Repression of pattern-triggered immune responses by hypoxia

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Abstract

Combined abiotic/biotic stresses frequently occur in nature. Yet, relatively little is known about how plants regulate the crosstalk between stress response pathways to trigger a coordinated response to combined stresses. Protein degradation by the ubiquitin/proteasome system (UPS) is central to the regulation of plant responses to multiple stresses, including the crosstalk between pathways. The Arg/N-degron pathway, a subset of the UPS, targets proteins based on their N-termini. In plants, it has been implicated in the responses to both biotic and abiotic stresses, while being a key regulator of hypoxia response through the degradation of ERF-VII transcription factors, which orchestrate the onset of the hypoxia response program. Because of its central position in regulating multiple (a)biotic stresses, we hypothesized that the Arg/N-degron pathway could act in the crosstalk between abiotic/biotic stresses. In exploring this possibility using the model pathogen-associated molecular pattern flg22 to elicit pattern-triggered immunity (PTI), we uncovered a link between the transcriptional response programs to hypoxia and to flg22. Combined hypoxia/flg22 treatments further showed that hypoxia represses the flg22 transcriptional program, as well as the expression of pattern recognition receptors, MAPK signalling and callose deposition during PTI, through mechanisms that are at least partly dependent on the ERF-VIIs. These findings are of relevance to understanding the trade-offs between plant responses to combined abiotic/biotic stresses in the context of our efforts to increase crop resilience to global climate change. Our results also show that the well-known repressive effect of hypoxia on innate immunity in animals also applies to plants.

Significance statement

Understanding how plants regulate the crosstalk between stress response pathways is key to our efforts to increase crop resilience and mitigate yield losses caused by global climate change. Despite the urgency to do so, relatively little remains known about how plants respond to combined stresses, which frequently occur in nature. Here, we show that the hypoxia response program and the basal layer of plant immunity (pattern-triggered immunity or PTI) share components. Our data also show that hypoxia represses several key aspects of the plant's innate immune response, a situation akin to that discovered in

animals decades ago. These findings have implications for our ability to develop resilient crops by limiting the negative trade-offs that exist between hypoxia response and immunity.

Introduction

A key question in biology is 'how do organisms perceive and respond to environmental cues, as well as stresses?'. While much has been learned about plant responses to individual cues and stresses, the question of how plants integrate information from multiple, combined, stresses to trigger a coordinated response has come to the forefront due to the negative impact of global climate change on crop yields. Studies have shown that the transcriptional response programs to combined stresses can differ considerably from those of the respective individual stresses (1-3). However, relatively little is known about the mechanisms underpinning such differential transcriptional outputs. The ubiquitin/proteasome system is a key player in coordinating the crosstalk between stress response pathways (reviewed in (4-6)) by regulating, for example, the stability of transcriptional regulators that coordinate the crosstalk between phytohormone signalling pathways, such as ethylene/jasmonic acid or jasmonic acid/salicylic acid signalling (4, 7-9). The N-degron pathways are a subset of the ubiquitin/proteasome system that target substrate proteins for degradation based on their N-termini (reviewed in (10, 11)). These pathways have been specifically implicated in the responses to a wide range of biotic and abiotic stresses (4, 12-16). However, their roles (and that of their substrates) in the regulation of the crosstalk between abiotic/biotic stress response pathways have not been examined.

The arginylation-dependent Arg/N-degron pathway requires the sequential activity of several enzymatic components to modify the N-terminus of substrate proteins before degradation (Fig. 1A). As some of these components are oxygen-dependent, the pathway functions as a *de facto* oxygen sensor that suppresses hypoxia responses in oxygenated conditions *via* the turnover of group VII ETHYLENE RESPONSE FACTOR (ERF-VII) transcription factors, which act as master regulators of the hypoxia response program (17-20). In this case, the N-terminal cysteine (Cys) residue of ERF-VIIs is oxidized by PLANT CYSTEINE OXIDASE enzymes (PCOs) (21-23), followed by the conjugation of arginine (Arg) to their N-

terminus by so-called Arg-transferases (ATE1 and ATE2 in Arabidopsis) (24, 25). This N-terminal Arg (a destabilizing residue) is an N-degron that can be recognized by the PROTEOLYSIS6 (PRT6) E3 ligase, resulting in the degradation of the target proteins (11, 25-27). Arabidopsis *ate1 ate2* (noted *a1a2*) and *prt6* mutants accumulate the ERF-VII transcription factors and consequently display a constitutive activation of the hypoxia response program (18).

The Arg/N-degron pathway has also been implicated in the response of plants to biotic stresses, with varying susceptibility/resistance phenotypes (13-16). The *a1a2* and *prt6* mutants were found to be more susceptible to clubroot gall caused by the biotrophic protist pathogen *Plasmodiophora brassicae* and this susceptibility was dependent on the accumulation of the ERF-VIIs in the Arg/N-degron pathway mutant backgrounds (14). Alternatively, overexpression of some ERF-VIIs correlates with increased resistance to the necrotrophic fungus *Botrytis cinerea* (28). This points to a potential double role of ERF-VIIs in the regulation of hypoxia response and plant defenses against pathogens, but their role in plant innate immune pathways or in the crosstalk between hypoxia response and plant immunity have not been explored in detail.

Considering that Arg/N-degron pathway mutants are affected for their defenses against a range of pathogens with different lifestyles, one hypothesis is that a core aspect of the plant innate immune response, such as pattern-triggered immunity (PTI), is mis-regulated in these mutants. The onset of PTI relies on the detection of conserved Pathogen-Associated Molecular Patterns (PAMPs) at the cell-surface by Pattern Recognition Receptors (PRRs) and triggers a transcriptional reprogramming to activate defense-related genes. Here, using flg22 (derived from bacterial flagellin) as a model PAMP and elicitor of PTI, we sought to determine if (i) the Arg/N-degron pathway contributes to the regulation of PTI; (ii) ERF-VIIs play a role in PTI; and (iii) there is a crosstalk between plant responses to hypoxia and to flg22, which could be regulated by the ERF-VIIs. Our transcriptomic analyses of *a1a2* and wild-type seedlings in response to flg22 treatment indicate that ERF-VIIs, as well as part of the downstream hypoxia response program, play a role in the transcriptional response to flg22. The use of combined hypoxia/flg22 treatments further showed that hypoxia represses flg22 transcriptional responses, as well as multiple aspects of PTI, such as MAPK signalling and callose deposition, through mechanisms that are at least partly independent from the ERF-VIIs. In sum, our data reveal a previously unknown crosstalk between hypoxia signalling

and PTI, with hypoxia exhibiting a repressive effect on PTI, which is analogous to the repression of innate immunity by hypoxia in animal cells, such as macrophages (29). This finding is also of relevance to the trade-offs between plant responses to abiotic and biotic stresses in our efforts to increase crop resilience to mitigate the yield losses caused by global climate change.

Results

Transcriptomic response of *a1a2* seedlings to flg22

To explore whether the Arg/N-degron pathway plays a role in the regulation of PTI, we first used RNA-sequencing (RNA-seq) to compare the genome-wide expression changes of *a1a2* and wild-type seedlings treated with 1 µM flg22 for 1 hr (Fig. S1A). When comparing flg22 to mock-treated seedlings (flg/m), the total number of differentially expressed genes (DEGs; adj. *p*-value<0.05 and |log2(FC)|>0.585) was similar in wild-type (Col-0) and in *a1a2* seedlings (~4,400 DEGs), as well as the number of up- and down-regulated genes (~2,800 up-regulated and ~ 1,500 down-regulated genes) (Fig. 1B). Our two datasets had a large overlap with a previously published transcriptomic dataset obtained under comparable experimental conditions (Fig. S1B). As expected, PTI-associated Gene Ontology (GO) terms were also found to be over-represented among the DEGs in wild-type and in *a1a2* mutant seedlings after flg22 treatment (*e.g.* 'innate immune response', 'response to salicylic acid stimulus', 'callose deposition in cell wall', 'MAPKKK cascade', or 'response to oxidative stress') (Fig. S1C and Supplemental Dataset 1). Differences in the transcriptional response of the *a1a2* mutant compared to the wild type were also found, with 700 and 507 DEGs being specific to either *a1a2* or the wild type, respectively, after flg22 treatment (Fig. S1B).

For a more stringent comparison between *a1a2* and wild-type seedlings, we re-analyzed the RNA-seq data by comparing directly the two genotypes under either mock (a1a2/Col-0 (m)) or flg22 treatment (a1a2/Col-0 (flg)) (cut-offs applied: adj. *p*-value<0.05 and |log2(FC)|>0.585). This analysis revealed 104 DEGs in a1a2/Col-0 (m) and 97 DEGs for a1a2/Col-0 (flg) (Fig. 1C). A GO analysis of all DEGs in *a1a2 versus* wild-type seedlings retrieved GO categories related to known functions of the Arg/N-degron pathway (Fig. S1D and Supplemental Data 1), including 'response to hypoxia', 'regulation of seed germination', 'regulation of lipid metabolic process' and 'glycoside metabolic process' (13, 18, 30-32). The

directionality of the gene expression changes was also as expected, with the expression of several known hypoxia response genes being constitutively induced in *a1a2* (Fig. S1E). Of the DEGs identified in both a1a2/Col-0 datasets, 59 were differentially expressed in *a1a2* independently of the flg22 treatment (Fig. S1F) and these common DEGs were associated with GO categories known to be constitutively mis-regulated in *a1a2* mutants, such as 'protein arginylation' and 'response to hypoxia' (13, 18, 30). Relatively few genes (28) were specifically mis-regulated in the *a1a2* mutant in response to flg22 (Fig. S1F). The majority of these 28 DEGs were repressed in *a1a2* compared to the wild type after flg22 treatment (Fig. S1G), with several having known functions in the regulation of plant defenses against pathogens (*e.g. PLANT DEFENSIN2.1* (*PDF2.1*); *VEGETATIVE STORAGE PROTEIN1* (*VSP1*); *CYSTEINE-RICH RLK25* (*CRK25*); *CHITINASE A* (*CHIA*)). We also tested whether there were differences in the amplitude of gene expression changes in DEGs that were common to the two genotypes, and found that in response to flg22, the directionality and amplitude of gene expression changes were comparable in *a1a2* and Col-0 (Fig. S1H).

The *a1a2* mutant is expected to accumulate N-degron pathway substrates, which could result in protein abundance differences without transcriptional changes. We hence also conducted a proteomic comparison of *a1a2* and wild-type seedlings: 74 proteins accumulated differently in at least one of the four comparisons (a1a2 (flg/m); Col-0 (flg/m); a1a2/Col-0 (m) or a1a2/Col-0 (flg); cut-off applied: multi-sample ANOVA with permutation-based FDR< 0.05, followed by Tukey´s *post-hoc* test with FDR<0.05). A comparison of the fold-changes in the proteomic and RNA-seq datasets indicated a strong positive correlation (Pearson r ~ 0.8) (Fig. 1D,E). This included proteins/genes up-regulated by hypoxia response (*e.g.* ADH1 and HB1), and proteins involved in glucosinolate biosynthesis (*e.g.* BCAT4, TGG2, IGMT5), which were found to be less abundant in *a1a2* in agreement with their lower expression (13). A small number of proteins/genes behaved differently in the mock-treated *a1a2* mutant compared to the wild type, with some having known roles in plant defense (*e.g.* SENESCENCE-ASSOCIATED GENE 21 (SAG21) (33)).

Finally, we examined whether the transcriptional differences identified between flg22-treated *a1a2* and wild-type seedlings could be sufficient to affect PTI. We first tested the effect of prolonged flg22 exposure using seedling growth inhibition (Fig. 1F) and root growth inhibition assays (Fig. S1I). Both indicated that the *a1a2* mutant and the wild type were affected in a similar manner by flg22. The production of apoplastic reactive oxygen

species (ROS) upon flg22 treatment was also not affected in the mutant compared to the wild type (Fig. S1J). In contrast, callose deposition assays showed that *a1a2* was negatively affected for callose deposition in response to flg22 treatment (Fig. 1G). This could potentially be a consequence of (i) reduced up-regulation of *GLUCAN SYNTHASE-LIKE5* (*GSL5*), an important callose synthase during flg22 response (34), in *a1a2* seedlings ($\log_2$(flg/m)=0.815) compared to the wild-type ($\log_2$(flg/m)=1.023); or (ii) lower glucosinolate levels in *a1a2* (13), since glucosinolates are important for callose biosynthesis (35) and we also observed lower accumulation and expression of glucosinolate biosynthesis enzymes in flg22-treated *a1a2* seedlings (Fig. 1E). Alternatively, the constitutive activation of hypoxia response in *a1a2* could also contribute to the decreased callose deposition phenotype (*e.g.* callose content changes upon hypoxia treatment of wheat seedlings (36, 37)).

Hypoxia and flg22 transcriptional response programs overlap

Our data with the *a1a2* mutant suggest a potential connection between hypoxia and flg22 responses. In addition, our RNA-seq analysis revealed that genes associated with the GO categories 'response to oxygen levels' and 'response to hypoxia' were enriched among flg22-responsive genes in the wild type (Col-0 flg/m) and in *a1a2* (a1a2 flg/m) (Fig. 2A and Supplemental Dataset 1), further suggesting potential links between hypoxia and flg22 responses. We carried out a similar GO analysis using (i) the similar Arabidopsis flg22 response dataset from Denoux *et al*. (38); and (ii) a dataset composed of conserved flg22-responsive genes in 4 different species of Brassicaceae (termed 'Brassicaceae core PTI') (39). Notably, a similar enrichment for the 'response to hypoxia' and 'response to oxygen levels' was found (Fig. 2A), with several hypoxia-related DEGs common to at least 2 datasets showing the same directionality of expression changes (Fig. S2A). In our Col-0 flg/m dataset, DEGs found in these GO categories included *ERF73/HRE1* (AT1G72360), *ERF71/HRE2* (AT2G47520; Fig. S1E), *HEMOGLOBIN1* (*HB1*; AT2G16060; Fig. S1E) or *Alanine AMINOTRANSFERASE1* (*AlaAT1*; AT2G47520), as well as *ENHANCED DISEASE SUSCEPTIBILITY 1* (*EDS1*) and *PHYTOALEXIN DEFICIENT 4* (*PAD4*).

These observations suggest an overlap between the transcriptional responses to flg22 and to hypoxia. This overlap was further explored by comparing our Col-0 flg/m dataset with a list of hypoxia responsive genes in 7-day old Arabidopsis seedlings treated

with 2 hrs of hypoxia (O_2<2%) compared to normoxia (|$\log_2$(FC)|>1.0 and FDR<0.05) (40)). This analysis revealed a statistically significant overlap (*p*-value<3.384e-133) of 384 DEGs (Fig. 2B). The majority of these common DEGs had the same directionality of gene expression change in response to hypoxia and to flg22 (Fig. 2C). This was the case for typical flg22 response genes (*e.g. WRKY33* or *CPK28*), hypoxia response genes (*e.g. LBD41* or *HRE2*), or genes known to be involved in both response pathways (*e.g. RBOHD, MPK3* or *HB1*). One notable exception was *FLS2*, which codes for the flg22 PRR, and whose transcription was repressed under hypoxia, while being up-regulated upon flg22 treatment. GO analysis of the 384 common DEGs indicated an enrichment for genes associated with defense-related processes (*e.g.* 'defense response by callose deposition', 'indole glucosinolate metabolic process' or 'defense response') and the response to oxygen levels. In addition, there was an over-representation of response genes to a range of other abiotic stresses (*e.g.* osmotic stress, temperature), and to stress-related hormone signalling pathways (*i.e.* ethylene, salicylic acid and jasmonic acid) (Supplemental Dataset 4). To determine if the overlap of hypoxia and flg22 responses could be a conserved feature of the flg22 transcriptional response program, we also compared the Brassicaceae core PTI dataset (39) and hypoxia response genes (40). A statistically significant overlap (*p*-value<5.247e-52) of 118 DEGs was found (Fig. S2B), and similarly to the comparison with our Col-0 flg/m dataset, most common genes had the same directionality of gene expression change (Fig. S2C). Hence, the common regulation of a subset of hypoxia and flg22 response genes upon flg22 treatment appears to be a conserved feature, at least within the Brassicaceae family.

Because the ERF-VII transcription factors are the master regulators of the transcriptional response program to hypoxia, we explored whether the common regulation of some genes by both flg22 or hypoxia requires the ERF-VIIs. To this end, we took advantage of the fact that *a1a2* mutants constitutively accumulate ERF-VIIs, and identified flg22-responsive genes in the wild type that were also mis-regulated in flg22-treated *a1a2* compared to the flg22-treated wild type (Fig. 2D). About half of the common 17 DEGs were up-regulated in *a1a2* compared to the wild type after flg22 treatment (Fig. 2E). These up-regulated genes were mostly hypoxia response genes, including *ERF71/HRE2*, *HB1*, *LBD41* or *HRA1*. In addition, the promoter of some of these 17 genes, such as *OSM34, NAC047, AT5G46295,* are bound by the ERF-VII transcription factor HRE2 in ChIP-seq experiments (40). This may be relevant in light of the up-regulation of *ERF71/HRE2* upon flg22 treatment

(Fig. S1E). Attempts to further examine the role of ERF-VIIs in the regulation of some flg22 responsive genes using RT-qPCR on specific genes with the quintuple *erfVII* mutant and its combination with a *prt6-1* mutant (*prt6-1 erfVII* sextuple mutant) (41) suggest that the ERF-VIIs likely partially contribute to the expression defects of some of the flg22 responsive genes that are mis-regulated in Arg/N-degron pathway mutants (Fig. S2D-G). Altogether, these results indicate a crosstalk between hypoxia and flg22 responses, and that ERF-VIIs may be involved in the regulation of some flg22 response genes.

Hypoxia represses the flg22 transcriptional response program

To examine the potential crosstalk between hypoxia and flg22 responses, the genome-wide expression changes that occur under individual (hypoxia or flg22) and combined hypoxia/flg22 treatments were determined in wild-type Col-0 seedlings. We focused on a relatively short 1 hr treatment that enabled monitoring of the onset of both flg22 and hypoxia responses (Fig. S3A). The DEGs (cut-offs: adj. *p*-value<0.05 and $|\log_2(FC)|>0.585$) identified under individual hypoxia (HM v NM; hypoxia/mock v normoxia/mock) or flg22 (NF v NM; normoxia/flg22 v normoxia/mock) treatments compared to untreated seedlings overlapped with those obtained in other similar published datasets (38, 40) (Fig. S3B-C), and retrieved an enrichment for expected GO categories (Suppl. Dataset 5). Analysis of the number of DEGs relative to control conditions indicated that flg22 treatment alone induced greater transcriptional changes (NF v NM; 1906 DEGs) than hypoxia alone (HM v NM; 145 DEGs), likely because of the short 1 hr treatment (Fig. 3A). Combining hypoxia/flg22 resulted in even larger transcriptional changes (HF v NM; 2612 DEGs) than flg22 treatment alone (Fig. 3A). A comparison of the three datasets showed a large overlap between the responses to flg22 alone (NF v NM) and to combined hypoxia/flg22 treatment (HF v NM; 1601 DEGs in common), and a smaller overlap between hypoxia and combined hypoxia/flg22 treatments (131 DEGs) (Fig. 3B). In addition, 59 genes were commonly differentially expressed in all conditions, with an over-representation of genes associated with GO categories relating to metabolic processes (*e.g.* 'response to carbohydrate stimulus'), hormone signalling (ethylene, abscisic acid and brassinosteroids), immunity ('response to chitin') and development ('root hair elongation' or 'developmental maturation') (Suppl. Dataset 5). The vast majority of these 59 DEGs showed the same directionality of gene expression change irrespective of the stress applied (Fig. S3D). For the 53 genes that were up-regulated in all

conditions (Fig. 3C), the combined hypoxia/flg22 treatment enhanced the amplitude of up-regulation (Fig. 3D). While there were only 2 common genes being down-regulated (Fig. 3E), the same trend was observed, such that combined hypoxia/flg22 resulted in a stronger repression of these 2 genes compared to hypoxia or flg22 alone (Fig. 3F).

Strikingly, 939 genes were uniquely regulated in response to combined hypoxia/flg22 (HF v NM), indicating that the combined stress elicits a novel transcriptional response. GO analysis of these 939 hypoxia/flg22-specific DEGs revealed an enrichment for genes associated with expected defense and phosphorylation-related functional GO categories. GO analysis for molecular functions revealed an over-representation of transcription factor-coding genes (Suppl. Dataset 5), with bHLH, Zn finger and homeobox transcription factors accounting for a larger number of differentially expressed transcription factors (Fig. S3E). The expression of these transcription factors was mostly repressed by hypoxia/flg22 treatment, while NAC, WRKY and bZIP transcription factors were up-regulated. In contrast, the 304 DEGs specific to flg22 treatment did not have an over-representation of transcription factor coding genes, so this appears to be a unique feature of the response program to combined hypoxia/flg22 (Suppl. Dataset 5). Separate GO analyses for uniquely down-regulated genes upon hypoxia/flg22 revealed an enrichment for terms associated with developmental processes, such as 'morphogenesis of polarized epithelium', including genes associated with phytohormone signalling pathways with roles in cell division and differentiation (*i.e.* auxin and cytokinin signalling) (Fig. 3G). In contrast, genes that were uniquely up-regulated after the combined treatment were enriched for genes associated with stress responses, including GO terms for other stresses (*e.g.* response to heat), suggesting that the response to combined stresses could be broader and overlap with that of other individual stresses (Fig. 3G).

To further characterize the crosstalk between hypoxia and flg22 responses, we used three metrics established to study the crosstalk between the transcriptional programs to combined abiotic stresses in *Marchantia polymorpha* (2) (see also Fig. S3F for calculations). Specifically, these metrics allow the quantification of (i) shared aspects of the transcriptional response programs (similarity score ranges from 0 to 1, with 1 corresponding to identical response programs), (ii) stress dominance (suppression score varies between -1 and 1, with a negative score indicating a suppression of flg22 response by hypoxia), and (iii) novel components of combined treatments (novel interaction score; varies between 0 and 1, with

1 indicating that combined stresses results in an entirely new transcriptional program) (2). For both up- and down-regulated genes, the negative suppression score obtained suggests that the hypoxia response program represses flg22 response at the transcriptional level (Fig. 3H). In addition, the higher novel interaction score for down-regulated genes indicates that the repression of specific genes under combined hypoxia/flg22 might be an important aspect of the unique response to this combined stress (Fig. 3H). A GO analysis of the 480 down-regulated genes under hypoxia/flg22 only (Fig. 3G) suggests an over-representation for genes associated with development. Strikingly, there is also an enrichment for genes coding for cytochrome P450 enzymes, which use molecular oxygen to catalyze the oxidation of other molecules (Suppl. Dataset 5).

The metrics derived from our RNA-seq dataset analysis suggest that hypoxia represses some aspects of flg22 response. We therefore examined more carefully the 304 DEGs (Fig. 3B) identified only after flg22 treatment (*i.e*. these genes no longer respond to flg22, when combined with hypoxia). A GO analysis of these DEGs showed an enrichment for molecular functions associated with phosphorylation ('kinase activity'), 'carbohydrate binding' and 'transmembrane receptor protein kinase'. Notably, genes in the latter GO category included PRRs such as *FLS2* and *EFR*. Hence, our data suggest that one particular aspect of the repression of flg22 response by hypoxia could occur at the level of PRR expression.

Hypoxia suppresses PTI

Next, we tested whether different aspects of PTI were also repressed by combined hypoxia/flg22 as opposed to flg22 or hypoxia treatments alone, and examined if ERF-VII transcription factors played a role in the repression of PTI. We first used root growth inhibition assays (Fig. 4A) because both flg22 and hypoxia suppress root elongation, so this assay could reveal additive or synergistic effects of the combined treatment. Either flg22 (NF) or hypoxia (HM) treatments resulted in decreased root elongation compared to the same genotype in control conditions (normoxia/mock (NM)). Combined hypoxia/flg22 (HF) did not enhance the inhibitory effects of hypoxia on root elongation in Col-0. In contrast, the *erfVII* quintuple mutant was more severely affected by the combined stress, thus suggesting that expression of ERF-VIIs in the wild type may contribute to protecting the root meristem under combined hypoxia/flg22 treatment. Expression analysis by RT-qPCR of selected

immunity-related genes confirmed that hypoxia dampens the up-regulation of flg22 response genes (Fig. 4B), including PRR-coding genes such as *FLS2* and *EFR*, through mechanisms that are likely partly dependent on the ERF-VIIs. However, immunoblot analysis of FLS2 protein levels using seedlings of a *FLS2$_{pro}$:FLS2-3xmyc-GFP* line (42) suggested that FLS2 protein levels are unchanged after 1 hr of combined hypoxia/flg22 treatment compared to individual treatments (Fig. S4A).

Activation of MAPK signalling is another key feature of PTI. Immunoblots with anti-phosphorylated MPK antibodies indicated that the phosphorylation of MPK3 and MPK6 is reduced in Col-0 seedlings under combined hypoxia/flg22 treatment compared to flg22 treatment (Fig. 4C,D). A reduction in MPK3/6 phosphorylation was also observed with the *erfVII* mutant (Fig. 4C,D), although the repressive effect of combined hypoxia/flg22 on MPK activation seemed weaker in this mutant. In contrast, *MPK3/4/6* expression was not repressed under hypoxia/flg22 compared to flg22 treatment alone (Fig. S4C). Finally, callose deposition was significantly decreased under combined hypoxia/flg22 conditions in Col-0 and *erfVII* seedlings, compared to treatment with flg22 alone (NF) (Fig. 4E). Notably, no statistically significant differences between Col-0 and *erfVII* were found, suggesting that ERF-VIIs are not responsible for the decreased callose deposition under combined hypoxia/flg22.

Discussion

In animals, the complex crosstalk between hypoxia and immunity has been known for decades and some of the underlying mechanisms have been dissected in different cell types. For example, using murine macrophages as a model system, hypoxia has been shown to dampen innate immune signalling pathways (29, 43, 44). In plants, it has been shown that infection with the necrotrophic fungus *Botrytis cinerea* can result in localized hypoxic niches as a consequence of increased respiration and/or water soaking (45). Other pathogens trigger local hypoxic environments *via* the formation of galls/tumors (e.g. *Agrobacterium tumefaciens*) (46). In this context, the ERF-VII transcription factors have been shown to play a role in the ability of plants to defend themselves (14, 46). Despite these potential links between hypoxia and infection by necrotrophic and gall-forming pathogens, the connection between hypoxia and innate immune signalling pathways had not been investigated in

detail. Here, we used transcriptomics datasets to identify connections between hypoxia and PTI, and show that hypoxia represses PTI. We also used genetic backgrounds (Arg/N-degron pathway mutants such as *a1a2*, and *erfVII* quintuple mutants) to examine whether ERF-VIIs play a role in the crosstalk between hypoxia response and PTI.

The genome-wide comparison of the transcriptional changes induced by flg22 treatment in wild-type Col-0 and *a1a2* seedlings revealed that the flg22 transcriptional response program includes sets of hypoxia response genes, and that this overlap is a conserved feature, at least within the Brassicaceae (see model in Fig. 4F). Our transcriptomic data with the *a1a2* mutant, that constitutively accumulates ERF-VII transcription factors (18), as well as with an *erfVII* quintuple mutant, suggest that ERF-VIIs could play a role in the transcriptional regulation of some flg22-responsive genes. In fact, the analysis of an existing ChIP-seq dataset for HRE2 suggests that a proportion of genes that respond to flg22 are bound by HRE2. Together with our finding that HRE2 expression is up-regulated by flg22 treatment, this overlap reinforces the idea that some ERF-VIIs may transcriptionally regulate some flg22-responsive genes (40). However, the role of ERF-VIIs in flg22 response needs to be further explored directly in the context of PTI.

A key question is whether the hypoxia-related genes identified in flg22 transcriptomics datasets are regulated due to the formation of a hypoxic environment during PTI, or require alternative hypoxia-independent mechanisms for their regulation. Our data indirectly suggest that flg22 treatment does not trigger hypoxia in seedlings, as we did not detect the up-regulation of many well-characterized hypoxia marker genes, such as *ALCOHOL DEHYDROGENASE1* (*ADH1*) or *PLANT CYSTEINE OXIDASE1* (*PCO1*). This is in agreement with previous data suggesting that flg22 concentrations of up to 10 µM (a concentration higher than those used in this study) are not sufficient to trigger local hypoxic niches in 3-4-week old plants (45). Hence, the regulation of common genes by either hypoxia or flg22 treatment may not require the formation of a hypoxic environment, but instead might involve other mechanisms.

Considering the overlap between the transcriptional responses to flg22 and hypoxia, and also the fact that hypoxic niches form during infection by necrotrophic and gall-forming pathogens (45, 46), we examined more carefully the potential crosstalk between hypoxia and PTI by first comparing the genome-wide transcriptional changes in wild-type seedlings treated with individual stresses (hypoxia or flg22 alone) or combined hypoxia/flg22. The use

of metrics developed to compare the crosstalk between stresses (2) revealed that hypoxia represses the transcriptional flg22-response program, and that this repression is particularly relevant for genes that are down-regulated by flg22. Indeed, the negative suppression score was lower for down-regulated genes (-0.16 compared to -0.031 for up-regulated genes), and importantly was also within the same range as the suppression scores obtained when combined abiotic stresses were applied to *Marchantia* (2). Unique features associated with the response of wild-type seedlings to combined hypoxia/flg22 emerged in our datasets, with over 900 unique DEGs upon hypoxia/flg22 treatment, compared to hypoxia or flg22 treatments alone. The novel interaction score obtained for down-regulated genes upon combined hypoxia/flg22 was higher than the novel interaction scores found for the range of 18 combined abiotic stresses in *Marchantia* (2), thus suggesting that the repression of specific sets of genes is an important and unique feature of plant responses to combined hypoxia/flg22. These unique features include the repression of developmental processes, which could correlate with a resource/allocation problem and potential energy crisis that is typical of hypoxia stress. In addition, we observed the repression of genes coding for enzymes (cytochromes P450) that use oxygen as co-substrate, suggesting a limitation of enzymatic processes that consume oxygen (Fig. 4F).

Another unique feature of the combined hypoxia/flg22 response was the over-representation of genes associated with transcription factors, with some families being up-regulated (NAC, WRKY, bZIP), while others tended to be predominantly down-regulated (bHLH, Zn finger, homeobox). Altogether, the data suggest that the coordination of plant responses to combined hypoxia/flg22 requires the orchestration of gene expression changes under the control of specific transcription factor families. Notably, some of the up-regulated transcription factor families, such as NAC or WRKY, are typically associated with stress responses (47), while some of the transcription factor families that are mostly repressed (*e.g.* homeobox transcription factors) are generally associated with developmental processes (48). This suggests again an increased need to regulate resource allocation under combined stress, with a stronger prioritization of stress response pathways as opposed to developmental processes. Our core findings with combined hypoxia/flg22 (*i.e.* the preponderance of a novel transcriptional response, the relevance of transcription factors and the stronger prioritization of stress responses) are reminiscent of previous findings in studies with combined abiotic/biotic stresses (1, 3, 49-53). However, such studies have thus

far focused on combinations between pathogens and abiotic stresses such as heat and drought, and have not included hypoxia.

Notably, the repression of PTI by hypoxia is not observed just at the transcriptional level, but also for different key aspects of PTI. Our data show that MAPK signalling and callose deposition are repressed under combined hypoxia/flg22. Similarly, the expression of key PTI components, such as the PRRs *FLS2* and *EFR*, is repressed under combined hypoxia/flg22 compared to flg22 alone. The use of *erfVII* mutants suggests that these master regulators of the hypoxia response program could play a role in regulating plant responses to combined hypoxia/flg22 (e.g. root growth ingibition assays, transcriptional differences and to some extent MPK signalling). However, other aspects of PTI such as callose deposition appear to be repressed in an ERF-VII independent manner. In the case of MAPK, the inhibitory effects appear to be independent of the transcriptional regulation of *MPK3/4/6*, and occurs either post-transcriptionally on MPK proteins, or further upstream in the flg22-dependent signalling events originating from FLS2. Altogether, our data strongly suggest that ERF-VII transcription factors play a role in regulating the crosstalk between hypoxia and flg22 responses when both stresses are combined. However, other, yet to be identified, genes are also likely to play a role.

Connections and crosstalk between hypoxia response and immunity/plant defenses are also likely to evolve in the course of development, as the levels of ERF-VIIs and the regulation of innate immunity change with developmental stages and age (54-58). Such developmentally-dependent crosstalk between abiotic and biotic stresses was identified in the context of combined mild salt stress and pathogen infection with the biotrophic oomycete *Hyaloperonospora arabidopsidis* or with the hemibiotrophic bacterium *Pseudomonas syringae* (59). In the latter study, the hormonal crosstalk between abscisic acid (ABA) and salicylic acid (SA) played an important age-dependent role. The hypoxia/immunity crosstalk may also be particularly complex because, in contrast to other abiotic stresses, hypoxia itself has a dual nature, in that it may be considered as either a stress (*e.g.* during flooding; termed 'acute hypoxia' in this context), or a physiological condition (*e.g.* in meristems, which are hypoxic; termed 'chronic hypoxia') (60, 61). Hence, the output of the crosstalk between hypoxia and immunity could differ between tissues and cell types, as observed in mammals (reviewed in (29)) and as suggested by a recent single-cell RNA-seq study (62).

Finally, combined hypoxia/flg22 treatment indicates that plants or tissues experiencing acute hypoxia are likely to be more susceptible to pathogens. The latter is important to explore and consider in terms of agricultural applications, as it suggests that flooding may have a negative impact on the ability of plants to fight-off pathogens. This could be exacerbated by the fact that flooding also causes an increased risk of pathogen infection, likely as a result of increased dampness and changes to the soil and plants' microbiome (63, 64). Hence, the crosstalk between hypoxia and immunity is a new trait to consider in our endeavour to generate more climate resilient crops.

Materials and Methods

Detailed Materials and Methods are available in the Supplementary Information.

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Figures and Figure legends

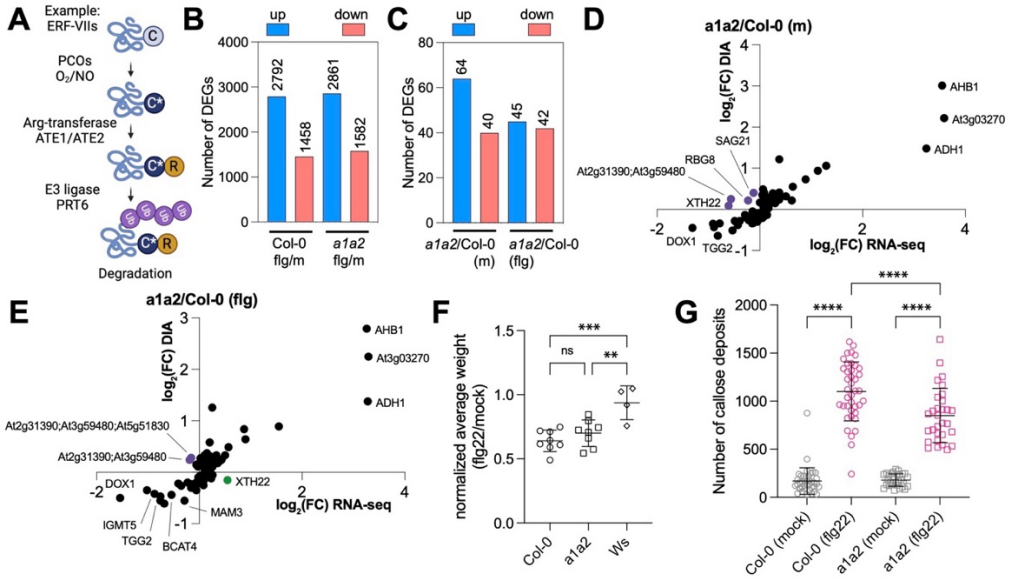


Figure 1: Response of *a1a2* mutant and wild-type Col-0 seedlings to flg22. (A) The oxygen-dependent Arg/N-degron pathway. O_2: oxygen; NO: nitric oxide. **(B)** Number of up and down-regulated DEGs when comparing flg22 to mock-treated samples (flg/m) for wild-type (Col-0) and *a1a2* (a1a2) mutant seedlings. **(C)** Number of up and down-regulated DEGs when comparing *a1a2* to wild-type Col-0 seedlings, either mock (a1a2/Col-0 (m)) or flg22-treated (a1a2/Col-0 (flg)). **(D)** Comparison of differential protein accumulation and differential gene expression in mock-treated Col-0 and *a1a2* seedlings. **(E)** Comparison of differential protein accumulation and differential expression in flg22-treated Col-0 and *a1a2* seedlings. (D) and (E) Purple: lower mRNA levels and higher protein accumulation in *a1a2* compared to the wild type. Green: higher expression in *a1a2* seedlings, but lower protein levels in this mutant compared to the wild type. The $\log_2$ of the fold changes (FC) are shown. **(F)** Seedling growth inhibition assays. Ws: Wassilewskija (flg22-insensitive control (65)). Means and standard deviations are shown from 8 independent replicates (8 seedlings/genotype/replicate). Results of relevant statistical tests are indicated after one-way ANOVA with Tukey's multiple comparison. **(G)** Quantitation of callose deposits following flg22 treatment. Means and standard deviations from 4 and 3 biological replicates are shown for Col-0 and *a1a2*, respectively, with 10 seedlings/replicate/condition. The results of one-way ANOVA and Tukey tests are shown for relevant comparisons. Asterisks: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$; ns: not statistically significant.

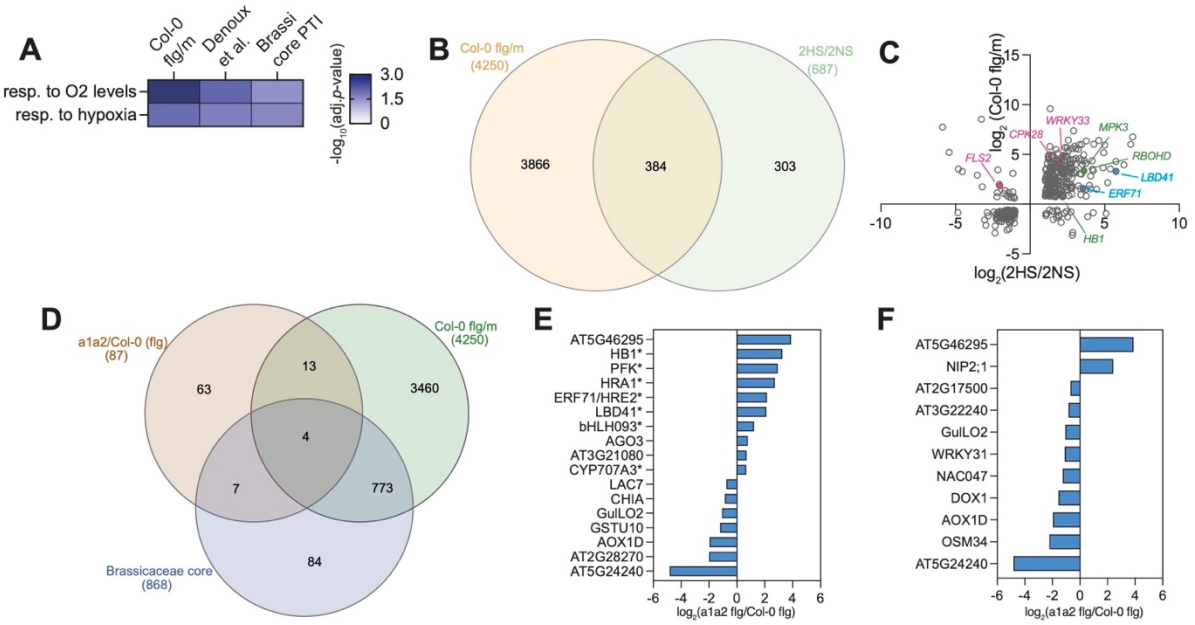
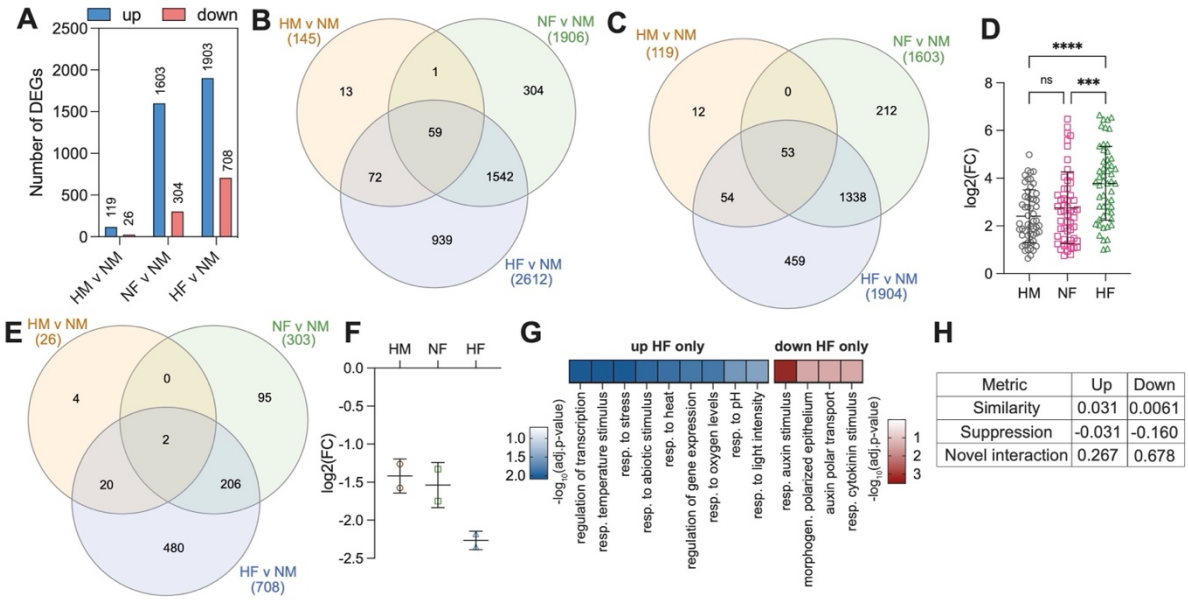


Figure 2: Overlap between the flg22 and hypoxia transcriptional response programs. (A) Enrichment for GO categories associated with oxygen levels in different datasets: Col-0 flg/m (this study); Denoux *et al.* (38) and Brassicaceae core PTI (39). The -$\log_{10}$ of the corrected *p*-values are shown for the GO categories 'response to oxygen levels' (GO#70482) and 'response to hypoxia' (GO#1666). See also Supplemental Dataset 1. **(B)** Overlap between flg22 response genes identified in Col-0 and differentially regulated genes after 2 hrs of hypoxia (2HS/2NS; O_2<2%; cut-off applied: |log(FC)|>1.0 and FDR<0.05) (40). **(C)** Comparison of the directionality and amplitude of gene expression change for the 384 genes common to Col-0 flg/m and 2HS/2NS. **(D)** Comparison of DEGs in *a1a2* compared to Col-0 in the presence of flg22 (a1a2/Col-0 (flg)) with flg22-responsive genes identified in Col-0 (Col-0 flg/m) and Brassicaceae core PTI genes (39). **(E)** Differential expression (presented as $\log_2$ of the fold change) of the 17 DEGs in both a1a2/Col-0 (flg) and Col-0 flg/m. Asterisks denote known hypoxia response genes (31). **(F)** Differential expression (presented as $\log_2$ of the fold change) of the 11 DEGs in both a1a2/Col-0 (flg) and Brassicaceae core PTI genes (39).



Metric	Up	Down
Similarity	0.031	0.0061
Suppression	-0.031	-0.160
Novel interaction	0.267	0.678

Figure 3: Effects of combined hypoxia/flg22 treatments on genome-wide transcription. (A) Number of up- and down-regulated genes. NM: normoxia/mock; HM: hypoxia/mock; NF: normoxia/flg22; HF: hypoxia/flg22. **(B)** Overlap between the 3 different datasets using all DEGs. **(C)** Overlap between the up-regulated DEGs in each of the 3 datasets. **(D)** Average expression of the 53 up-regulated DEGs common to all 3 datasets. Asterisks: * p≤ 0.05; ** p≤0.01; *** p≤0.001; **** p≤0.0001; ns: not statistically significant. **(E)** Overlap between the down-regulated DEGs in each of the 3 datasets. **(F)** Average expression of the 2 down-regulated DEGs common to all 3 datasets. **(G)** Selected GO terms enriched (corrected *p*-value<0.05) amongst uniquely up- and down-regulated DEGs upon combined hypoxia/flg22. See Suppl. Dataset 5 for full list of GO terms. **(H)** Metrics to characterize the transcriptional interaction between individual (hypoxia or flg22) stresses and combined hypoxia/flg22 treatment (see Fig. S3 for calculations).

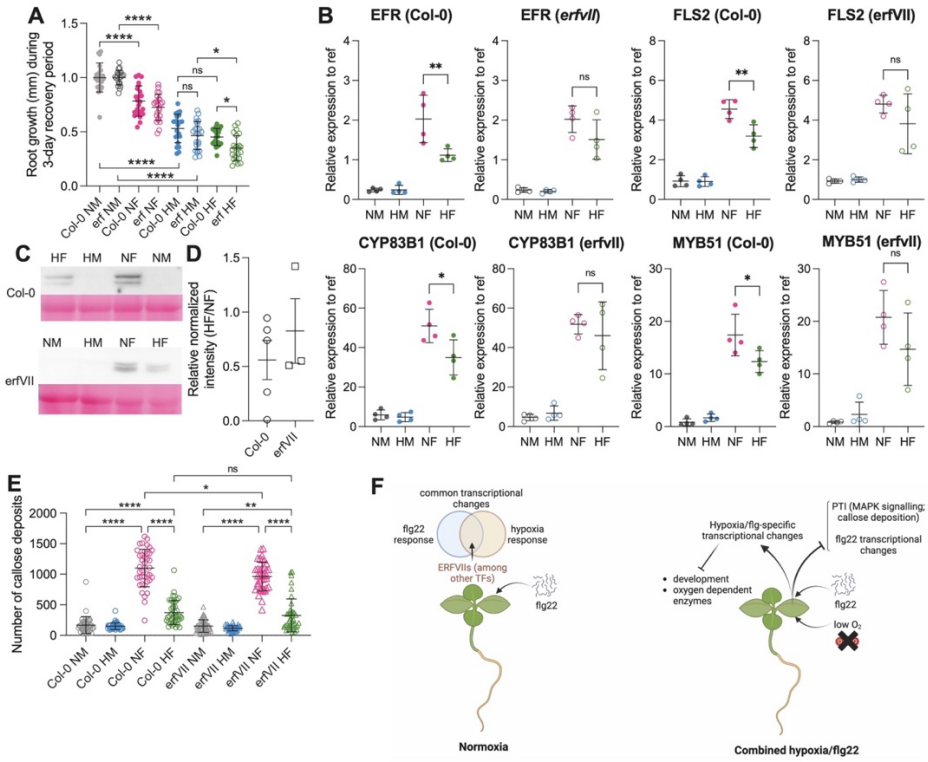


Figure 4: Repression of PTI by hypoxia in combined hypoxia/flg22 experiments. (A) Root growth inhibition assays with wild-type (Col-0) and *erfVII* mutant seedlings under combined hypoxia/flg22 treatment. Means and standard deviations of 4 biological replicates with 5-6 seedlings/genotype/condition in each biological replicate are shown. The results of statistical tests (2-way ANOVA with Tukey test) for relevant comparisons are shown. NM: normoxia/mock; NF: normoxia/flg22; HM: hypoxia/mock; HF: hypoxia/flg22. **(B)** Transcriptional suppression of immunity-related genes under combined hypoxia/flg22 treatment compared to normoxia/flg22 in Col-0 and *erfVII*. Means and standard deviations from 4 biological replicates are shown. The results of one-way ANOVA and Tukey tests are shown for the NF to HF comparison. **(C)** Anti-phosphorylated MPK immunoblots using Col-0 and *erfVII* mutant seedlings. Note different labelling of the lanes between the two genotypes. See also Fig. S4. **(D)** Relative intensity of MPK signals under combined hypoxia/flg22 compared to normoxia/flg22 after normalization with Ponceau intensity. Mean and standard error of the mean are shown. **(E)** Quantitation from callose deposition experiments. Mean and standard deviation from 4 biological replicates are shown, with 10 seedlings/genotype/condition in each biological replicate. The results of ANOVA and Tukey tests are only shown for the NF to HF comparison for each individual genotype. **(F)** Model summarizing the crosstalk between hypoxia and PTI under normoxia, and upon combined

hypoxia/flg22. Asterisks: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$; ns: not statistically significant.

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Authors contributions

BCM, CMD, MM, PG, PFH and EG designed the work, conducted experiments, analyzed data and wrote the manuscript.

Data Sharing

All data from this study are included in the article and/or in the supporting information. All lines are also available upon request. RNA-seq data have been deposited with the Gene Expression Omnibus (GEO) repository (at http://www.ncbi.nlm.nih.gov/) under GSE246173 and GSE246848. *Note to reviewers*: the data has been submitted and the authors are waiting for publication before release. Reviewer access can be requested. *Note to reviewers*: the RNA-seq data has been submitted and the authors are waiting for publication before public release. Reviewer access can be requested). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (66) via the PRIDE (67) partner repository with the dataset identifier PXD046263 (Reviewer account username: reviewer_pxd046263@ebi.ac.uk; Password: JdeljrD3).