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# Transcriptomic analysis of two wheat genotypes in the presence of the pathogen *Zymoseptoria tritici* and the biological control agent *Clonostachys rosea*

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ABSTRACT

Biological control agents (BCAs) are reported to control plant diseases by directly targeting pathogens or indirectly by enhancing the plant's immune system. It has also been reported that plants exhibit genetic variation for compatibility with BCAs, ultimately impacting biocontrol efficacy. This study explored transcriptomic host responses of two winter wheat genotypes differing for biocontrol efficacy of the fungal BCA *C. rosea* in controlling septoria tritici blotch disease caused by the fungus *Zymoseptoria tritici*. Leaves of winter wheat genotypes NGB6704 (high biocontrol efficacy) and NGB348 (low biocontrol efficacy) were spray inoculated with *C. rosea*, *Z. tritici,* or their co-inoculation and were harvested at 8 h, 16 h, 32 h, and 40 h for differential gene expression analysis. The results indicate genotype-dependent and time-dependent responses in gene expression towards *C. rosea* and *Z. tritici*. Induction of several defense-related genes associated with pattern-triggered immunity and effector-triggered immunity was also observed in interactions with *C. rosea* exclusively and in the presence of *Z. tritici*. NGB348 showed a stronger expression of defense-related genes when inoculated with *C. rosea* at early time points, while NGB6704 exhibited a stronger response at 40 h, emphasizing the differential responses to the presence of *C. rosea* by the two genotypes, ultimately affecting STB disease development. Cross-referencing differentially expressed genes with genes segregating for *C. rosea* biocontrol efficacy identified genes associated with receptor-like protein kinases, chitinases, oxalate oxidases, and E3 ubiquitin-protein ligases. Further microscopic and functional validation studies are recommended to determine the intricate nature of plant genotype-specific interactions.

## 1. Introduction

Wheat (*Triticum aestivum* L.) is one of the most significant cereal crops in Europe. It covers the largest European arable land area, and Europe contributes a third of all wheat production globally (269.26 million tonnes out of 798.98 million tonnes globally) in 2023 [1]. However, wheat production is negatively affected by several diseases that are estimated to cause up to 25 % yield losses in northwestern Europe [2]. A major disease affecting European wheat production is septoria tritici blotch (STB), caused by the fungal plant pathogen *Zymoseptoria tritici* (teleomorph *Mycosphaerella graminicola*). Septoria tritici blotch is estimated to cause higher yield loss in northwestern Europe (5.51 %) compared to the global average (2.44 %), with yield losses of up to 50 % in Europe during severe epidemics [2,3]. *Zymoseptoria tritici*, a hemibiotroph with a long asymptomatic phase, goes through several cycles of sexual and asexual reproduction during the growing season, resulting in repeated infection of new plants through airborne sexual ascospores and mainly rain-dispersed asexually produced pycnidia [4,5]. Wheat disease management in Europe primarily relies on the use of pesticides and resistant cultivars [6], with an estimated 70 % of all cereal fungicides targeted specifically at controlling *Z. tritici* [7]. Pesticide resistance development in pathogens is a severe problem affecting efficacy and future crop security [8,9], affecting wheat production. *Zymoseptoria tritici* exhibits a high evolutionary

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potential, enabling it to develop resistance to single-target fungicides [7,10,11]. Moreover, *Z. tritici* has been shown to rapidly overcome plant resistance in major wheat growing regions of the UK, Europe and Australia [4,12–15].

Hence, incorporating additional strategies for STB disease control, in an integrated pest management (IPM) context, is essential to complement the use of resistant cultivars and fungicide applications and to reduce selection pressure within pathogen populations. The European Union Framework Directive 2009/128/EC asks all plant production professionals to comply with IPM principles [9,16,17]. Within IPM, the use of biological control methods for pest and disease management is one of the key approaches. Bioprotection, including biocontrol and the use of natural substances, is a rapidly growing industry with a global market value of 7.54 billion USD in 2023 and projections of up to 28.61 billion USD by 2032 [18]. Stenberg et al. [19] defines biological control as "the exploitation of living agents (including viruses) to combat pestilential organisms (pests and pathogens), directly or indirectly, for human good". Biological control agents (BCAs) can act against pathogens by one or more of the following mechanisms: competing for resources and space, through antibiosis and hyperparasitism, as well as indirectly by inducing plant immune responses [20,21]. Depending on BCA, pathogen, plant, and environmental factors, one or more modes of action can be exhibited by BCAs [22]. Certain BCAs can also tolerate fungicides, making them suitable to be used simultaneously or in rotation with fungicides for disease management [23–27]. Numerous bacterial, fungal, oomycete, and viral BCAs, including the fungus *Clonostachys rosea*, have been successfully commercialized in the EU, US, UK and other parts of the world for management of above-ground as well as below-ground root and soil-borne diseases [21].

Certain strains of *C. rosea* (phylum Ascomycota, order Hypocreales) are successful BCAs against various plant pathogenic fungi and oomycetes [22]. Owing to its generalist lifestyle, *C. rosea* employs different strategies to combat pathogens, such as competition for nutrition and space [28], antibiosis [29,30], direct parasitism [22,31], and induction of plant defense responses [32,33]. In this study, we used *C. rosea* strain IK726, a highly effective BCA originally isolated from barley roots in 1992 in Denmark [34]. The genome of *C. rosea* IK726 was sequenced in 2015 [35], and it has been reported to exhibit biocontrol properties against various plant diseases and causal pathogens such as *Alternaria dauci*, *A. radicina*, *Bipolaris sorokiniana*, *Botrytis cinerea*, *Fusarium oxysporum*, *F. culmorum*, *F. graminearum*, *F. avenaceum*, *F. coeruleum*, *Helminthosporium solani*, *Plasmodiophora brassicae*, *Pythium tracheiphilum,* and *Zymoseptoria tritici* [22].

Although the critical role of plant genotypic variation in disease resistance is long recognized in plant breeding, plant genotypic variation may also influence how plants respond to beneficial microorganisms, including BCAs [21,36–38]. A limited number of studies report that plant genotypes influence biocontrol efficacy against pathogens, as observed in various crops, including pine, tomato, potato, wheat, sugar beet, and lentils [36,39–46]. In our previous work, we also show significant variation in the biocontrol efficacy of *C. rosea* IK726 in controlling fusarium foot rot caused by *F. graminearum* and STB in a large winter wheat population [47,48]. In these studies, we also identified wheat genomic regions and alleles significantly associated with biocontrol efficacy. However, the mechanistic basis of this biocontrol compatibility trait is unknown. Therefore, using transcriptome profiling to better understand the molecular responses triggered during plant-pathogen-BCA tri-partite interactions can be a useful approach.

Research has been conducted on transcriptome changes in *Z. tritici* and wheat during compatible and incompatible interactions [as reviewed in 49]. In infection with *Z. tritici*, transcriptional downregulation of wheat defense was observed during the early symptomless colonization phase [50]. However, during the transition phase between 7 and 14 days, upregulation of genes encoding receptor-like kinases, pathogenesis-related (PR) proteins, and other defense-related genes was observed [50,51]. While comparing susceptible and resistant wheat cultivars, resistant plants showed strong upregulation of defense-related genes during incompatible interactions, including PR genes (PR-1, PR-2, PR-5), peroxidases, chitinases, protein disulfide isomerases, and phenylalanine ammonia-lyase, providing critical insights into the mechanisms underlying resistance [49,52]. Additionally, strain-specific gene expression of small secreted proteins, secreted peroxidases, proteases, plant cell wall-degrading enzymes in *Z. tritici* have been documented, enhancing our understanding of the infection processes used by this pathogen [53,54]. A few studies have also explored the transcriptome response in plants induced by *C. rosea*. Multiple plant factors were observed to respond to *C. rosea*-biocontrol of the grey mold pathogen *B. cinerea* on tomatoes, including the activity of protective enzymes, accumulation of reactive oxygen species (ROS), and the regulation of stress response genes such as mitogen-activated protein kinase (MAPK), WRKY transcription factor, β-xylanase, and ATP synthase CF1 α-subunit [32]. *Clonostachys rosea* IK726 also modulated the expression of defense-related genes in potato tubers directly as well as in the presence of the fungal pathogen *Helminthosporium solani* [55]. Similarly, transcriptional reprogramming of wheat genes involved in stress responses and growth was demonstrated during root colonization by *C. rosea* IK726 [56]. However, the extent to which plant genotypes differ in their transcriptional response towards BCAs remains largely unexplored.

In the current study, we performed a transcriptomic analysis of two different winter wheat genotypes differing in their ability to benefit from *C. rosea*-mediated biocontrol of STB. We hypothesized that (i) gene expression differs between plant genotypes in the presence of the biocontrol agent *C. rosea* and the pathogen *Z. tritici*, (ii) variation in biocontrol efficacy of *C. rosea* between plant genotypes is associated with distinct sets of differentially expressed genes and that (iii) *C. rosea* induces plant genotype-specific defense-related genes directly and in the presence of *Z. tritici*. The results suggest that low efficacy of *C. rosea*-mediated biocontrol may be connected with a rapid and exaggerated defense response in wheat, potentially hindering the effectiveness of *C. rosea* as a BCA.

## 2. Materials and methods

### 2.1. Plant and fungal material

Winter wheat genotype NGB6704, supporting high *C. rosea*-mediated biocontrol efficacy of STB (HE), and genotype NGB348, exhibiting low *C. rosea*-mediated biocontrol efficacy of STB (LE), as described previously [48], were used in the current work. The genotypes used were originally obtained from the Nordic Genetic Resource Centre (Alnarp, Sweden).

*Zymoseptoria tritici* strain Alnarp 1 (named here for clarity), originally isolated from a wheat field in southern Sweden in 2015 [57], was revived from a 50 % glycerol conidial suspension stored at −80 °C and maintained on yeast malt sucrose (YMS) agar plates [57,58]. Plates were incubated in the dark at 20 °C for 10–12 days. Conidia were harvested by adding sterile water to the plates, scraping the plate surface, and filtering through miracloth (Merck KGaA, Darmstadt, Germany). The conidia concentration was determined using an improved Neubauer hemacytometer (Hausser Scientific, Horsham, PA) and was adjusted to $1 \times 10^6$ cfu/ml in the final water suspension.

*Clonostachys rosea* strain IK726 [34] was inoculated on potato dextrose agar (PDA; BD Difco Laboratories, France) media plates and were incubated in the dark at 20 °C for 18 days. Similar to the *Z. tritici* suspension, conidia of *C. rosea* were harvested from the plates by adding sterile water to release conidia, filtering, and determining the concentration using a hemacytometer. The final concentration of *C. rosea* was adjusted using water to $1 \times 10^7$ cfu/ml.

### 2.2. Bioassay setup

Surface-sterilized seeds (detergent and 2 % NaOCl, with rinsing during and after treatment) of the two wheat genotypes NGB6704 and NGB348 were sown in plastic pots (9 × 9 × 8 cm) containing potting soil (Såjord, Hasselfors Garden AB, Sweden), with 7–8 seeds per pot. Plants were grown under controlled conditions with 60 % relative humidity with 16 h light (250 μmol/$m^2$/s with mounted white tubular LED lights) at 22 °C and 8 h dark at 16 °C. Fully developed 2nd leaf from each plant was fixed horizontally with the adaxial side facing upward for treatment application. Four treatments were applied to each genotype: (1) Control (mock treatment, water sprayed), (2) Cr (*C. rosea* at 1 × $10^7$ cfu/ml), (3) Zt (*Z. tritici* at 1 × $10^6$ cfu/ml), and (4) ZtCr (*C. rosea* at 1 × $10^7$ cfu/ml and *Z. tritici* at 1 × $10^6$ cfu/ml). *Clonostachys rosea* was sprayed (until runoff) first and allowed to incubate for 24 h, during which the trays were covered with plastic bags and kept in darkness to maintain high relative humidity. Leaf samples were collected at 8 h and 16 h post-*C. rosea* inoculation. At 24 h, *Z. tritici* was sprayed (until runoff), followed by the same incubation conditions, and further samples were collected at 32 h and 48 h post-*C. rosea* inoculation. At each time point, leaves were harvested from two separate plants within the same pot (pooled to constitute one biological replicate), and four biological replicates were collected per genotype, treatment, and time point, as shown in Supp. Fig. 1. All samples were collected from a single experiment.

### 2.3. Phenotypic data analysis

Plants remaining (n = 6 to 15) after leaf harvest for RNA extraction (described below) were used to score the disease development. As described previously [48], the disease was visually assessed as the percentage of necrotic leaf area as a proxy from 0 to 100 % with a 5 % interval. Disease scoring was performed on the 2nd leaf of each plant at 10, 14, 16, 19, 22, 25, and 28 days post inoculation (dpi) with *Z. tritici*. Using these time points of disease scoring, the relative area under the disease progress curve (rAUDPC) was estimated using the R package agricolae v1.3-7 [59] as below:

$$rAUDPC = \sum_{i=1}^{n-1} \frac{y_i + y_{i+1}}{2} \times (t_{i+1} - t_i) \Big/ 100 \times (t_n - t_1)$$

where $y_i$ is the disease score in percent at timepoint $t_i$, $t_{i+1} - t_i$ is the time interval between two scorings, and $n$ is the total number of scoring time points. $100 \times (t_n - t_1)$ is the AUDPC maximum used in the denominator to estimate the relative AUDPC.

To check for the genotypic differences between treatments, analysis of variance (ANOVA) was performed using a linear model with genotype and treatment interaction. The analysis was performed in R v4.4.1 using the stats package [60]. The model is as follows:

$$y_{ijkl} = \mu + p_{lj} + g_i + t_l + (gt)_{il} + n_{ljk} + \varepsilon_{il}$$

where $y_{ijkl}$ denotes the rAUDPC estimate of the $i$-th genotype in the $l$-th treatment, $\mu$ denotes the overall mean, $g_i$ is the effect of the $i$-th genotype, $t_l$ is the effect of $l$-th treatment, $(gt)_{il}$ is the interaction effect between $i$-th genotype and $l$-th treatment, $p_{lj}$ is the effect of the $j$-th pot nested within the $l$-th treatment, and $\varepsilon_{il}$ is the residual term for which homogenous variance was assumed and was subjected to normal distribution. For multiple comparisons, a post-hoc Tukey's test among genotypes across treatments was performed using the packages emmeans v1.10.5 [61] and multcomp v1.4-26 [62]. Moreover, for each genotype, the contrasts between treatments were calculated as the difference between Zt (disease severity with *Z. tritici* alone) and ZtCr (disease severity with both *Z. tritici* and *C. rosea*), were used as measures of biocontrol efficacy (Zt - ZtCr). These contrasts served as indicators of the effect of *C. rosea* in reducing disease severity for each genotype.

### 2.4. RNA extraction, quality control, and sequencing

Leaf samples were harvested in 2 ml screw cap tubes containing three sterile glass beads (2 mm diameter) and were immediately frozen in liquid nitrogen prior to storing at −70 °C until further use. Stored samples were freeze dried for 48 h prior to RNA extraction. Freeze dried samples were homogenized using Percellys 24 Tissue homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) at 5000 rpm for 2 × 30 s. Total RNA was extracted from the ground leaf samples using the Qiagen RNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA) following the manufacturer's instructions. Genomic DNA was removed from the samples using DNase I (Thermo Fisher Scientific Inc., Waltham, MA) treatment in 20 μl reactions. The yield, purity, and integrity of RNA extractions were determined using Nanodrop 1000 (Thermo Fisher Scientific Inc., MA) and Agilent Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany). Five hundred ng of total RNA for four biological replicates per treatment condition (96 samples) were submitted to the SNP&SEQ Technology Platform (National Genomics Infrastructure, Sweden and SciLifeLab, Uppsala, Sweden) for library preparation using TruSeq Stranded Total RNA kit (Illumina Inc., San Diego, CA) with polyA selection and sequencing 150 bp paired-end reads on a NovaSeq X Plus system. One sample (genotype NGB6704 in ZtCr at 32 h) was excluded at this stage due to poor RNA quality.

### 2.5. Transcriptome quality control and alignment

Raw RNA-seq data was processed using nf-core/rnaseq v3.14.0 [63] of the nf-core collection of workflows [64] using the "–skip_alignment" and "–pseudo-alignment salmon" options. The pipeline was executed with Nextflow v23.10.1 [65] on the high-performance computing cluster Dardel (KTH Royal Institute of Technology, Sweden) using the singularity container [66] and the appropriate institute's configuration (https://nf-co.re/configs/pdc_kth). Briefly, adapters from raw sequences were trimmed using TrimGalore v0.6.7 [67], ribosomal RNA was removed using SortMeRNA v4.3.4 [68] followed by pseudoalignment using Salmon v1.10.0 [69] with added options "–dumpEq –gcBias –posBias –seqBias". Reads were aligned to the merged reference genomes of *T. aestivum* cultivar Chinese Spring v2.1 (https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_018294505.1/), *Z. tritici* strain IPO323 v2.0 (https://www.ncbi.nlm.nih.gov/datasets/genome/GCA_000219625.1/) and *C. rosea* strain IK726 v2.0 (https://www.ncbi.nlm.nih.gov/datasets/genome/GCA_902827195.2/). Salmon's output of quantification files (quant.sf files) was used for downstream differential gene expression analysis. Details regarding the pipeline parameters and options are given in Supp. File 1.

### 2.6. Mapping of fungal transcriptomes across treatments

Reads mapping only to *C. rosea* and *Z. tritici* were filtered and used as a proxy for the biomass of these fungi across treatments. Additionally, reads mapping specifically to housekeeping genes of *C. rosea* and *Z. tritici* were used as a proxy for estimating biomass. For *C. rosea*, five housekeeping genes were selected: actin (*act*), beta-tubulin-like gene (*tub*), elongation factor 1-alpha (*tef1*), glyceraldehyde-3-phosphate dehydrogenase (*gpd1*) and protein kinase C (*pkc1*), as previously suggested in the literature [70,71]. Similarly, for *Z. tritici*, nine housekeeping genes were selected: actin (*act*), beta-tubulin-like gene (*BTUB*), calmodulin (*cal*), cyclophilin (*cyp*), elongation factor 1-alpha (*EF1*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), heat stress protein 80-1 (*hsp80-1*), protein kinase C (*PKC*) and transcription factor class C (*TFC1*), following previous recommendations [72,73]. Generalized linear models were applied using a quasi-binomial distribution for the percent of total read counts dependent on the genotype, hour, and treatment terms, as well as their two-way and three-way interactions. The model estimates for read counts were back-transformed for interpretation.

**Table 1**
Differential gene expression contrasts. Each cell indicates the contrast of a given treatment against the control for the corresponding genotype and time point.

| Time | Genotype | |
|---|---|---|
| | NGB6704 | NGB348 |
| 8 h | Cr v Control | Cr v Control |
| 16 h | Cr v Control | Cr v Control |
| 32 h | Cr v Control | Cr v Control |
| | Zt v Control | Zt v Control |
| | ZtCr v Control | ZtCr v Control |
| 40 h | Cr v Control | Cr v Control |
| | Zt v Control | Zt v Control |
| | ZtCr v Control | ZtCr v Control |

Treatments: Control: Mock water treatment, Cr: *Clonostachys rosea,* Zt: *Zymoseptoria tritici*, ZtCr: *Zymoseptoria tritici* + *Clonostachys rosea*.

### 2.7. Differential gene expression analysis

Quality control, normalization, and differential gene expression analysis were performed in R v4.4.1 [60] using scripts (v20231215) modified from the Umeå Plant Science Centre bioinformatics facility [74]. Briefly, Salmon's quant.sf files were imported using tximport v1.32.0 [75]. Reads mapping only to *T. aestivum* genes were filtered and used for the analysis. Reads were normalized using the variance stabilizing transformation. Differential gene expression analysis was performed using DESeq2 v1.44.0 [76]. To identify differentially expressed genes (DEGs), relevant contrasts were run separately, whereby a condition with a specific genotype, treatment, and time point was compared to its respective control treatment for the given genotype and time point (Table 1). Genes were considered differentially expressed (DEG) with absolute log2 fold change >1, false discovery rate (FDR) adjusted *P* value < 0.05 [77]. Summaries of DESeq2 runs for each contrast are provided in Supp. Files 2, 4, and 6.

### 2.8. Gene ontology enrichment analysis

Gene Ontology (GO) IDs assigned to genes were retrieved from the wheat reference genome project (https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_018294505.1/). GO enrichment was performed using topGO v2.56.0 [78] using the parent-child Fisher's test with FDR-adjusted *P* value < 0.05. GO enrichment analysis was conducted separately for upregulated and downregulated genes within each contrast whereby all the expressed genes of that particular contrast were used as the background population. Definitions associated with the GO terms were extracted using the package GO.db v3.19.1 [79].

### 2.9. Cross-referencing differentially expressed genes with prior association findings

Differentially expressed genes identified in the current study were compared to the genes located within wheat genomic regions previously reported to segregate with the efficacy of *C. rosea*-mediated biocontrol of STB [48]. Genes within a ±1.6 cM interval flanking each significantly associated SNP marker were extracted for cross-referencing. Previously, gene nomenclature was based on Ensembl Traes IDs; however, for cross-referencing, the genomic regions were re-scanned using the NCBI database.

## 3. Results

### 3.1. Phenotypic evaluation of disease development

To confirm the development of STB disease and the biocontrol efficacy of *C. rosea* in our two selected wheat genotypes, a bioassay was performed under controlled conditions. Analysis of variance (ANOVA) of the STB disease development on the NGB6704 (HE) and NGB348 (LE) winter wheat genotypes revealed a significant treatment effect on rAUDPC ($F = 8.99$, $P < 0.05$), while the genotype effect ($F = 3.31$, $P = 0.07$) and genotype-by-treatment interaction ($F = 0.81$, $P = 0.49$) were not significant. Post-hoc estimates showed no significant disease development ($P > 0.05$) in the Control and Cr treatments for either genotype, and similar susceptibility was shown for both genotypes in the Zt treatment (Fig. 1a). In contrast, significant disease development ($P < 0.05$) was observed in the Zt treatment for genotype NGB6704 (HE), with a marked reduction in disease development upon spraying *C. rosea*

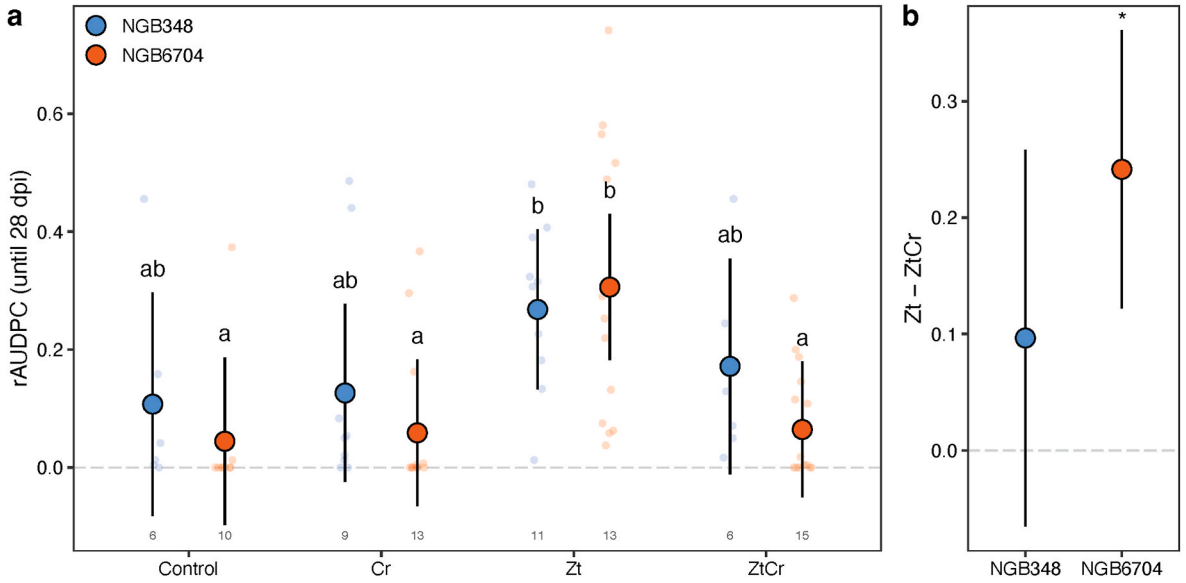


**Fig. 1.** (a) Relative area under disease progress curve (rAUDPC) estimates of disease severity across treatments Control, Zt (*Zymoseptoria tritici*), Cr (*Clonostachys rosea*) and ZtCr (*Z. tritici* and *C. rosea*). Points with opacity represent raw estimates of rAUDPC values. Bold points represent the model estimated means, and error bars represent 95 % confidence intervals. Points sharing the same letters indicate a non-significant difference ($P > 0.05$) as determined by Tukey's post-hoc comparisons test. (b) rAUDPC-based biocontrol efficacy estimates (Zt - ZtCr) of *C. rosea* in controlling septoria tritici blotch in genotypes NGB348 and NGB6704. Bold points represent the model estimated contrast (Zt – ZtCr), and error bars represent 95 % confidence intervals. Biocontrol efficacy estimates with error bars not overlapping with 0 are significant at $P < 0.05$.

**Table 2**
Sequence reads (in million) summary statistics of total reads and reads mapping to *Triticum aestivum*, *Clonostachys rosea,* and *Zymoseptoria tritici* across genotypes, treatments and hour.

| Genotype | Treatment | Hour | Total reads: Mean ± SD (Median) | *T. aestivum* reads: Mean ± SD (Median) | *C. rosea* reads: Mean ± SD (Median) | *Z. tritici* reads: Mean ± SD (Median) |
|---|---|---|---|---|---|---|
| NGB6704 | Control | 8 | 32.4 ± 10.7 (31.0) | 32.4 ± 10.7 (31.0) | 0.0 ± 0.0 (0.0) | 0.0 ± 0.0 (0.0) |
| | | 16 | 28.5 ± 9.6 (26.7) | 28.5 ± 9.6 (26.7) | 0.0 ± 0.0 (0.0) | 0.0 ± 0.0 (0.0) |
| | | 32 | 26.3 ± 4.1 (25.8) | 26.3 ± 4.0 (25.7) | 0.0 ± 0.1 (0.0) | 0.0 ± 0.0 (0.0) |
| | | 40 | 33.2 ± 8.0 (33.1) | 33.2 ± 8.0 (33.1) | 0.0 ± 0.0 (0.0) | 0.0 ± 0.0 (0.0) |
| | Cr | 8 | 32.3 ± 9.1 (33.1) | 31.8 ± 9.1 (32.3) | 0.5 ± 0.4 (0.5) | 0.0 ± 0.0 (0.0) |
| | | 16 | 24.6 ± 5.1 (25.9) | 23.5 ± 4.9 (24.9) | 1.0 ± 0.2 (1.0) | 0.0 ± 0.0 (0.0) |
| | | 32 | 27.0 ± 9.1 (25.7) | 26.4 ± 8.9 (25.0) | 0.6 ± 0.2 (0.7) | 0.0 ± 0.0 (0.0) |
| | | 40 | 26.0 ± 4.6 (26.0) | 24.7 ± 4.2 (25.1) | 1.3 ± 0.7 (1.2) | 0.0 ± 0.0 (0.0) |
| | Zt | 32 | 26.3 ± 6.5 (28.9) | 26.2 ± 6.5 (28.8) | 0.0 ± 0.0 (0.0) | 0.1 ± 0.0 (0.1) |
| | | 40 | 42.9 ± 13.9 (48.0) | 42.6 ± 13.7 (47.6) | 0.0 ± 0.0 (0.0) | 0.3 ± 0.2 (0.3) |
| | ZtCr | 32 | 25.4 ± 13.4 (20.4) | 22.8 ± 14.2 (17.8) | 2.3 ± 0.7 (2.4) | 0.3 ± 0.1 (0.2) |
| | | 40 | 27.6 ± 9.9 (23.0) | 18.0 ± 6.9 (16.3) | 8.7 ± 3.1 (8.1) | 0.9 ± 0.4 (0.8) |
| NGB348 | Control | 8 | 31.1 ± 12.3 (29.2) | 31.1 ± 12.3 (29.2) | 0.0 ± 0.0 (0.0) | 0.0 ± 0.0 (0.0) |
| | | 16 | 23.5 ± 2.2 (23.3) | 23.5 ± 2.2 (23.3) | 0.0 ± 0.0 (0.0) | 0.0 ± 0.0 (0.0) |
| | | 32 | 30.3 ± 7.8 (28.7) | 30.3 ± 7.8 (28.7) | 0.0 ± 0.0 (0.0) | 0.0 ± 0.0 (0.0) |
| | | 40 | 28.8 ± 6.1 (30.9) | 28.8 ± 6.1 (30.9) | 0.0 ± 0.0 (0.0) | 0.0 ± 0.0 (0.0) |
| | Cr | 8 | 40.3 ± 8.5 (41.4) | 39.6 ± 8.3 (40.6) | 0.7 ± 0.3 (0.7) | 0.0 ± 0.0 (0.0) |
| | | 16 | 32.8 ± 15.3 (26.4) | 31.1 ± 15.6 (25.6) | 1.7 ± 1.0 (1.6) | 0.0 ± 0.0 (0.0) |
| | | 32 | 35.6 ± 13.6 (35.9) | 34.4 ± 13.8 (34.8) | 1.2 ± 0.6 (1.2) | 0.0 ± 0.0 (0.0) |
| | | 40 | 27.9 ± 4.2 (29.6) | 25.1 ± 3.4 (25.2) | 2.8 ± 2.1 (2.5) | 0.0 ± 0.0 (0.0) |
| | Zt | 32 | 28.1 ± 9.8 (25.8) | 28.0 ± 9.8 (25.8) | 0.0 ± 0.0 (0.0) | 0.1 ± 0.0 (0.1) |
| | | 40 | 32.3 ± 4.1 (32.2) | 32.1 ± 4.1 (32.0) | 0.0 ± 0.0 (0.0) | 0.2 ± 0.1 (0.1) |
| | ZtCr | 32 | 25.8 ± 4.9 (24.8) | 23.7 ± 5.7 (22.6) | 1.9 ± 0.7 (2.0) | 0.2 ± 0.1 (0.2) |
| | | 40 | 31.4 ± 13.0 (33.0) | 28.0 ± 13.0 (30.1) | 3.1 ± 0.9 (3.4) | 0.2 ± 0.1 (0.2) |

Treatments: Control: Mock water treatment, Cr: *Clonostachys rosea,* Zt: *Zymoseptoria tritici*, ZtCr: *Zymoseptoria tritici* + *Clonostachys rosea.*
Reads are averaged across biological replicates.

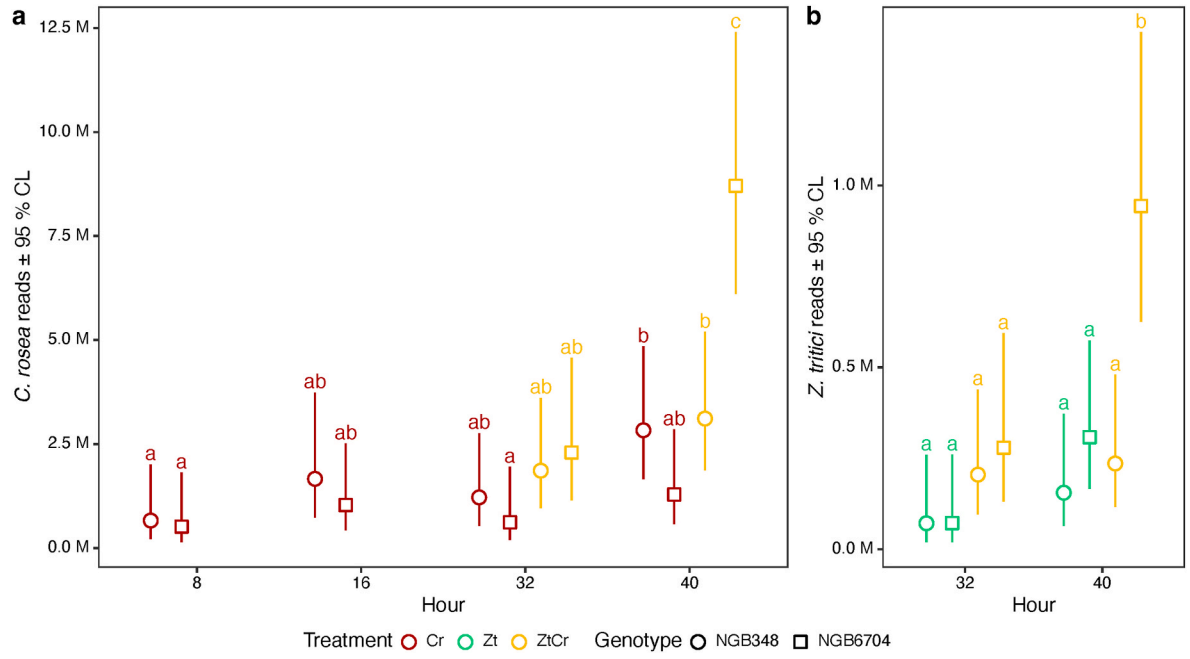


**Fig. 2.** Reads mapping to (a) *Clonostachys rosea* IK726 in treatments Cr (*C. rosea*) and ZtCr (*Zymoseptoria tritici* and *C. rosea*) in two genotypes at 8 h, 16 h, 32 h and 40 h and (b) *Zymoseptoria tritici* IPO323 in treatments Zt (*Z. tritici*) and ZtCr (*Z. tritici* and *C. rosea*) in two wheat genotypes at 32 h and 40 h. Points represent the model mean estimate and error bars represent 95 % confidence interval. Treatments sharing the same letters indicate a non-significant difference ($P > 0.05$) as determined by Tukey's post-hoc comparisons test.

in the ZtCr treatment. This pattern was further highlighted in the biocontrol efficacy estimation, where the Zt - ZtCr contrast showed a significant reduction in disease for genotype NGB6704 (HE, $P < 0.05$) but not for NGB348 (LE) (Fig. 1b).

### 3.2. Transcriptome depth and variation in wheat genotypes across treatments

The transcriptomic analysis was performed with four replicates in all conditions, except for NGB6704 at 32 h and 40 h in treatment ZtCr and NGB348 at 16 h in treatment Cr, where only three replicates were used due to low RNA or sequencing quality. Mapping percentages following

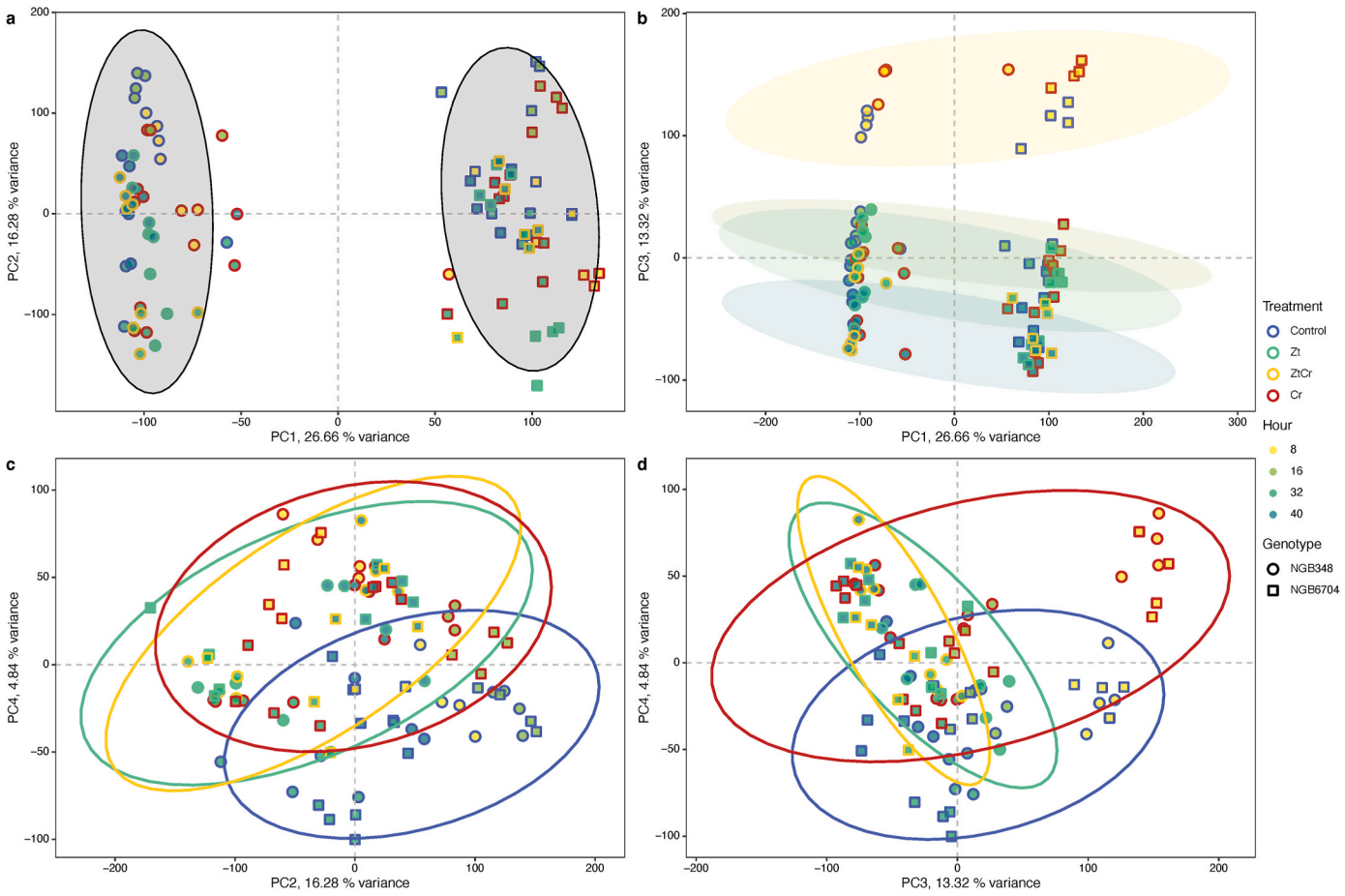


**Fig. 3.** Principal component analysis (PCA) plots of variance stabilized wheat transcriptome data set showing sample distribution in PC1 and PC2 (a), PC1 and PC3 (b), PC2 and PC4 (c), and PC and PC4 (d). The point shape represents two genotypes (NGB6704 and NGB348), and the border color represents four treatments (Control, Zt – *Zymoseptoria tritici*, Cr – *Clonostachys rosea*, ZtCr – *Z. tritici* and *C. rosea*) and fill color represents four time points (8 h, 16 h, 32 h, and 40 h). Ellipses cluster genotypes (a), time points (b), and treatments (c and d). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

alignment to the merged genome of the three organisms ranged from 79.1 % to 89.2 %, with an average mapping ranging from 80.6 % (in NGB348 in treatment Control at 8 h) to 87 % (in NGB6704 in treatment ZtCr at 40 h) across conditions.

Reads mapping to the *Z. tritici* and *C. rosea* genomes were filtered and used as a proxy for fungal biomass on wheat leaves over time. Reads mapping to *C. rosea* in the Control and Zt treatments and reads mapping to *Z. tritici* in the Control and Cr treatments were found to be essentially absent (Table 2). Reads that mapped to *C. rosea* in the treatment Cr showed no significant ($P > 0.05$) differences between the two wheat genotypes at various time points (Fig. 2a). However, in the treatment ZtCr at 40 h, NGB6704 (HE) had significantly ($P < 0.05$) more reads mapped to *C. rosea* than NGB348 (LE). Similarly, reads mapped to *Z. tritici* showed no significant ($P > 0.05$) differences across two genotypes in the treatment Zt at 32 h and 40 h, but NGB6704 (HE) had significantly ($P < 0.05$) more reads mapped to *Z. tritici* than NGB348 (LE) at 40 h in the ZtCr treatment (Fig. 2b). This pattern was also supported by individual housekeeping genes of *C. rosea* and *Z. tritici* (Supp. Fig. 2).

Most samples had >90 % of total reads mapped to the *T. aestivum* genome, based on the absolute read counts shown in Table 2. The rest of the quality control and analysis was performed on the subset of reads that mapped only to *T. aestivum*. Principal component analysis (PCA) of the entire dataset across 16 experimental conditions revealed distinct clustering of samples (Fig. 3). PC1 primarily distinguished between the two winter wheat genotypes (Fig. 3a). PC2, PC3, and PC4 clustered samples based on treatment, specifically separating *Z. tritici* treatments (Zt and ZtCr) from non-*Z. tritici* treatments (Control and Cr) (Fig. 3c – d). Moreover, PC3 clustered samples according to time points (Fig. 3b).

### 3.3. Transcriptional response differences between wheat genotypes in response to C. rosea inoculation

The expression profiles of wheat transcripts during the interaction with *C. rosea* were compared with those within the Control treatment for each genotype at each time point, making a total of eight contrasts (Table 1). With absolute log2 FC > 1 and FDR adjusted $P < 0.05$, the two winter wheat genotypes showed differences in their response to *C. rosea* across the four tested time points (Supp. File 2), with relatively few genes regulated in common (Fig. 4). A total of 2090 DEGs were identified with the highest number of DEGs in NGB348 (LE) at 8 h (1066 upregulated and 60 downregulated) and in NGB6704 (HE) at 40 h (503 upregulated and 204 downregulated) (Fig. 4a). Both genotypes showed an increased number of upregulated genes over time in the Cr treatment.

There was also a genotype effect on temporal expression; NGB6704 (HE) showed fewer DEGs compared with NGB348 (LE) at early time points (8 h, 16 h, and 32 h), but more DEGs at 40 h (Fig. 4). Among the genes uniquely associated with genotypes, NGB348 notably showed upregulation of predicted WRKY transcription factors, germin-like proteins, LRR receptor-like proteins, and a pathogenesis-related protein 1-like protein (Fig. 5). In contrast, NGB6704 showed upregulation of genes putatively encoding pathogenesis-related (PR) protein PRB1-2-

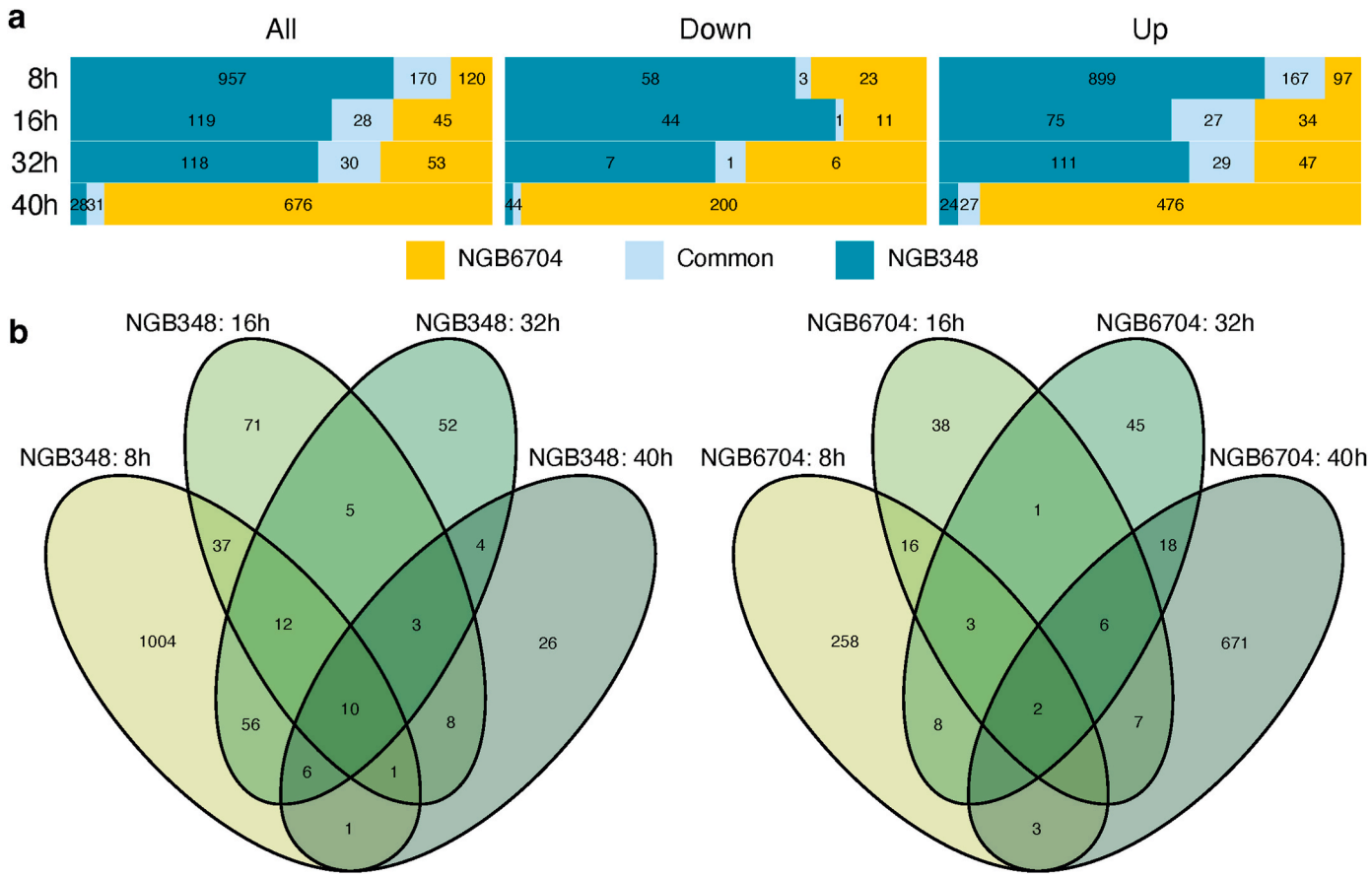


**Fig. 4.** (a) Number of differentially expressed genes (DEGs) in winter wheat genotypes in response to *Clonostachys rosea* at time points 8 h, 16 h, 32 h, and 40 h. Venn diagram (b) shows the total number of unique or shared DEGs between different time points in genotypes NGB348 (left) and NGB6704 (right). Genes were considered differentially expressed with absolute log2FC $> 1$ and FDR-adjusted $P < 0.05$.

like protein, peroxidase, lipoxygenase, and endochitinase.

GO enrichment analysis of DEGs revealed common and genotype-specific enrichment (Supp. File 3). Both genotypes had the highest number of enriched GO terms at 8 h, highlighting transcriptomic changes related to *C. rosea* inoculation. Both genotypes showed enrichment for defense response to fungus, response to oxidative stress, secondary metabolic process, amino acid metabolism and catabolism, macromolecule modification, ammonium transmembrane transporter activity, iron ion binding, enzyme inhibitor activity, hydrolase activity, oxidoreductase activity, and molecular function inhibitor activity at 8 h. GO terms were enriched for manganese ion binding at all time points in genotype NGB348 (LE) but only at later time points (32 h and 40 h) in genotype NGB6704 (HE). Similarly, GO enrichment for the external encapsulating structure was enriched only at later time points in NGB6704 (HE) but at all time points in NGB348 (LE). Antioxidant activity was enriched in NGB348 at timepoints 8 h, 32 h, and 40 h but only at 8 h and 16 h in NGB6704. Regulation for RNA metabolic process, gene expression, and macromolecule biosynthesis were enriched at 8 h in NGB348 but at 40 h in NGB6704.

### 3.4. Transcriptional response differences between wheat genotypes in response to Z. tritici infection

Application of *Z. tritici* at time points 32 h and 40 h was compared to the control treatment separately in each genotype. Genotype NGB6704 (HE) showed a strong response to the application of *Z. tritici* with 1516 and 543 DEGs at 32 h and 40 h respectively (Fig. 6). On the contrary, NGB348 (LE) had relatively few DEGs, with only 135 and 6 at 32 h and 40 h, respectively. Moreover, we also observed a differential response between genotypes (Supp. File 4). Two genes predicted to encode UNC93-like proteins, an ion channel regulatory protein, were upregulated in NGB348 but downregulated in NGB6704 (Fig. 7). Similarly, gene LOC123076790, putatively encoding for an β-fructofuranosidase, was upregulated in NGB348 and downregulated in NGB6704. Gene LOC543422 associated with PR protein PRB1-3 also showed differing expression between genotypes, whereby it was downregulated in NGB348 but upregulated in NGB6704. Additionally, NGB6704 showed upregulation of genes predicted to encode ABC transporter G family protein, G-type lectin S-receptor-like serine/threonine protein kinase, alcohol dehydrogenase, endochitinase, cytokinin dehydrogenase, peroxidase, and germin-like protein 8-5. NGB6704 further showed downregulation of 29 kDa ribonucleoprotein A, ribonuclease 1-like, and rubisco large subunit-binding protein. These genes were not DE in the NGB348 genotype.

GO enrichment from the DEGs showed enrichment of common terms between the two genotypes (Supp. File 5). GO terms related to amino acid catabolic and metabolic process and protein phosphorylation, protein kinase activity, chitinase, and chitin-binding activity were enriched in both genotypes at 32 h for the upregulated genes subset. No significantly enriched terms were present for NGB348 at 40 h due to the low number of DEGs. NGB6704 exhibited consistent enrichment of most GO terms related to the metabolism of amino acids in the upregulated genes, while in the downregulated genes, it showed enrichment of metabolic process related to sulfur, proteinogenic amino acid, glutathione as well as amino acid biosynthesis.

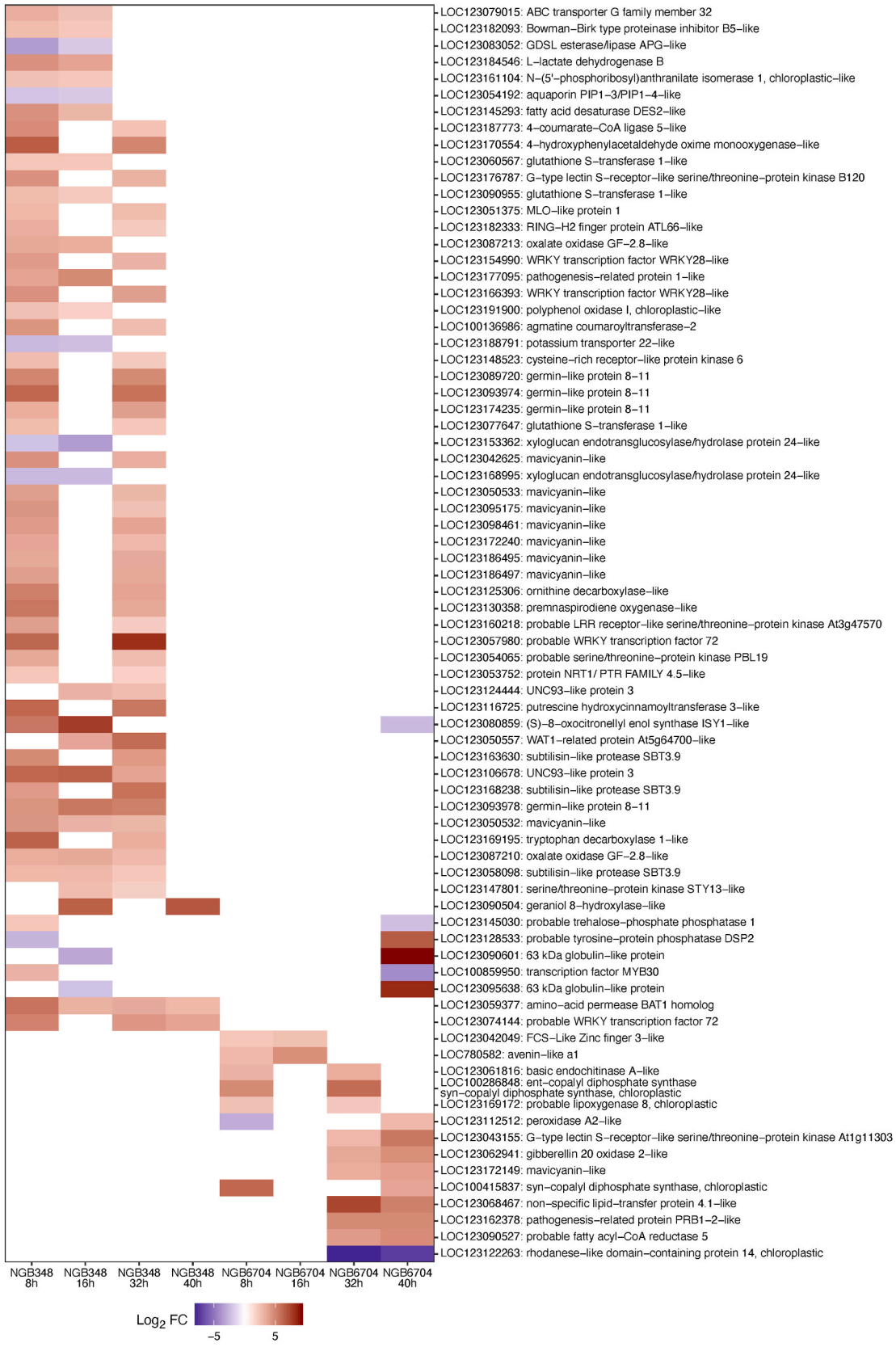


**Fig. 5.** Heatmap showing the log2 fold change (FC) of selected differentially expressed genes (relative to respective control) in response to *Clonostachys rosea* at time points 8 h, 16 h, 32 h, and 40 h. Genes shown were either unique to genotypes NGB348 and NGB6704 or with differing expression patterns between genotypes. Genes were considered differentially expressed with absolute log2FC $> 1$ and FDR-adjusted $P < 0.05$.

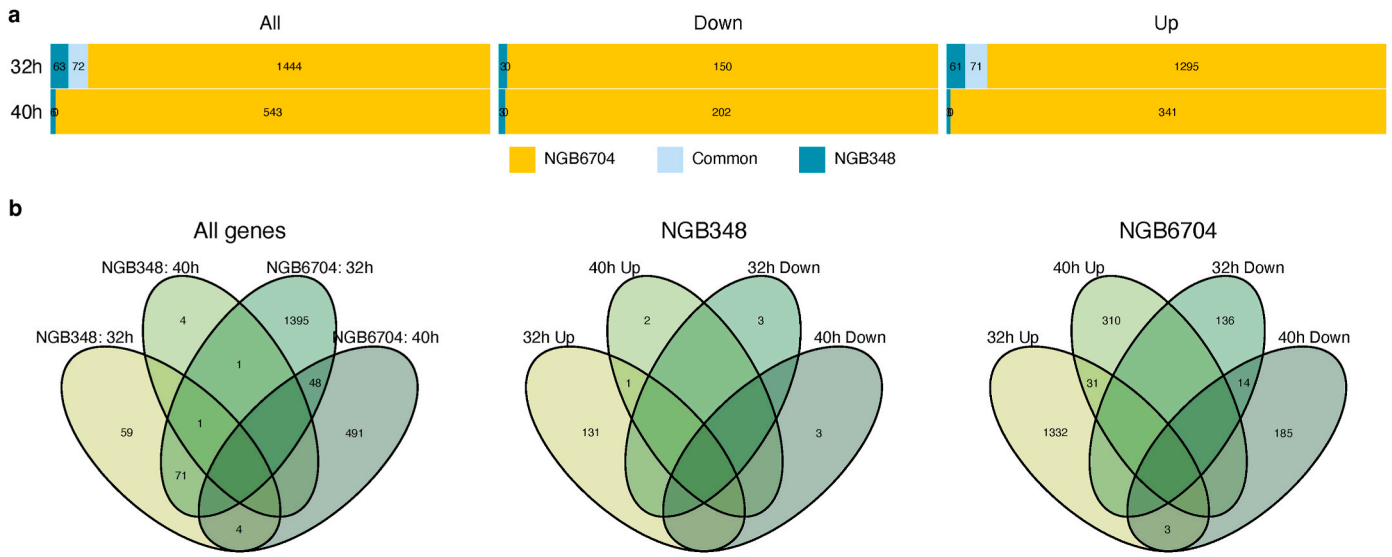


**Fig. 6.** (a) Number of differentially expressed genes (DEGs) in winter wheat genotypes in response to *Zymoseptoria tritici* at time points 32 h and 40 h. Venn diagram (b) shows the total number of unique or shared DEGs between samples across genotypes (left), NGB348 (middle), and NGB6704 (right). Genes were considered differentially expressed with absolute log2FC $> 1$ and FDR-adjusted $P < 0.05$.

### 3.5. Transcriptional response differences between wheat genotypes in response to co-inoculation with C. rosea and Z. tritici

Sequential application of *C. rosea* and *Z. tritici* at 32 h and 40 h was compared against the control treatment for each genotype. In a similar manner to the results seen with the application of *C. rosea* only, NGB348 (LE) showed more DEGs at 32 h (n = 343), and NGB6704 (HE) showed more DEGs (n = 179) at 40 h (Fig. 8a). In total, 27 DEGs and 24 DEGs were shared between genotypes at 32 h and 40 h, respectively. Most DEGs were upregulated in both genotypes (Fig. 8b, Supp. File 6). Among the genotype-specific DEGs, NGB348 showed upregulation of genes related to germin-like protein, PR protein PRB1-2-like protein, and wall-associated receptor kinase 3-like protein (Fig. 9). Downregulation of genes predicted to encode cold-shock proteins and chlorophyll *a-b* binding protein was also observed. In NGB6704, upregulation of genes related to ABC transporter G family member 32, gibberellin protein, gibberellin 20 oxidase 2-like protein, non-specific lipid transfer protein, and fatty acyl-CoA reductase was observed. Moreover, downregulation of ribonuclease 1-like was also observed.

Commonly enriched GO terms between the two genotypes were related to processes of cell wall macromolecule catabolism, aminoglycan metabolism and catabolism, chitin binding, manganese ion binding, and oxidoreductase activity (Supp. File 7). Additionally, NGB6704 uniquely showed enrichment of lipid transport and localization at 32 h and 40 h.

### 3.6. Shared genes between DEGs and segregating genomic regions

Cross-referencing genes located in genomic regions segregating with the efficacy of *C. rosea*-mediated biocontrol of STB [48] and the current DEG dataset identified multiple genes in common (Table 3).Three E3 ubiquitin-protein ligase PUB23-like genes were significantly linked to biocontrol efficacy in the genome-wide association study (GWAS) [48], from which one gene (LOC123134991) was differentially expressed between the NGB348 and NGB6704 genotypes (Table 3).

A selected number of genes displayed upregulation in NGB348 after the *C. rosea* application and in NGB6704 after the *Z. tritici* application. Genes encoding for leaf rust disease-resistance locus receptor-like protein kinase-like genes, associated with *Z. tritici* treatment in the GWAS study, showed differential expression. Interestingly, seven of these genes were upregulated in NGB348 at 8 h post-*C. rosea* application, while only one was upregulated in NGB6704. However, NGB6704 exhibited upregulation of these genes post-*Z. tritici* inoculation at 32 h and 40 h. Another receptor-like protein kinase showed upregulation in the presence of *C. rosea* in NGB348 and the presence of *Z. tritici* in NGB6704. Similarly, genes related to ABC transporter G family member 32-like and oxalate oxidase, associated with *Z. tritici* treatment in the GWAS study, showed upregulation in NGB348 in the presence of *C. rosea* and in NGB6704 in the presence of *Z. tritici*. A chitin elicitor receptor kinase 1-like gene, associated with *Z. tritici* + *C. rosea* treatment in the GWAS study, was upregulated in NGB348 in response to *C. rosea* and in NGB6704 in response to *Z. tritici*.

Some of the overlapping genes between the DEG dataset and the GWAS dataset were upregulated exclusively in NGB6704. Non-specific lipid-transfer protein genes associated with *Z. tritici* in the GWAS study were upregulated exclusively in NGB6704 following *C. rosea* treatment and co-inoculation with *Z. tritici* and *C. rosea*. Another gene associated with *Z. tritici* in the GWAS study related to 3-isopropylmalate was upregulated exclusively in NGB6704 in treatments with *C. rosea* and *Z. tritici*. Furthermore, six genes (LOC123064258, LOC123064272, LOC123064492, LOC123068204, LOC123086769, and LOC123142257), differentially regulated exclusively in NGB6704 following *Z. tritici* and/or *C. rosea* treatment, were associated with *Z. tritici* or *Z. tritici* + *C. rosea* treatment in the GWAS study.

## 4. Discussion

In this study, we explored the transcriptomic responses of two wheat genotypes exhibiting varying efficacy to *C. rosea* in controlling STB. Phenotypic assessment of the two wheat genotypes, NGB6704 and NGB348, showed similar susceptibility to *Z. tritici* infection and STB disease development, confirming previous results [48,80]. However, when applying the BCA *C. rosea*, a significant reduction in STB disease was only observed in genotype NGB6704. This difference was also noted when comparing biocontrol efficacy (Zt - ZtCr), where genotype NGB6704 (HE) is able to benefit more from the *C. rosea* application compared with NGB348 (LE). These results are consistent with previous findings [48], where NGB6704 also responded positively to *C. rosea*-mediated biocontrol of STB. While NGB348 was previously reported to exhibit increased STB severity when co-inoculated with *Z. tritici* and *C. rosea* [48], this negative effect was not observed in the current study.

Based on overall read counts as well as read counts specific to

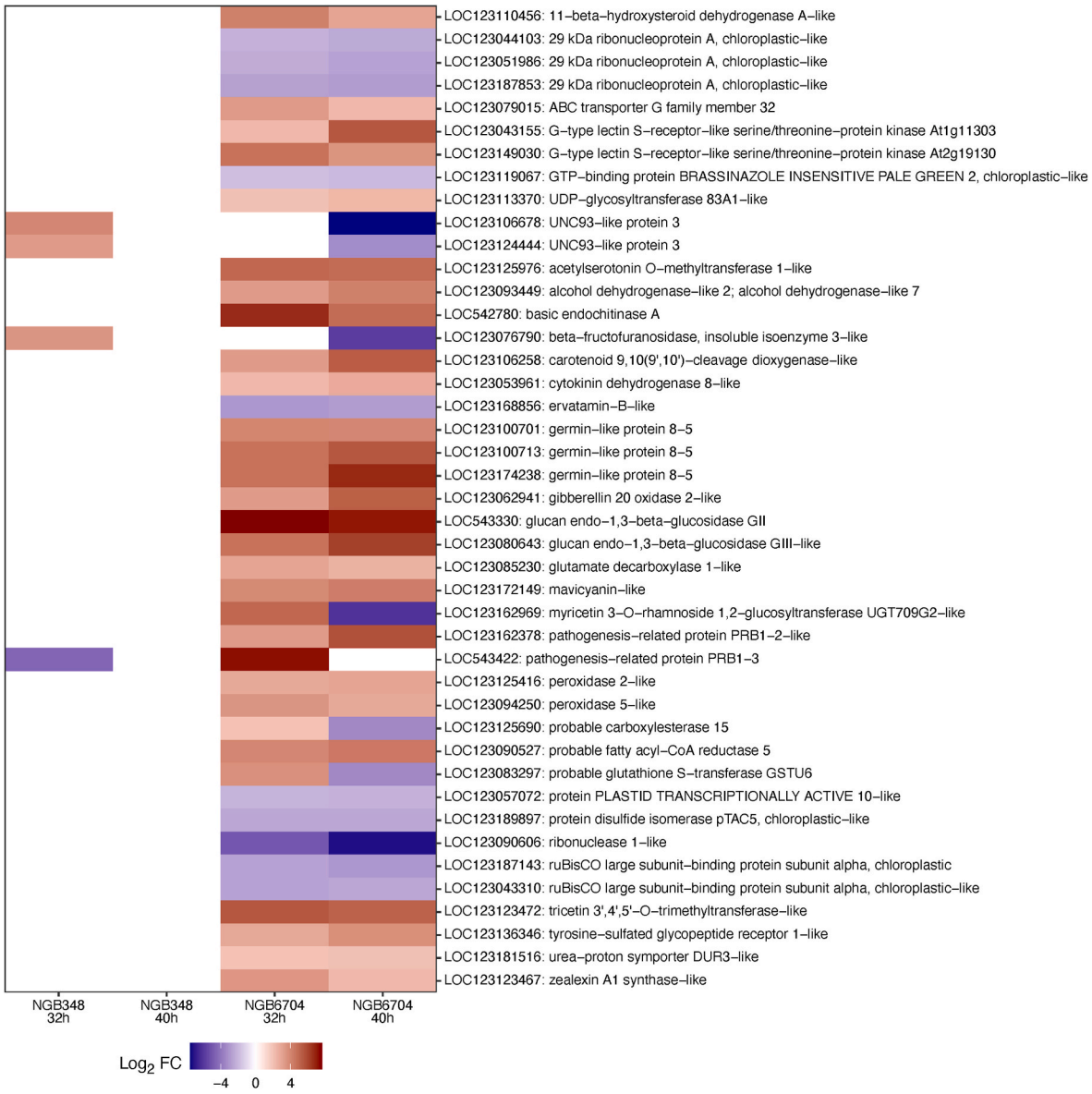


**Fig. 7.** Heatmap showing the log2 fold change (FC) of selected differentially expressed genes (relative to respective control) in response to *Zymoseptoria tritici* at time points 32 h and 40 h. Genes shown were either unique to genotypes NGB348 and NGB6704 or with differing expression patterns. Genes were considered differentially expressed with absolute log2FC $> 1$ and FDR-adjusted $P < 0.05$.

housekeeping genes, used as a proxy for fungal biomass, neither *C. rosea* nor *Z. tritici* showed significant differences between genotypes at any time point when applied separately. Overall, *Z. tritici* read counts were comparable with previously reported levels during the asymptomatic phase within the initial 24 h [50]. An increase in *Z. tritici* read counts was only observed during the transition from the asymptomatic phase to the symptomatic phase, occurring approximately 7–9 days post-inoculation [50,54]. While *C. rosea* naturally resides in the soil and the rhizosphere, foliar survival is also established, with microscopy demonstrating successful germination, growth, and sporulation on barley leaves by 7 days post-inoculation [81]. In this study, similar *C. rosea* read counts were observed across genotypes when applied alone. However, a significant increase in the biomass of both *Z. tritici* and *C. rosea* was detected when co-applied to NGB6704 (HE) but not NGB348 (LE) after 40 h. This suggests a genotype-specific interaction effect, which is further supported by a stronger transcriptional response and a higher number of DEGs in NGB6704 compared to NGB348 at 40 h. Importantly, this increased fungal biomass on NGB6704 ultimately correlates with stronger biocontrol and reduced STB disease severity at 28 days post-inoculation, compared to NGB348.A microscopic investigation of *C. rosea* and *Z. tritici* on these genotypes could provide further insights into the survival, growth and interaction dynamics between the two fungi.

The major source of variation in the overall wheat transcriptomic response was the plant genotype, suggesting intrinsic differences between NGB6704 and NGB348 that influence the outcome during interactions with *C. rosea* and/or *Z. tritici*. Previously, plant genotype has been shown to be the primary source of transcriptomic variation in plants treated with resistance inducers, including defense hormones [82], and more recently in peas infected by the pathogen *Aphanomyces euteiches* and in sugar beet infected by *A. cochlioides* [83,84]. The plant genotype has been reported to constitute a major source of variation in the biocontrol efficacy of *C. rosea* against both STB and fusarium foot rot in wheat [47,48].

The application of *Z. tritici* was the next major influencer on the transcriptome response, followed by the application of *C. rosea* and time points. The transcriptomic analysis revealed distinct gene expression patterns in response to *C. rosea*, *Z. tritici*, and their co-application in two

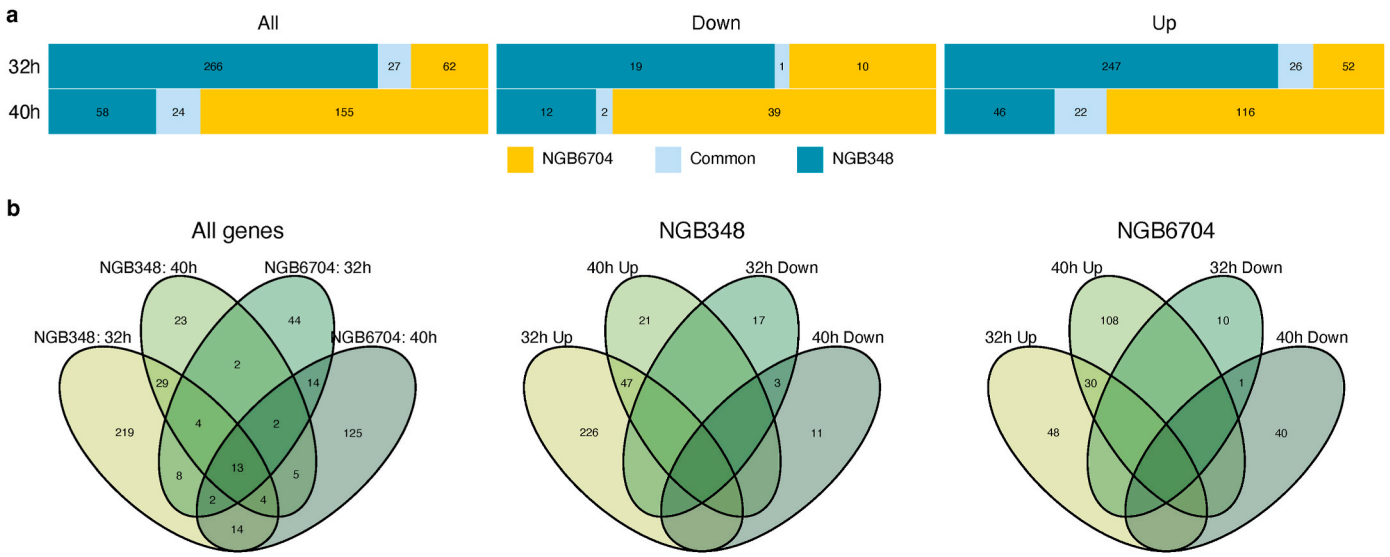


**Fig. 8.** (a) Number of differentially expressed genes (DEGs) in winter wheat genotypes in response to combined application of *Clonostachys rosea* and *Zymoseptoria tritici* (ZtCr) at time points 32 h and 40 h. Venn diagram (b) shows the total number of unique or shared DEGs between samples across genotypes (left), NGB348 (middle), and NGB6704 (right). Genes were considered differentially expressed with absolute log2FC $> 1$ and FDR-adjusted $P < 0.05$.

winter wheat genotypes, NGB6704 (HE) and NGB348 (LE), exhibiting contrasting *C. rosea*-mediated biocontrol efficacy against STB. Distinct genes and variations in the degree of regulation of shared genes were observed between genotypes.

Both genotypes responded to *Z. tritici* inoculation but showed distinct transcriptomic profiles despite exhibiting similar susceptibility to STB. NGB6704 showed a high number of DEGs, while NGB348 exhibited very few, suggesting ineffective pathogen detection, suppression of defenses, or a delayed response not captured at the sampled time points. In NGB6704, upregulated genes were dominated by immune defense responses, including serine/threonine-protein kinases, cysteine-rich receptor-like kinases, ABC transporters, chitinase, germin-like proteins, PR proteins, WRKY transcription factors, and MYB transcription factors Receptor-like kinases are key components of cell-surface immunity in plants that detect non-self as signs of infection [85], are also encoded by resistance genes such as *Stb6*, *Stb15*, and *Stb16q* [49]. In addition, WRKY transcription factors regulate defense signaling in both pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) [86]; and were previously reported to be upregulated at 1 dpi following *Z. tritici* infection [50]. Similarly, germin-like proteins, which are highly ubiquitous glycoproteins involved in developmental processes and plant defense against biotic and abiotic stresses [87,88], were previously shown to be upregulated during *Z. tritici* infection, albeit at later infection stage [50]. These functions together suggest a strong immune response involving direct attack on *Z. tritici*, synthesis and efflux of specialized metabolites, and cross-linking of plant cell wall components.

Genes putatively encoding PR proteins are among the few genes upregulated in both genotypes upon *C. rosea* exposure. PR proteins are indicative of a generalized recognition of microbial ingress and activation of defenses in plants [89]. PR protein family members are induced not only by pathogens but also by BCAs and other beneficial microbes. For example, arbuscular mycorrhizal fungi induce the expression of PR genes in potatoes, and is further amplified in the presence of the pathogens *Alternaria solani* [90] or *F. sambucinum* [91]. *Clonostachys rosea* has also been shown to induce PR genes in wheat, with amplification in the presence of *F. culmorum* [92], in pine both alone and in the presence of *F. circinatum* [43] and in oat, where *C. rosea* is also able to detoxify toxins produced by *F. graminearum* [93].

Beyond the common PR response to *C. rosea* application, the two wheat genotypes showed distinct transcriptomic responses, with relatively few shared DEGs and marked differences in the timing and extent of plant defense-related gene expression. NGB348 (LE) displays a strong early response (8 h post-*C. rosea* application) with induction of germin-like proteins, serine/threonine protein kinases, lectin domain-containing kinases, cysteine-rich receptor-like kinases, wall-associated receptor-like kinases, receptor-like protein kinases, WRKY transcription factors, auxin-responsive proteins, disease resistance proteins, PR proteins, E3 ubiquitin-protein ligases, and chitinases. Unique to NGB348 were several WRKY transcription factors and germin-like proteins, both central to defense regulation and stress tolerance [86,94–97]. The higher expression of these genes suggests rapid activation of PTI by *C. rosea*. In contrast, NGB6704 (HE) shows a less intense and delayed response (40 h post-*C. rosea* application), characterized by induction of genes predicted to encode defense proteins, including chitinase, peroxidase, and lipoxygenase. This delayed response in the genotype with high *C. rosea* efficacy may indicate a more modulated or controlled defense activation.

Comparison of *C. rosea* response with *Z. tritici* response reveals that several genes upregulated by *Z. tritici* in NGB6704 (HE) at later stages (32 h and 40 h), including protein kinases, germin-like proteins, and PR-proteins, are instead induced at earlier time points by *C. rosea* in NGB348 (LE) at earlier time points, but not in NGB6704. When exposed to *Z. tritici* alone, NGB6704 showed an induction of genes from the same defense-related categories, though involving different DEGs, indicating distinct regulatory strategies between the two genotypes. This may suggest that *C. rosea* specifically suppresses early defense activation in NGB6704. Taken together, these patterns suggest that *C. rosea* exposure in NGB348 (LE) triggers an initial activation of defense genes that may return to baseline, whereas in NGB6704 (HE) *C. rosea* initially suppresses immune responses followed by a stronger PTI response at later stages.

During co-inoculation with *C. rosea* and *Z. tritici*, NGB348 (LE) exhibited a strong defense response at 32 h, potentially driven by *C. rosea*, suggesting that *C. rosea* may stimulate a more dominant defense response, even in the presence of *Z. tritici*. The genes induced in both wheat genotypes reflected similar functional categories, including genes related to direct fungal attack (chitinase, subtilisin-like protease, peroxidase, laccase), production and efflux of defense specialized metabolites (tau-cadinol synthase-like protein, ABC transporter G family), plant cell wall strengthening (peroxidase, laccase), and other defense-related proteins including germin-like proteins, and different types of

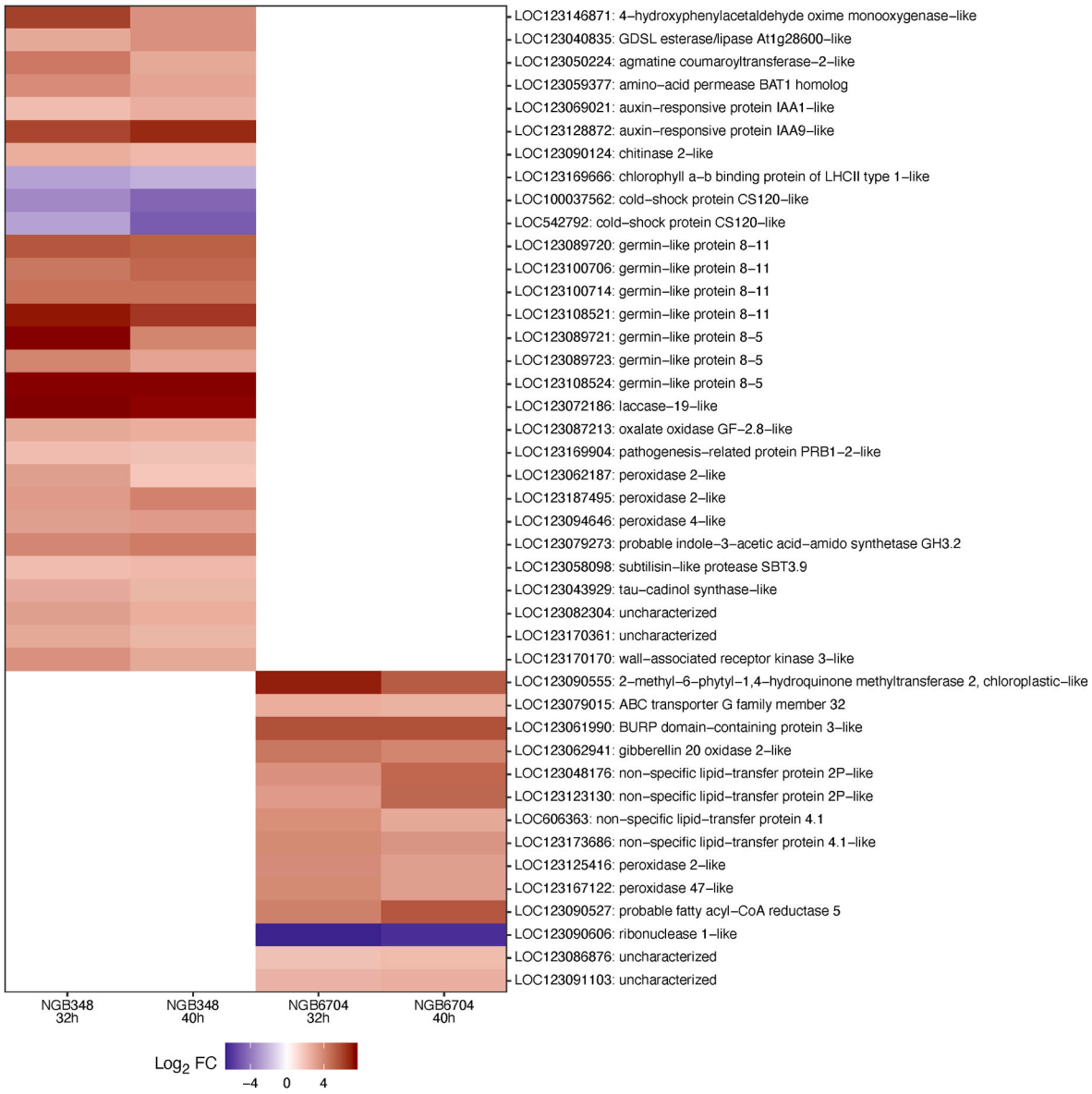


**Fig. 9.** Heatmap showing the log2 fold change (FC) of selected differentially expressed genes (relative to respective control) in response to combined application of *Clonostachys rosea* and *Zymoseptoria tritici* (ZtCr) at time points 32 h and 40 h. Genes shown were either unique to genotypes NGB348 and NGB6704 or with differing expression patterns. Genes were considered differentially expressed with absolute log2FC threshold >1 and FDR-adjusted $P < 0.05$.

PR proteins (PRB1-2-like, non-specific lipid-transfer protein 2P-like). Despite these shared functions, a strong temporal difference between the genotypes is observed: defense gene expression peaked at 32 h in NGB348 but at 40 h in NGB6704, potentially also due to higher fungal biomass at the later time point in NGB6704.

Cross-referencing wheat DEGs from the current study and genes physically located in genomic regions segregating with exposure to *C. rosea*, *Z. tritici*, or their combination [48] highlighted several defense-related genes, including receptor-like protein kinases, an ABC transporter, an oxalate oxidase, and a chitin elicitor receptor kinase, all showing genotype-specific expression with early induction in NGB348 (LE) and delayed activation in NGB6704 (HE). Non-specific lipid--transfer protein 2P-like PR genes were instead specifically upregulated in NGB6704 and associated with *Z. tritici* resistance regions, [48], suggesting roles in in pathogen defense and *C. rosea*-mediated biocontrol. Three E3 ubiquitin-protein ligase PUB23-like genes were previously reported to segregate with *C. rosea*-mediated biocontrol efficacy [48]. Two of these genes were upregulated in both genotypes at 8 h following *C. rosea* treatment, suggesting allelic variation in protein structure rather than transcriptional regulation as the basis of genotype-specific responses. Given the central role of ubiquitination in regulating plant immunity [98,99] and evidence that E3 ligases interact with *Z. tritici* effectors [100], these findings suggest that both transcriptional and structural variation in defense genes contribute to wheat responses to *C. rosea* and fungal pathogens, warranting further functional characterization.

In conclusion, this study revealed clear genotype-specific differences in wheat gene expression responses to *C. rosea* and *Z. tritici*. With the low-efficacy genotype NGB348 showing stronger and earlier defense activation that may undermine biocontrol benefits, several scenarios could explain these observations. Firstly, while *C. rosea* may exert a comparable biocontrol effect against *Z. tritici* in both genotypes, NGB348 (LE) might perceive *C. rosea* as a greater threat, triggering an excessive defense response that may diminish biocontrol efficacy. Secondly, the strong defense activation in the low biocontrol efficacy genotype could involve a hypersensitive-like response that restricts pathogen spread, but could also potentially stress the plant, hindering its ability to benefit from biocontrol by *C. rosea*. Although *C. rosea* alone did not induce

**Table 3**
Log2 fold change of differentially expressed wheat genes (FDR-adjusted $P < 0.05$) physically located in ±1.6 cM genomic regions segregating with the efficacy of *Clonostachys rosea*-mediated biocontrol of septoria tritici blotch caused by *Zymoseptoria tritici*, as previously reported in Chaudhary et al. [48]. Trait denote Zt (*Z. tritici* alone), ZtCr (*Z. tritici* + *C. rosea*) and biocontrol efficacy (Zt - ZtCr) as reported in Chaudhary et al. [48]. Empty cells denote gene not differentially expressed at absolute log2 fold change threshold >1 and FDR-adjusted $P < 0.05$.

| Trait | Chromosome | Description | Gene ID | Cr v Control | | | | | | | | Zt v Control | | | | ZtCr v Control | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | NGB348 | | | | NGB6704 | | | | NGB348 | | NGB6704 | | NGB348 | | NGB6704 | |
| | | | | 8h | 16h | 32h | 40h | 8h | 16h | 32h | 40h | 32h | 40h | 32h | 40h | 32h | 40h | 32h | 40h |
| Biocontrol efficacy | 6B | E3 ubiquitin-protein ligase PUB23-like | LOC123134976 | 3.6 | | | | 3.1 | | | | | | | | | | | |
| | | | LOC123134991 | 7.3 | | | | | | | | | | | | | | | |
| | | | LOC123134993 | 3.7 | | | | 3.2 | | | | | | | | | | | |
| Zt | 2B | Trimethyltridecatetraene synthase-like | LOC123047413 | | | | | | | | | | | −3.2 | | | | | |
| | 3B | 3-isopropylmalate dehydrogenase | LOC123064221 | | | | | | | | 3.6 | | | | 3.4 | | | | |
| | | Receptor-like Protein kinase-like (RLK); Leaf rust 10 | LOC123064589 | 4.7 | | | | 2.6 | | | | | | | | | | | |
| | | Disease resistance locus | LOC123068455 | 3.3 | | | | | | | | | | 1.9 | | | | | |
| | | | LOC123068451 | 6.4 | | | | | | | | | | 2.9 | | | | | |
| | | | LOC123068417 | | | | | | | | | | | 1.5 | | | | | |
| | | | LOC123068430 | | | | | | | | | | | | 6.1 | | | | |
| | | | LOC123068450 | 3.9 | | | | | | | | | | 2.6 | | | | | |
| | | | LOC123068454 | 1.9 | | | | | | | | | | | | | | | |
| | | | LOC123068429 | | | | | | | | | | | 1.8 | | | | | |
| | | | LOC123067231 | | | | | | | | | | | 2.1 | | | | | |
| | | | LOC123068447 | 5.9 | | | | | | | | | | 2.7 | | | | | |
| | | NADPH-dependent aldehyde reductase- | LOC123068392 | | | | | | | | | | | 1.5 | | | | | |
| | | Beta-fructofuranosidase | LOC123068213 | 8.6 | | | | 3.9 | | | | | | 2.6 | | | | | |
| | | | LOC123068294 | 4.4 | 4.4 | | | 2.9 | | | | | | | | | | | |
| | | Cysteine-rich receptor-like protein kinase | LOC123068286 | 3 | | | | | | | | | | 2.3 | | | | | |
| | | Non-specific lipid-transfer protein | LOC606363 | | | | | | | | | | | | | | | 3.7 | 2.8 |
| | | | LOC123068467 | | | | | | | 7.3 | 5.1 | | | | | | | | 7 |
| | | | LOC123068469 | | | | | | | | | | | | | | | | 5.6 |
| | | | LOC123068477 | | | | | | | | | | | | | | | | 1.7 |
| | | Probable receptor-like protein kinase (RLK) | LOC123067243 | 2.3 | | | | | | | | | | | | | | | |
| | | Probable_serine/threonine-protein kinase | LOC123064257 | | | | | | | | 5.7 | | | | | | | | |
| | | Proline-rich transmembrane protein | LOC123064265 | 3 | | | | 2.4 | | | | 3.6 | | 2.9 | | | | | |
| | | Putative glutamine amidotransferase | LOC123064606 | 4.6 | | | | | | | | | | | | | | | |
| | | Putative pentatricopeptide repeat-containing protein | LOC123068239 | | | | | | | | | | | −5.3 | | | | | |
| | | ABC transporter G family member | LOC123070611 | 4 | | | | | | | | | | 3.5 | | | | 2.3 | |
| | | GDSL esterase/lipase | LOC123070474 | | | | | | | | | | | 1.6 | | | | | |
| | | Receptor-like Protein kinase-like (RLK); Leaf rust 10 Disease resistance locus | LOC123070520 | 2.2 | | | | | | | | | | | | | | | |
| | | MAPK kinase substrate protein | LOC123065542 | | | | | | | | | | | 2.8 | | | | | |
| | | VQ motif-containing protein | LOC123070619 | | | | | | | | | | | 1.7 | | | | | |
| | | Anthocyanidin 5,3-O-glucosyltransferase | LOC123065626 | 6 | | | | | | | | | | | | | | | |
| | | Basic endochitinase | LOC123070508 | | | | | | | | | | | 6.4 | | | | 4.1 | |
| | | Mitogen-activated protein kinase | LOC123067546 | | | | | | | | | 3 | | | | | | | |
| | | Oxalate oxidase | LOC543323 | | 3.6 | | | | | | | | | 4.6 | | | | | |
| | | | LOC123070398 | | | | | | | | | | | 4.1 | | | | | |
| | | Probable glutamate carboxypeptidase | LOC123070655 | | | | | | | | | | | | 3.4 | | | | |
| | | Receptor-like protein kinase 5 (RLK 5) | LOC123070648 | 1.9 | | | | | | | | | | | | | | | |
| ZtCr | 1B | LEC14B protein | LOC123107986 | | | | | | | | | | | 1.9 | | | | | |
| | | UDP-glycosyltransferase | LOC123107683 | | | | 2.4 | | | | | | | | | | 2.6 | | |
| | | | LOC123107676 | | | | | | | | | | | | | | 2.4 | | |
| | | Pentatricopeptide repeat-containing protein | LOC123107532 | −2.2 | | | | | | | | | | | | | | | |
| | 6D | Chitin elicitor receptor kinase | LOC123142266 | 3.2 | | | | | | | | | | 2.7 | | | | | |

disease symptoms in either genotype, its interaction with NGB348 might trigger a localized, defense-driven response that, paradoxically, could be detrimental to the plant's overall health. Another possibility is that interactions between *C. rosea* and *Z. tritici* alter pathogen behavior, as suggested by higher fungal biomass in the high-efficacy genotype. By integrating transcriptomic and population genetic data, this study underscores the complexity of tripartite interactions among wheat, pathogens, and biocontrol agents. Further work should test these hypotheses under different environmental conditions and across genotypes to clarify underlying mechanisms and optimize biocontrol strategies.

## CRediT authorship contribution statement

**Sidhant Chaudhary:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Edoardo Piombo:** Supervision, Software, Formal analysis, Data curation. **Mukesh Dubey:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization. **Dan Funck Jensen:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Laura Grenville-Briggs:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization. **Magnus Karlsson:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization.

## Ethics approval and consent to participate

Not applicable.

## Availability of data and materials

Raw transcriptome data can be accessed on the European Nucleotide Archive (ENA, https://www.ebi.ac.uk/ena/browser/search) under Bioproject "PRJEB87780".

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## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Magnus Karlsson reports financial support was provided by SLU Grogrund. Magnus Karlsson and Dan Funck Jensen have patent BCA control of STB licensed to Lallemand. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pmpp.2025.103013.

## Data availability

All data is available in the manuscript or in public repositories.

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