis.

219 Protein extracts were heated in SDS sample buffer with 50 mM DTT at 80°C for 10 minutes
220 and resolved by SDS-PAGE. Proteins were transferred to a nitrocellulose membrane via wet
221 transfer at 20 V overnight. Transfer efficacy was verified with Ponceau staining. Membranes
222 were blocked with 5% milk in PBST for 2 hours, then incubated with primary antibodies
223 (Poly-ubiquitin P4D1 at 1:2500, Santa Cruz Biotechnology; Actin at 1:5000, Agrisera; actin
224 was used as a loading control for normalisation) for 1.5 hours, followed by three 5-minute
225 washes in PBST. Secondary antibody, anti-mouse 1:5000 from cell signalling, incubation
226 lasted 1 hour, followed by three additional washes. Detection was performed using a
227 chemiluminescent substrate (SuperSignal™ West Pico PLUS, ThermoFisher Scientific) and
228 visualised with LI-COR® Odyssey Fc. With band intensity quantified using Image Studio 6.1.

229 **Statistical analysis**

230 Physiological and molecular data were analysed using two-way ANOVA, followed by Tukey's
231 HSD post-hoc tests ($P < 0.05$). RNA-seq analyses employed FDR-corrected thresholds
232 (FDR < 0.05, FC > 2, logCPM > 2). All data are presented as mean ± standard error.

233 **Results**

234 **AMF alone does not reduce rust infection or improve biomass under pathogen**
235 **challenge**

236 Microscopy revealed abundant arbuscules and hyphae in inoculated roots, and qPCR
237 confirmed AMF colonisation, while controls showed negligible amplification (Figure 2 A-B).
238 One week after *P. hordei* infection, AMF presence did not significantly affect rust severity.
239 Both AMF-colonised and non-colonised plants developed similar densities of rust pustules.
240 *P. hordei*-specific gene measurements (PhEF2) showed only a slight, non-significant
241 increase in Puccinia in AMF-infected plants (Figure 2 C).

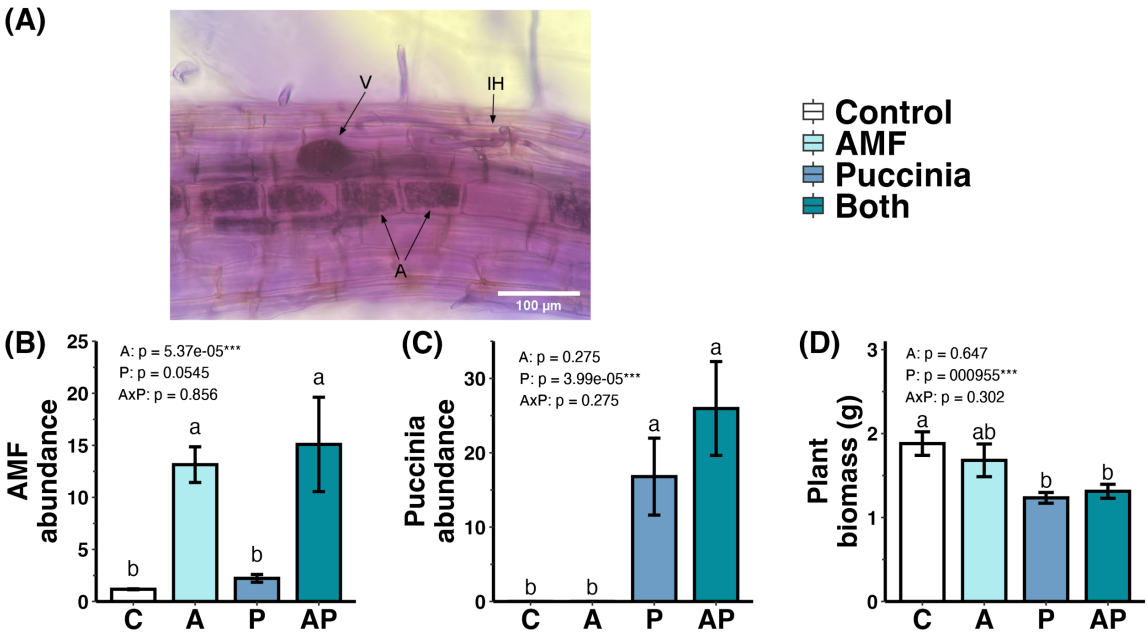

Figure 2 Endpoint measurements of AMF colonisation, Puccinia infection, and plant biomass after one week of Puccinia hordei infection. (A) Photograph of barley roots stained to show AMF colonisation, highlighting vesicles – V, intraradical hyphae – IH, and arbuscules – A. (B) Relative AMF DNA in barley roots, quantified as AMF copy number normalized to HvEMF1α, five weeks post-inoculation. (C) Relative abundance of Puccinia in barley leaves, quantified as PhEF2 copy number normalized to HvACT, one-week post-infection. (D) Total dry biomass of shoots and roots after five weeks of growth. Bars sharing different letters indicate significant differences (P < 0.05). C, control; A, AMF addition; P, Puccinia addition; AP, AMF plus Puccinia addition (mean ± SE, n = 6).

All infected plants, whether AMF-colonised or not, had ~20% less biomass than uninfected controls (p = 0.000955), with no significant AMF x pathogen interaction on biomass (Figure 2D). The root:shoot ratio remained unchanged (Figure S1), indicating a proportional reduction in overall growth rather than specific resource allocation effects.

Importantly, barley growth was not adversely affected by AMF (Figure 2 D). There were no significant differences in shoot or root dry weights between AMF-inoculated and control plants. This suggests AMF-barley partnerships can form without compromising growth, satisfying a key condition for using AMF in agriculture.

Overall, *P. hordei* remained virulent, causing substantial biomass losses regardless of AMF presence. Although, anecdotally, pustule formation appeared slightly delayed by ~1–2 days in AMF plants, disease progression was similar within a week. This lack of a strong phenotypic resistance prompted further investigation into molecular interactions to see if AMF might prime immune responses not reflected in infection or growth metrics.

AMF primes the expression of barley defence genes during *P. hordei* infection

Although AMF did not reduce rust severity, we hypothesised that it might prime host defences. To test this, we measured transcript levels of salicylic acid (SA)-responsive PR genes and a WRKY transcription factor. The expression patterns were consistent with classic priming: AMF alone did not activate these defence genes, with PR1, PR2 and PR3 remaining at low levels in healthy leaves, comparable to non-colonised controls (Figure 3).

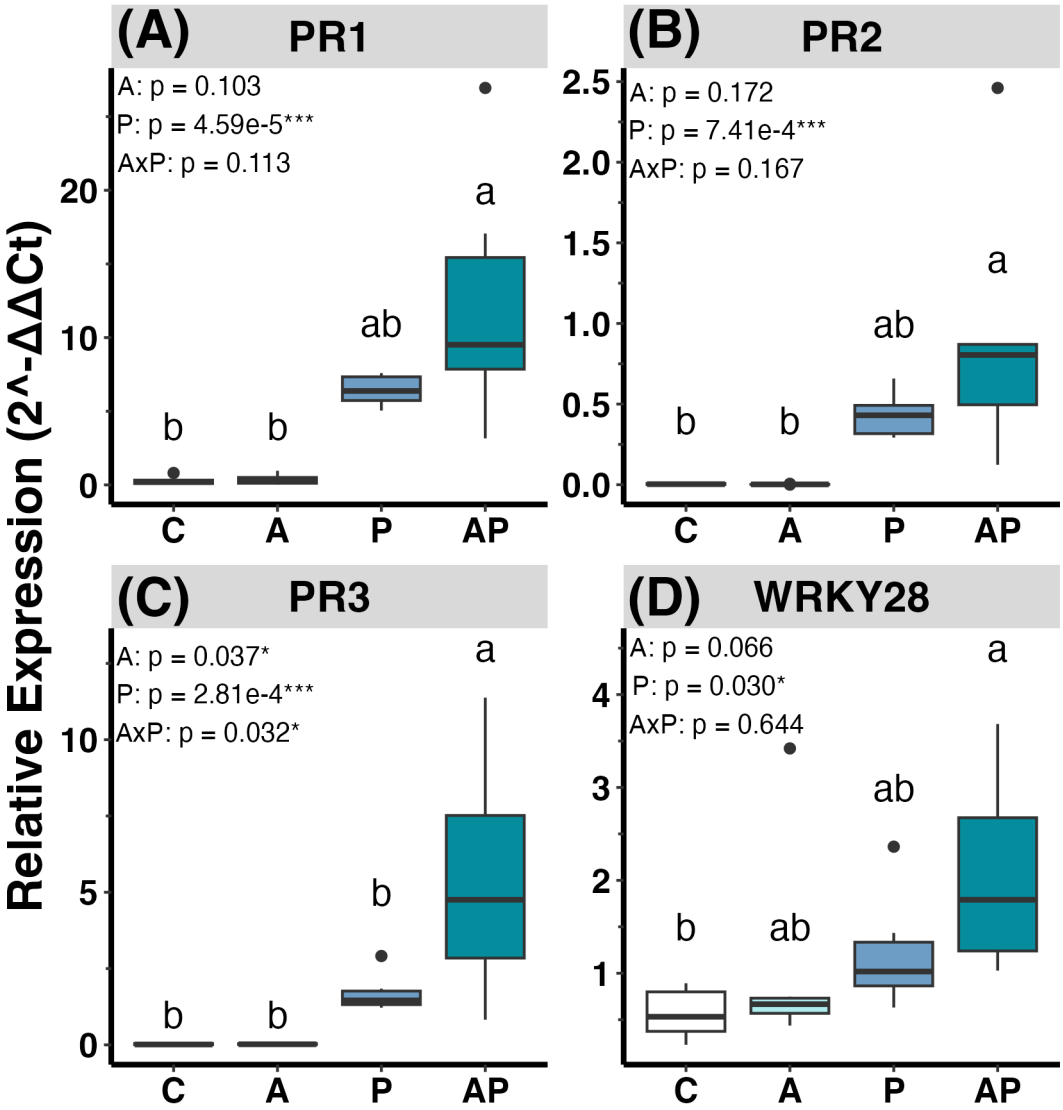

Figure 3 Relative expression of barley immune marker genes (A) PR1, (B) PR2, (C) PR3, (D) WRKY28. Gene expression is relative to actin (HvACT); Bars sharing different letters indicate significant differences (P < 0.05). C, control; A, AMF addition; P, Puccinia addition; AP, AMF plus Puccinia addition (median ± 1.5 IQR, n = 6).

Upon *P. hordei* infection, however, AMF-colonised plants showed a stronger induction of defence genes than pathogen-only plants. While rust infection elevated PR1, PR2 and PR3 transcripts in non-mycorrhizal plants, their expression was significantly higher in AMF-colonised infected plants (Figure 3 A-C). For example, PR1 was barely detectable in healthy leaves, strongly induced by rust in non-AMF plants, and even more elevated in AMF-colonised infected plants. These synergistic increases illustrate that AMF amplifies pathogen-triggered SA defences.

We observed a similar trend for the regulatory transcription factor *WRKY28*, which showed minimal expression in healthy leaves, induction by rust, and approximately doubled expression in AMF-colonised infected plants (Figure 3 D). This suggests that mycorrhizal priming not only boosts PR gene induction but also activates higher-order immune regulators.

Together, these results show that priming occurs at the transcriptional level. AMF alone did not trigger defences but prepared plants to mount a stronger response upon infection. Although this did not reduce pathogen load within the one-week timeframe, it may slow

disease progression, with rust symptoms appearing slightly delayed. The lack of immediate protection despite strong PR induction suggests that *P. hordei* continues to suppress or evade host defences, or that timing and physiological constraints limit the effectiveness of the primed response.

AMF reprograms the barley leaf transcriptome

For a comprehensive view of the transcriptomic changes, we then performed RNA-sequencing on the leaves. AMF colonisation of healthy leaves altered 526 genes (344 upregulated, 182 downregulated; Figure 4 A). Upregulated genes were enriched for defence, stress responses, and protein modification, notably ubiquitination, including multiple E3 ligases (Figure 4 B). This suggests AMF enhances the host's capacity to regulate immunity via protein turnover. Induction of sugar transporters and amino acid synthesis genes likely reflects increased nutrient exchange, while downregulated transcripts related to growth, photosynthesis, and gibberellin signalling indicate a moderated growth programme. These shifts did not affect overall biomass (Figure 2 D), consistent with a primed but balanced state.

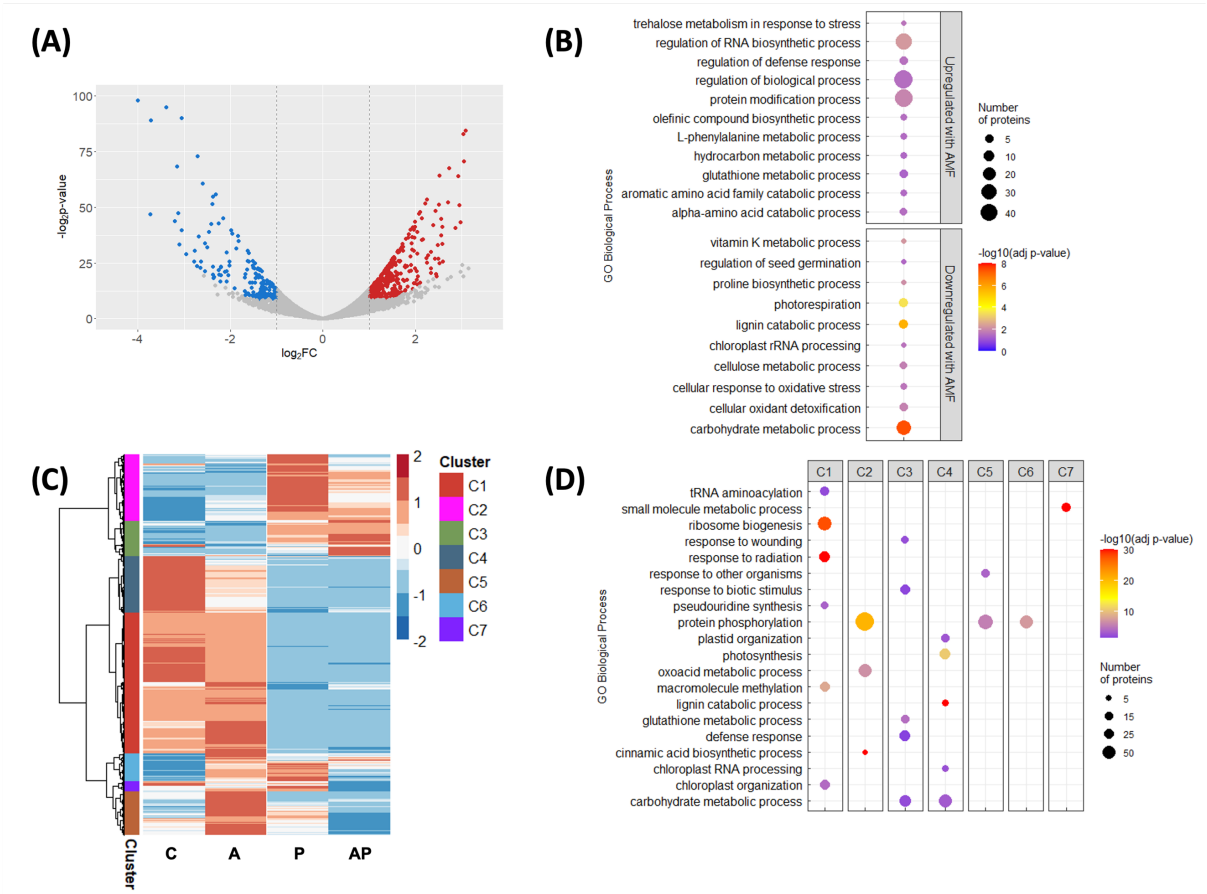

Figure 4 AMF induced transcriptional changes in leaves in uninfected (A-B) and P. hordei infected plants (C-D) n=3. (A) Differential expression analysis identified 526 genes significantly regulated by AMF in uninfected leaves (Control vs AMF; fold change > 2, FDR < 0.05, logCPM > 2). (B) GO enrichment of AMF-responsive genes in AMF plants relative to Control, using g:Profiler with Benjamini–Hochberg correction (adjusted P < 0.05, term size > 2). (C) Scaled expression of genes differentially expressed across treatments (C, control; A, AMF addition; P, Puccinia addition; AP, AMF plus Puccinia addition), based on all pairwise comparisons (FC > 2, FDR < 0.05, logCPM > 2). Genes were clustered into seven co-expression groups using Ward's method and Euclidean distance (dissimilarity threshold = 20). (D) GO enrichment of clusters from (C), using g:Profiler with g:SCS correction (adjusted P < 0.05).

313 During *P. hordei* infection, 231 genes were differentially expressed in AMF-colonised plants
314 compared with non-mycorrhizal controls (163 upregulated, 68 downregulated). AMF reduced
315 the overall scale of rust-induced transcriptional reprogramming (~15% fewer induced and
316 ~30% fewer repressed genes), but the pattern of expression was distinct. Cluster analysis
317 revealed that AMF amplified defence-related genes, including PRs and glutathione-
318 associated transcripts (Cluster 3), while dampening broad kinase- and receptor-driven
319 signalling cascades (Clusters 2 and 7; Figure 4 C-D). Genes induced by AMF alone but
320 suppressed during co-infection (Clusters 5 and 6) may reflect negative feedback on pre-
321 activated signalling modules. GO analysis of AMF-specific DEGs during infection highlighted
322 targeted upregulation of fungal defence, chitinase activity, and hormone signalling, while
323 genes involved in broader stress responses, vesicle trafficking, and metabolic processes
324 were suppressed. Together, these results suggest that AMF reduces global transcriptional
325 upheaval while sharpening immune outputs, allowing for a faster and more focused defence
326 response. Although this reprogramming did not lessen rust severity within one week, it
327 indicates a mechanism by which AMF may delay pathogen development and strengthen
328 resilience under field conditions. We next examined WRKY transcription factors to explore
329 potential regulatory mechanisms underlying this response.

330 **AMF regulates immune signalling pathways**

331 To investigate AMF-mediated modulation of plant immune regulation, we examined key
332 components of canonical immune signalling pathways. Given the strong induction of
333 transcription factors in AMF-colonised plants, we focused on the WRKY family, which are
334 known regulators of biotic and abiotic stress responses. WRKYs were highly enriched, with
335 nearly 50% of annotated barley WRKY genes showing differential expression. In healthy
336 plants, AMF generally upregulated WRKY expression, whereas in infected plants, AMF
337 suppressed most infection-induced WRKYs (Figure 5 A). Notable exceptions included
338 *WRKY27*, *WRKY63*, and *WRKY1/38*, which were induced even more in co-infected plants.
339 Most differentially expressed WRKYs belonged to Classes II and III, consistent with a role in
340 biotic stress regulation.

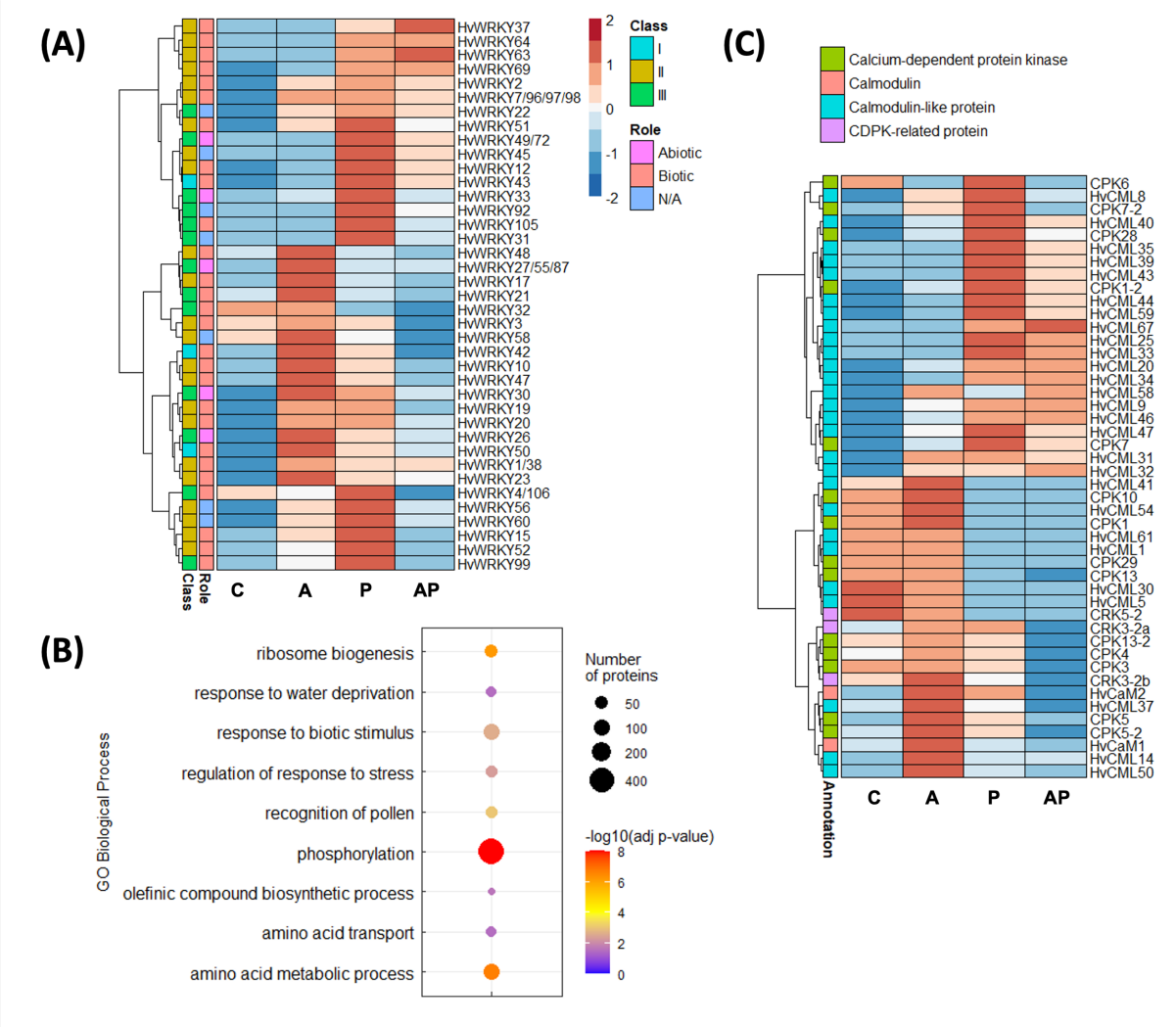

Figure 5 AMF modulation of WRKY transcription factor expression and calcium signalling; n=3. (A) Scaled expression of differentially expressed WRKY genes across four treatments (C, Control; A, AMF; P, Puccinia; & AP, AMF and Puccinia), based on all pairwise comparisons (FC > 2, FDR < 0.05, logCPM > 2). Genes were annotated by WRKY class (Kan et al. 2021) and known roles in biotic or abiotic stress responses. (B) GO enrichment of 4,416 DEGs containing a predicted WRKY-binding motif within 500 bp upstream promoter regions (FIMO, P < 0.001). (C) Scaled expression of differentially expressed calcium signalling genes across the same four treatments (FC > 2, FDR < 0.05, logCPM > 2). Calmodulin and calmodulin-like genes were annotated based on (Cai et al. 2022); calcium-dependent protein kinase (CDPK) and CDPK-like genes were annotated based on their closest Arabidopsis homologues.

To assess the regulatory reach of WRKY factors, we then scanned the 500 bp upstream promoter regions of all DEGs for W-box elements (TTGACY), the canonical WRKY binding motif. Approximately 80% of DEGs contained at least one W-box. This is significantly more than random expectation, suggesting that WRKY transcription factors are major drivers of the transcriptomic changes we observed in MIR. Gene ontology enrichment analysis showed over 400 genes involved in phosphorylation, alongside terms related to biotic stimulus, stress response, and olefinic compound biosynthesis (Figure 5 B).

Since WRKYs are themselves regulated via phosphorylation by calcium-dependent protein kinases (CDPKs), and AMF colonisation upregulated calcium-binding proteins, we also examined calcium signalling components (Figure 5 C). Several CDPKs, calmodulin, and calmodulin-like genes were upregulated in AMF-colonised healthy plants, suggesting that AMF primes WRKY activity through calcium-dependent pathways.

To assess how AMF modulates hormone-related pathways, we analysed transcriptional changes in the ethylene, SA, and JA pathways. In healthy AMF-colonised plants, ethylene-responsive genes such as *EIN3* were upregulated, while defence-associated ethylene responses remained unchanged (Figure S2), indicating a shift towards metabolic rather than immune regulation. In contrast, several JA pathway genes, including *LOX* and downstream JA-responsive transcripts, were expressed at lower levels in AMF-colonised diseased plants (AP) than in plants infected with *P. hordei* alone, suggesting that AMF dampens JA-associated defences during pathogen challenge. By comparison, SA signalling was enhanced. AMF colonisation increased the induction of SA-responsive PR genes during infection, consistent with priming. Expression of *NPR1*, the central SA co-activator, was also upregulated in AMF plants but suppressed by *P. hordei*, regardless of AMF status (Figure S3). This contrast, strong PR gene induction despite reduced *NPR1* transcripts, indicates that AMF primes SA defences, while the pathogen may actively target *NPR1* as a virulence strategy. Overall, AMF reshaped transcriptional profiles of hormone pathways, modulating transcription factors and protein turnover without impairing immune activation.

AMF induces expression of ubiquitination machinery genes

GO analysis showed the enrichment of ubiquitination-related processes among AMF-induced genes, identifying numerous altered E3 ubiquitin ligase genes. To validate and extend this observation, we assessed global protein ubiquitination across treatments. Western blotting showed that *R. irregularis* colonisation decreased ubiquitinated protein conjugates in barley leaves (Figure 6 A/S4). Healthy AMF-colonised plants showed a slightly lower ubiquitin signal compared to healthy controls. This reduction was more pronounced in Puccinia-infected AMF plants, which displayed visibly lower ubiquitination levels than Puccinia-only treated plants. Although this trend was not statistically significant due to variability, the visual observation is clear: ubiquitination is highly reduced in AMF-colonised infected plants. This could mean that proteasomal degradation of proteins is less active or that specific proteins are protected from ubiquitin-tagging in the mycorrhizal state.

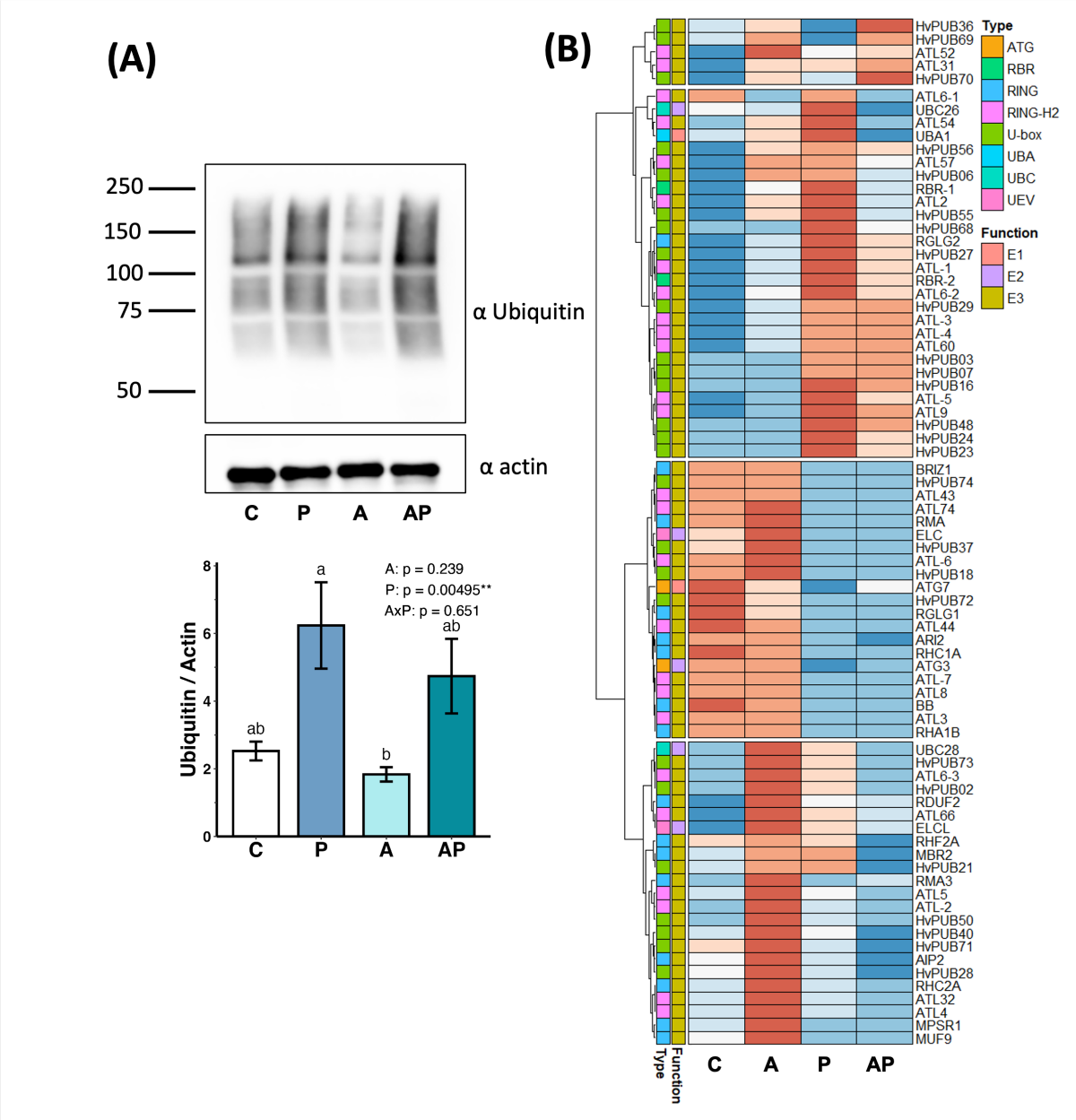

Figure 6 AMF influence on ubiquitination at protein and gene levels. (A) Western blot analysis of ubiquitinated proteins, normalised to actin (all replicates shown in Fig. S2). Bars with different letters indicate significant differences (P < 0.05; mean ± SE; n = 3). (B) Scaled expression of differentially expressed ubiquitin-related genes (FC > 2, FDR < 0.05, logCPM > 2) across treatments. Genes are annotated by function and type, with PUB genes classified according to Ryu et al. (2019) and closest Arabidopsis homologues. Treatments: C, control; A, AMF; P, Puccinia; AP, AMF + Puccinia.

To see whether ubiquitin changes were due to decreased protein turnover or changes to the ubiquitination pathway, we looked at expression of differentially expressed ubiquitination enzymes. Many E3 ligase genes were differentially expressed, as well as some ubiquitin E1 (activating enzyme) and E2 (conjugating enzyme) (Figure 6 B). Expression patterns clustered into five distinct groups, with one cluster upregulated by AMF in healthy and infected plants (Figure 6 B). This small group included several RING-type E3 ligases from the ATL family and PUB family, as well as a ubiquitin-conjugating enzyme**.** The consistent upregulation of these enzymes suggests that AMF actively enhances certain ubiquitination pathways. The specific increase of membrane-associated ATL/PUB E3s in AMF plants hints

that the symbiosis could be selectively promoting ubiquitination of specific membrane or signalling proteins, potentially to fine-tune immune signalling.

In contrast, the biggest cluster of ubiquitin-related DEGs were downregulated in AMF-colonised plants during infection (Figure 6 B); which correlates with the reduced global ubiquitin levels on the Western blots for co-infected plants (Figure 6 A). This cluster contained the E1 enzyme UBA1 and an E2 ubiquitin conjugating enzyme associated with formation of K48-linked chains UBC26. Downregulation of these enzymes suggests a reduction in proteasome-targeted degradation, which could stabilise immune proteins and impair pathogen exploitation of the ubiquitin-proteasome system, representing a mechanistic facet of AMF-induced immune modulation.

Discussion

Achieving global food security under increasing biotic stress demands sustainable strategies that reduce crop losses while minimising chemical input. In this study, we investigated how the AMF *R. irregularis* modulated barley immunity and growth during infection with the biotrophic foliar pathogen *P. hordei,* framing our findings within the context of UN Sustainable Development Goal 2: Zero Hunger.

Despite successful AMF colonisation, we observed no significant reduction in rust colonisation or mitigation of *P. hordei*-induced biomass loss after one week of infection. The lack of a strong reduction in rust formation or biomass loss in AMF-colonised barley might seem to contradict the dramatic increases we observed in PR gene expression and other defences. However, priming is often an anticipatory strategy that does not necessarily manifest as complete disease immunity; instead, it can lead to subtler benefits such as slower pathogen growth or reduced sporulation. This aligns with previous findings showing that MIR does not always result in immediate or visible phenotypic protection but confers partial resistance or tolerance (Pozo *et al.* 2002; Pozo and Azcón-Aguilar 2007; Jung *et al.* 2012).

Similarly, our anecdotal observation that rust appearance was slightly delayed in AMF plants hints at a disease retardation effect that our single time-point measurement did not fully capture. Future experiments quantifying rust development over time (e.g. latent period, spore production) could reveal benefits of MIR that static measurements miss. The absence of growth penalties supports the integration of AMF into cropping systems as a low-risk biostimulant, particularly if longer-term or field-based studies indicate delayed protective effects.

Transcriptomic profiling showed that AMF primes barley immunity at the molecular level. Defence-related genes, PR1, PR2, PR3 and WRKY28, were more strongly induced upon rust infection in AMF-colonised plants than in AMF-free controls. This transcriptional priming is characteristic of MIR, where immune responses are potentiated but not constitutively activated to avoid fitness costs associated with chronic defence (Pieterse *et al.* 2012; Conrath *et al.* 2015). Importantly, AMF alone did not induce PR gene expression, suggesting immune compatibility and minimal trade-offs under non-stress conditions.

AMF reprogrammed the leaf transcriptome of healthy plants towards enhanced immune readiness. Differentially expressed genes were enriched for responses to biotic stimuli, protein modification, and signalling pathways. Notably, several receptor-like kinases, secondary metabolism enzymes, and E3 ubiquitin ligases were upregulated. Concurrently,

transcripts linked to cell wall biosynthesis, gibberellin signalling, and photosynthesis were downregulated, reflecting a moderated growth programme. This shift suggests a reallocation of transcriptional investment from growth to immune preparedness, consistent with MIR (Jung *et al.* 2012; Huot *et al.* 2014). The transcriptional shift resembles a growth-vs-defence trade-off which in theory could reduce yield, though no biomass loss occurred.

Under pathogen challenge, AMF reduced the scale of Puccinia-induced transcriptional reprogramming. The total number of differentially expressed genes was lower in AMF-colonised plants, yet the magnitude of induction for specific defence genes was higher. This pattern suggests a more efficient, targeted immune response, consistent with prior reports that mycorrhizal plants activate key defence pathways more rapidly or strongly upon pathogen attack, even when global transcriptomic changes are limited (Cervantes-Gámez *et al.* 2016). Co-expression clustering highlighted both synergistic and antagonistic effects of AMF on pathogen-induced gene expression. Clusters enriched for defence genes (e.g. *PRs*, glutathione metabolism) were amplified by AMF, whereas clusters of signalling kinases and receptors were attenuated, suggesting AMF suppresses broad signalling cascades while enhancing specific responses.

AMF-induced priming also entails subtle shifts in hormone signalling. Colonised plants favoured SA-associated defences – evidenced by stronger PR gene induction – while downregulating certain JA pathway elements (e.g. a *lipoxygenase* gene) during rust infection. Such SA-JA antagonism is a well-established feature of plant immunity and is often exploited by pathogens to their advantage (Hou and Tsuda 2022). For example, in wheat, the stripe rust effector protein PNPi binds directly to the SA master regulator NPR1, blocking its ability to activate PR gene expression (Wang *et al.* 2016). This effector-mediated suppression of NPR1 suggests that *P. hordei* might employ a similar strategy to counteract the host's SA-dependent defences, which could explain why robust PR gene expression in AMF-colonised plants did not translate into significantly lower rust proliferation.

WRKY transcription factors emerged as key regulators of this primed immune state. Nearly half of annotated barley WRKYs were differentially expressed, and promoter motif analysis revealed enrichment of WRKY-binding sites among DEGs. Their preferential induction in mycorrhizal plants under rust challenge suggests that AMF colonisation does not indiscriminately activate the WRKY network but instead tunes a subset of stress-responsive regulators that are most relevant for immune priming. AMF modulated WRKY expression context-dependently, upregulating some WRKYs in healthy plants and dampening infection-induced WRKYs in co-infected plants, potentially to prevent immune overactivation. The involvement of calcium-dependent protein kinases and calmodulin-related genes further supports the role of calcium-WRKY signalling modules in MIR, consistent with recent findings in other systems highlighting calcium-dependent WRKY activation during MIR (Gu *et al.* 2025).

Strikingly, AMF also influenced ubiquitination pathways. E3 ligases, particularly PUB and ATL family members, were differentially expressed, and global ubiquitin levels were reduced in AMF-colonised plants during infection. Downregulation of key components of the K48-linked ubiquitin-proteasome degradation pathway (UBA1 and UBC26) suggests reduced proteasomal turnover. This may protect immune proteins from degradation and restrict pathogen hijacking of host ubiquitination machinery, representing a novel mechanism of MIR.

Conclusion

Although AMF colonisation did not reduce pathogen burden at the phenotypic level in this study, the extensive transcriptional and post-translational changes observed provide strong evidence for immune priming. These responses may confer delayed or context-dependent protection not captured within the experimental timeframe. Crucially, AMF colonisation occurred without compromising plant growth, underscoring the potential of *R. irregularis* or other AMF as a sustainable means of enhancing crop resilience. Our findings in the barley-*rust* pathosystem 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. Realising this potential will require integrating AMF-based strategies into crop production systems, alongside continued research into the ecological and mechanistic context-dependencies of MIR. In doing so, we may harness beneficial soil fungi to support plant health and productivity in ways that align directly with the goals of Zero Hunger and sustainable agriculture.

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Author Contributions

BO-P and TH designed the research; CM and KB performed the experiments and analysed data; CM and KB wrote the first draft. All authors contributed to the writing of the paper. CM and KB contributed equally to this work.

Conflict of Interest

The authors declared that there is no conflict of interest.

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Data availability

RNA-seq data generated in this study have been deposited in the NCBI Sequence Read Archive (SRA) under accession number PRJNA1328502. All other data supporting the findings of this study are available from the corresponding author upon request.