Title: Multiplexed effector screening for recognition by endogenous resistance genes using positive defense reporters in wheat protoplasts

Salome Wilson*, Bayantes Dagvadorj*, Rita Tam, Lydia Murphy, Nigel Heng, Emma Crean[$], Julian Greenwood, John P. Rathjen, and Benjamin Schwessinger

Research School of Biology, The Australian National University, Canberra, ACT, 2601, Australia

*These authors contributed equally to this work
[$]Current address: Institute for Plant Sciences, University of Cologne, Cologne, Germany

Corresponding author: Benjamin Schwessinger
Email: benjamin.schwessinger@anu.edu.au

Short Title:
Defense signaling in wheat protoplasts

Abstract

A key goal in agriculture is to create pathogen-resistant crop species via breeding or biotechnological approaches. Plant resistance (*R*) and pathogen avirulence (*Avr*) gene interactions play a vital role in pathogen resistance. Efficient molecular screening tools for crops lack far behind their model organism counterparts, yet they are essential to rapidly identify agriculturally important molecular interactions that trigger host resistance. To fill this important gap, we have developed a novel wheat protoplast assay that enables efficient screening of Avr/R interactions at scale. Our assay allows access to the extensive gene pool of phenotypically described *R* genes because it does not require the overexpression of cloned *R* genes. It is suitable for multiplexed *Avr* screening, with interactions tested in pools of up to fifty *Avr* candidates. Our

assay is based on newly identified Avr/R-induced defense genes which we used to create promoter-luciferase reporter constructs. We combined these reporter constructs with a synthetic biology based dual-color ratiometric reporter system that normalizes read-outs accounting for experimental variability and Avr/R-induced cell-death. In addition, we introduced a replicon-based plasmid derived from the wheat dwarf virus, which self-replicates in wheat reducing the amount of plasmid used in the assay. Our new assay increases the throughput of *Avr* candidate screening, accelerating the study of cellular defense signaling and resistance gene identification in wheat. We anticipate that the uptake of our assay by the community will significantly accelerate *Avr* identification for many wheat pathogens, leading to improved genome-guided pathogen surveillance and breeding of disease-resistant crops.

Introduction

Plant pathogenic fungi cause severe damage to cereal crops. These pathogens secrete hundreds of proteinaceous molecules called effectors during infection which manipulate plant processes to facilitate disease (Dodds and Rathjen, 2010). Some effectors can be recognized by resistance (R) proteins and are therefore also called avirulence (Avr) proteins. Avr recognition triggers a suite of defense-related host responses to fight off pathogen infection including the hypersensitive response (HR) that leads to cell-death at the site of infection and halts pathogen growth (Laflamme et al., 2020). The deployment of suitable *R* genes is a major breeding objective to reduce the negative impact of pathogens on crop production including in wheat. Recent years have made significant progress in identifying and cloning a wide variety of *R* genes in wheat (Wulff and Krattinger, 2022). Yet the identification of the corresponding *Avr* genes is lacking far behind. For example, there are only four confirmed *Blumeria graminis* f. sp. *tritici Avr* genes (Praz et al., 2017; Bourras et al., 2019; Hewitt et al., 2021; Müller et al., 2022), two for *Zymoseptoria tritici* (Zhong et al., 2017; Meile et al., 2018), and five for *Puccinia graminis* f. sp. *tritici* (Chen et al., 2017; Salcedo et al., 2017; Upadhyaya et al., 2021; Arndell et al., 2023). There are no confirmed *Avr* genes for *Puccinia triticina* and *Puccunia striiformis* f. sp. *tritici* despite both belonging to the top five wheat pathogens globally (Savary et al., 2019). This lack of confirmed *Avr* genes in wheat pathogen and especially rust fungi is confounded by the absence of an efficient screening tool for *Avr* recognition in wheat. Most current methods require

to have *Avr* and *R* gene candidates cloned, express them heterologously, and rely on HR as general read-out for Avr/R interactions. For example, the model plant *Nicotiana benthamiana* is used to validate Avr/R interactions as it produces visible HR upon transient expression of *Avr/R* genes through agroinfiltration. Virus-mediated overexpression (VOX) can be used to screen *Avr* candidates in wheat cultivars carrying the corresponding *R* gene as the Avr recognition will lead to visible HR symptoms on the infected leaves (Lee et al., 2012; Bouton et al., 2018). However, the method is only applicable to small proteins (Lee et al., 2012) and used viruses are not accessible globally due to biosecurity regulations (Bouton et al., 2018). Recently, HR-based assays in wheat protoplasts have emerged as a potential alternative to test Avr/R interactions (Saur et al., 2019). In this transient assay, wheat protoplasts are co-transfected with *Avr/R* genes along with a *luciferase* reporter gene. Avr/R interaction triggers cell-death causing significant decreases in luciferase enzyme activity. This assay has been used to validate *Avr* candidates from wheat rust pathogens (Saur et al., 2019; Upadhyaya et al., 2021; Luo et al., 2021; Ortiz et al., 2022). However, the HR-based wheat protoplast assay in its current form has several limitations that make it impractical for high-throughput *Avr/R* screening. Firstly, it requires the transgenic overexpression of both the *Avr* and *R* genes from plasmids (Saur et al., 2019). This prevents its use when the *R* gene has not yet been cloned thus stopping access to large numbers of phenotypically described but uncloned *R* genes (McIntosh et al., 1995). Moreover, it is well known that luminescence is highly variable between biological replicates and experiments (Saur et al., 2019; González-Grandío et al., 2021) which can make the results difficult to interpret for high-throughput screening purposes. In addition, the assay in its current form relies on the reduction of luciferase activity as read out which is unspecific and cannot be differentiated from cell-death that is unrelated to HR. Lastly, the assay requires large amount of target gene plasmids for the protoplast transfection to achieve observable Avr/R-triggered cell-death. These limitations encouraged us to develop a novel assay in wheat protoplast that relies on a positive defense specific read-out and accounts for experimental variability via normalization. Our new assay allows the research community to access the whole wheat gene pool to screen for Avr/R interactions and enables the investigation of downstream defense signaling directly in wheat.

Results

Cell-death triggered by Avr/R interactions in protoplasts is time sensitive

We first sought to characterize the time of HR onset in wheat protoplasts following *Avr/R* recognition. We used the two published *Avr/R* gene pairs, *AvrSr35/Sr35* (Salcedo et al., 2017; Saintenac et al., 2013) and *AvrSr50/Sr50* (Chen et al., 2017; Mago et al., 2015), from wheat stem rust *P. graminis* f. sp. *tritici* for initial method development. We aimed to identify the optimal time point for RNAseq analyses that captures transcriptional changes prior to the onset of HR triggered by Avr/R interactions. As previously described (Saur et al., 2019), we used luminescence as a proxy for cell viability after overexpression of the *luciferase* reporter gene in conjunction with each *Avr/R* combination and corresponding control. We expressed all *Avr* and *R* genes under control of maize ubiquitin promoter (*UBI*) (Christensen et al., 1992) when expressed from a plasmid. We used the wheat cultivar (cv.) Fielder for the analysis of *AvrSr35/Sr35* and expressed both genes from a plasmid. For *AvrSr50/Sr50*, we made use of the introgression of *Sr50* into cv. Gabo (GaboSr50) and generated protoplast from cv. Gabo and cv. GaboSr50 (Jensen and Saunders, 2023). We only expressed *AvrSr50* from a plasmid. We also included the controls, the reporter alone and the reporter with *Avr* or *R* alone (Zenodo Supplemental Data S1). We measured luminescence at 3, 4, 5, 6, and 18 h post-transfection (hpt). We observed a gradual increase in luminescence measurements in protoplast expressing luciferase alone or when expressed together with an *Avr* or *R* gene only (Figure 1, A and B). The luciferase activity was strongest at 18 hpt in all treatment groups except for the ones expressing *Avr/R* pairs. In cv. Fielder expressing *AvrSr35*/*Sr35* and in cv. GaboSr50 expressing *AvrSr50*, luminescence measurements only increased slightly early on during the time course when compared to mock treatments of protoplasts transfected with no DNA (Figure 1 C). At about 4 to 5 hpt we observed a divergence of luciferase activity from control treatment groups when compare with the Avr/R interaction treatment groups (Figure 1A and B). The lack of continuous increase of luciferase activity specifically in protoplast expressing *Avr*/*R* pairs suggests that their co-expressing leads to cell-death in wheat protoplasts soon after transfection.

Transfected protoplasts are suitable for replicable transcriptional analysis

We concluded that 4 hpt is a suitable time point to capture *Avr/R*-dependent transcriptional changes before strong HR induction based on our time course analysis (Figure 1). We repeated the experimental setup while omitting the reporter plasmid and collected protoplasts at 4 hpt for

RNA sequencing (Figure 2 A, Zenodo Supplemental Data S2). We included four biological replicates per treatment group and sequenced each sample on the Illumina platform generating >20 million 150 bp paired-end reads per replicate. We used kallisto (Bray et al., 2016) for transcript quantification and DESeq2 (Love et al., 2014) for downstream differential expression analysis. As a first quality control step we quantified the expression of all *Avr* and *R* genes to investigate their specific expression pattern at 4 hpt. This analysis clearly showed that all genes are correctly expressed and at similar levels, including in treatment groups that co-express *Avr/R* gene pairs (Figure 2 A). In addition, it revealed that the endogenous expression of *Sr50* in cv. GaboSr50 is much lower level than the overexpressed of *Sr35* in cv. Fielder from a plasmid under the *UBI* promoter. Next, we performed principal component analysis (PCA) of all sample replicates combined. This revealed that the factor "wheat cultivar" had the strongest impact on the observed variation in transcript abundance and accounted for close to 95% of all variation on PC1 and PC2 combined (Figure 2 B). Consequently, we analyzed each cultivar separately. The wheat cultivar specific PCA analysis showed a clear impact of Avr/R interaction on transcript abundance variation for both *AvrSr35/Sr35* and *AvrSr50/Sr50* (Figure 2 C and D). This impact was replicable because all four biological replicates clustered closely together and clearly separated treatment groups including Avr/R interactions sample types from their controls. This is consistent with the cell-death results seen in Figure 1 A and B. Overall, these results suggest that these data could be used to identify reporter genes specifically expressed downstream of Avr/R interactions in wheat protoplasts.

***Avr/R* recognition in wheat protoplasts leads to defense signaling**

Next, we investigated if the observed gene expression changes in *Avr/R* expressing protoplast overlapped with gene ontology (GO) terms commonly associated with defense signaling pathways in wheat and other plant species. We identified all genes that are specifically upregulated in response to Avr/R interactions in cv. Gabo/Gabo50 and cv. Fielder above a 2-fold change threshold. We identified 309 and 922 genes in *AvrSr35/Sr35* and *AvrSr50/Sr50* expressing protoplasts, respectively, when compared to all their control treatment groups. Complete gene lists are available on Zenodo as Supplemental Data S3. We performed a statistical enrichment analysis using these gene lists to test for overrepresentation of defense signaling associated GO terms. Our results revealed significant enrichment for GO terms related

to cellular components of cell periphery (GO:0071944) and plasma membrane (GO:0005886) in both cultivars (Figure 2 E). We identified defense response to other organisms (GO:0098542), response to other organisms (GO:0051707), and biological process involved in interspecies interaction between organisms (GO:0044419) when looking at cv. GaboSr50 alone (Zenodo Supplemental Data S4). We found molecular functions GO terms being enriched for calcium ion binding (GO:0005509), cation binding (GO:0043169), and calcium ion transmembrane transporter activity (GO:0015085) in both cultivars (Figure 2 E). Our GO terms analysis overall confirms that *Avr/R* expressing protoplasts undergo defense signaling before the onset of strong cell-death.

We were then interested in genes that are commonly upregulated by the Avr/R interaction in both cv. GaboSr50 and cv. Fielder. We identified 66 genes commonly upregulated in both cultivars, (Zenodo Supplemental Data S5) of which only 16 genes were associated with three different GO terms, all related to the molecular function of ion binding (Zenodo Supplemental Data S4). Our results showed that several of the identified genes have been previously implicated in plant defense signaling including TaNHL10 (TRAESCS3D02G368800) (Century et al., 1995; Aarts et al., 1998; Dagvadorj et al., 2022).

In summary, we observed the upregulation of genes involved in defense signaling in wheat protoplasts expressing *Avr/R* pairs. The promoters of commonly upregulated genes, by two *Avr/R* pairs and in two wheat cultivars (Figure 2F), are promising candidates to generate *Avr/R* inducible reporters.

Identification of positive defense reporters in wheat protoplasts

We further investigated the expression patterns of the 66 commonly upregulated genes to select a subset whose promoters we could test as positive defense signaling reporters. We selected 13 promoters (D1-D7, D10-D12, and D14-D16) based on their genes' expression patterns in both cultivars (Figure 2 F). To further evaluate these candidates, we cloned or synthesized their promoters, consisting of 600-800 bp upstream of their respective genes' start codons. We accounted for the impact of *Avr/R* induced HR upon luciferase activity (Figure 1) by adapting a dual-color green/red luciferase reporter system (González-Grandío et al., 2021). This ratiometric system uses green- and red-shifted luciferases (Sarrion-Perdigones et al., 2019), where green-shifted luciferase is fused to a conditionally responsive promoter and red-shifted luciferase

serves as a constitutively expressed reference for internal normalization, thus greatly reducing intra experimental variation.

To test the activity of our thirteen candidate defense promoters, we generated promoter fusion constructs driving the expression of green-emitting luciferase (E-Luc). These plasmids are named *pDefense* (*pD*[*1-7, 10-12, 14-16*]), respectively. We co-transfected these plasmids along with internal normalization plasmid *UBI-pRedf* and *UBI-AvrSr50* into protoplasts isolated from cv. GaboSr50 and Gabo. For the initial activity screening, we performed the test with two technical replicates. Ten of these promoters did not result in specific and reproducible promoter activation downstream of AvrSr50/Sr50 interactions when compared to their respective controls. In contrast, the three promoters D2, D14, and D15 showed a strong increase in normalized ratiometric luminescence specifically downstream of AvrSr50/Sr50 interactions (Supplemental Figure 1). Figure 3 shows an example of the specific activation of D14 downstream of AvrSr50/Sr50 in cv. GaboSr50 when compared to its control not expressing the *R* gene or the matching *Avr*. These results indicate that our new ratiometric reporter system is able to detect specific defense activation downstream the Avr/R interactions when the *R* gene is expressed endogenously.

The wheat dwarf virus-derived replicons-based system further increases the sensitivity of the positive defense reporter assay

The current wheat protoplast assay requires a large amount of DNA material for transfection to obtain observable cell-death. This makes its application prohibitive for large scale screening of many candidates or variants. We tested a replicon-based system using a disarmed version of the wheat dwarf virus (WDV) in our expression vector backbone (Gil-Humanes et al., 2017). We reasoned that this would generate self-replicating plasmids in wheat protoplasts and thereby reduce the amount of plasmids required to detect defense activation in our new ratiometric wheat protoplast assay. We assembled Golden Gate compatible WDV vector from Gil-Humanes et al. (2017) and denoted it as *pWDV1* (see Material and Methods). We expressed *AvrSr50* under ubiquitin promoter using *pWDV1* (*pWDV1-AvrSr50*) and used it to generate dose response curves using different amounts (0.1, 0.5, 1, 5 and 15 µg) of plasmid DNA for transfection. We measured induction of D14 induction (normalized luminescence) and cell viability (red

luminescence) and compared it to the dose response of the standard non-viral plasmid *pICH4772-AvrSr50* (Figure 4). We observed specific D14 promoter induction in all samples of cv. GaboSr50 protoplasts transfected with *pICH4772-AvrSr50* and *pWDV1-AvrSr50* when compared to controls in cv. GaboSr50 transfected with *pWDV1-AvrSr35* (1 and 15 µg) and cv. Gabo transfected with *pWDV1-AvrSr50* (1 µg) or *pWDV1-AvrSr35* (1 µg). We also observed that the promoter induction increases relative to the amount of *pICH4772-AvrSr50* used. At the same time, we were still able to observe induction of the defense reporter D14 even in the absence of cell-death at 0.1, 0.5 and 1 µg plasmid used. Using *pWDV1-AvrSr50* further improved the sensitivity of the assay as it increased defense reporter D14 activation at 0.1 and 0.5 µg plasmid and decreased cell viability when compared to the same amounts of plasmid for *pICH4772-AvrSr50*. Altogether, this result indicates that the ratiometric dual-color luciferase system combined with *pDefense* reporter and *pWDV1* is highly sensitive and specific requiring much less plasmid to detect Avr/R interactions than a single luciferase based wheat protoplast assays.

Positive defense reporters are highly sensitive to detect Avr/R interactions in wheat protoplast expressing *R* genes endogenously

Next, we explored if the defense reporters require overexpression of the *R* gene and if they are applicable to additional Avr/R interactions. We tested the induction of D2, D14 and D15 promoters expressing *AvrSr27, AvrSr35,* and *AvrSr50* under ubiquitin promoter in *pWDV1* using 1 µg plasmid in instead of the previously used 15 µg. We transfected cv. Coorong, C6969, and GaboSr50, with *pWDV1* encoding the recognized *Avr*, an unrecognized *avr*, or the empty vector control (Figure 5). Wheat cv. Coorong carries *Sr27* (Upadhyaya et al., 2021), and cv. C6969 (*Triticum aestivum*) carries *Sr35* which was introgressed from *Triticum monococcum* (McIntosh et al., 1984). Out of three promoters, we observed a strong induction of D14 specifically downstream of AvrSr27/Sr27, AvrSr35/Sr35, and AvrSr50/Sr50 interactions but not in any of the controls (Figure 5), validating the D14 defense reporter as well as *pWDV1* applicability in wheat protoplast assay. We decided to focus on D14 and switch all *Avr* expressions in *pWDV1* for the remainder of the study but to keep the reporter genes in the non-viral vector. These results support that the D14 promoter in combination with *pWDV1* is broadly applicable as a defense reporter in wheat independent of the wheat cultivar and the specific *Avr/R* combination used. In

addition, this suggests that our protoplast assay is applicable to testing wheat cultivars that carry endogenous *R* genes and does not require overexpression of a cloned *R* gene.

Positive defense reporters are broadly applicable for Avr/R interaction screening in many wheat cultivars

We aimed to illustrate the broad applicability of our new assay in a wide range of wheat cultivars as current cell-death based assays were mostly conducted in cv. Fielder (Saur et al., 2019, 5; Ortiz et al., 2022; Upadhyaya et al., 2021; Luo et al., 2021; Arndell et al., 2023). We tested the defense signaling reporter D14 for its inducibility by three *Avr/R* combinations in protoplasts of nine different wheat cultivars. We tested cultivars that carry *R* genes (cv. GaboSr50, cv. Coorong and cv. C6969) with their corresponding *Avr* construct *(AvrSr50, AvrSr27* and *AvrSr35*, respectively). The cultivars Clement, Compair, Avocet Yr8, (Dracatos et al., 2016) and Kenya W1483 (McIntosh et al., 1995) were tested with overexpression of *AvrSr50/Sr50* combination. The cultivars HeinesPeko (Dracatos et al., 2016), and Fielder were tested with *AvrSr27/Sr27* and *AvrSr35/Sr35* combinations, respectively. We include a negative control in all cultivars by expressing the unrecognized *avr, Avr* or *R* gene alone (Figure 4). In all cultivars we tested, a consistent robust induction of normalized luminescence of *pD14* was observed in protoplasts expressing cognate *Avr/R*-pair compared to the negative controls. The relative normalized luminescence and its induction in the *Avr/R* treatment group varied between the different cultivars. These results indicate that the new wheat protoplast assay enables the detection of Avr/R interactions in many wheat cultivars.

The positive defense reporter assay enables multiplexed screening of *Avr* candidates in bulk

To explore multiplexed screening of *Avr* candidates using the positive defense reporter assay, we transfected a pool of exemplifying ten or fifty *Avr* candidates into protoplasts isolated from cv. GaboSr50 and cv. Gabo. The pools include 1 µg of *pWDV1-AvrSr50*, and 4.5 µg of each *pWDV1-AvrSr35* and *pWDV1-AvrSr27* in the pool of ten, or 24.5 µg of each *pWDV1-AvrSr35* and *pWDV1-AvrSr27* in the pool of fifty so that one µg of *Avr* plasmid represents one Avr candidate. We used the same *Avr* pools excluding the *AvrSr50* as negative controls, and 1 µg of *pWDV1-AvrSr50* alone as a positive control. Each replicate included *pD14* reporter and *UBI-*

pRedf co-transfection to detect *Avr/R*-triggered defense promoter induction and normalized luminescence measurements. In cv. GaboSr50 protoplasts, *AvrSr50* alone and the pooled mixes containing *AvrSr50* induced a high level of normalized luminescence of *pD14* when compared to the pooled mix without *AvrSr50* and all other treatment groups including controls in cv. Gabo protoplasts (Figure 7). We observed a clear specific induction of the D14 promoter only in the presence of *AvrSr50* in cv. GaboSr50 when provided as pairs or as multiplexed mixtures with no statistical difference between the single or multiplexed treatment groups (Figure 7). Overall, these results show that our positive defense reporter protoplast assay can be used to screen a pool of *Avr* candidates against a wheat cultivar endogenously expressing the recognizing *R* gene.

Discussion

Here, we describe an improved wheat protoplast assay to screen avirulence effector candidates and study cellular defense signaling in wheat. We identify Avr/R-induced promoters and use them to generate reporter constructs that produce a positive readout during Avr/R interaction. We also reduce variability of luminescence measurement by adapting a ratiometric dual-color luciferase reporter system (Sarrion-Perdigones et al., 2019) and reduce the amount of required plasmids significantly enabling assays from simple minipreps. We show the broad applicability of the positive defense reporters by testing three *Avr/R* pairs from the wheat stem rust pathosystem on nine different wheat cultivars. The improved wheat protoplast assay is highly sensitive because it allows for multiplexed screening of *Avr* candidates in wheat cultivars expressing *R* genes endogenously.

This approach has several advantages over previous methods applied to identify Avr/R interactions. Firstly, it does not require a cloned and overexpressed *R* gene to test the interaction. We demonstrate that the positive defense reporter assay is suitable to test interactions directly in a broad range of wheat cultivars encoding specific *R* genes in their genome. This will open opportunities to screen *Avr* candidates directly on wheat-resistant cultivars, which was previously considered not amenable using the cell-death based wheat protoplast assays (Saur et al., 2019). Secondly, combining (generic) *Avr/R*-inducible promoters with the dual-reporter system allows for generating normalized positive signals, which enables a much more sensitive screening approach by greatly reducing variability between replicates. Furthermore, the high sensitivity of the assay allows to use 10-20 times lower amount of plasmid for transfections than

the former wheat protoplast assays, making the assay cost-effective, and easy to handle and prepare a large list of *Avr* candidates for screening. Thirdly, this approach unlocks screening multiple *Avr* candidates in a single transfection, which saves time and resources. Lastly, our new wheat protoplast reporter assay enables us to study a wide range of plant defense signaling directly in wheat. This is highlighted by our RNAseq analysis of defense signaling in wheat protoplasts. Our results show that genes responsible for calcium ion binding, cation binding and calcium ion transmembrane transporter activity along with other defense-related genes are specifically upregulated in response to both AvrSr35/Sr35 and AvrSr50/Sr50 interactions in two wheat cultivars. In line with our findings, there have been strong indications that plant defense signaling triggered by Avr/R interactions involves plasma membrane reorganization including formation of ion channels that regulate plant cell-death and disease resistance. Recent studies of *Arabidopsis* ZAR1 (Bi et al., 2021) and wheat Sr35 (Förderer et al., 2022) showed the activated ZAR1 and Sr35 proteins forming pentameric structures, so-called resistosomes, that appear to function as non-selective calcium channels responsible for Ca^{2+} uptake, leading to cell-death. Similarly, in *Arabidopsis*, an activated NRG1.1 protein, known as a downstream signaling component of plant *R* proteins, compose a Ca^{2+} permeable non-selective cation channel enabled by oligomerization, which controls cell Ca^{2+} influx and induces plant cell-death (Jacob et al., 2021). Therefore, our findings support that the channel activity, especially Ca^{2+} regulating channels, are conserved in broader Avr/R-induced downstream signaling.

Interestingly, one of our marker genes (the gene of promoter D14) encodes a predicted wheat NDR1/HIN1-like 10 (NHL10) protein. The NHL proteins that are grouped in a large gene family in *Arabidopsis thaliana* involved in plant defense, such as disease resistance (Aarts et al., 1998; Century et al., 1995) and pathogen sensing (Boudsocq et al., 2010; Zheng et al., 2004; Zipfel et al., 2004). The NHL proteins have one or two transmembrane domain(s) (Zheng et al., 2004) and several of them were shown to localize to the plasma membrane (Selote et al., 2014; Coppinger et al., 2004; Dagvadorj et al., 2022), which is thought to be crucial for their functioning in disease resistance pathways. Moreover, *Arabidopsis NHL10* expression, which is highly induced by flg22, is reported to be involved in Ca^{2+} signaling through the activation of calcium-dependent protein kinases (Boudsocq et al., 2010). In a recent study, wheat blotch disease effector ToxA was shown to interact with a wheat NHL protein, TaNHL10, to promote cell-death mediated by intracellular resistance like protein Tsn1 (Dagvadorj et al., 2022). Future

studies will benefit from our defense specific promoters, in particular D14, to investigate downstream defense signaling components in wheat.

Future studies will determine the capability of our positive defense reporter based Avr screening assay. Although we have performed exemplified test for ten and fifty *Avr* candidates screening in this study, we anticipate that the high sensitivity of the assay combined with self-replicative *pWDV1* plasmid can allow much higher number of *Avr* candidate screening in one wheat protoplast transfection. Moreover, we anticipate that the *pWDV1* plasmid will simplify overexpression of transient gene expression in wheat requiring only miniprep plasmid samples (Supplemental Figure 2), which facilitate functional gene and variant analysis directly in wheat protoplasts.

Our assay can be combined with a recent report that describes highly multiplexed avirulence effector recognition screening using cell-death as a negative enrichment method (Arndell et al., 2023). Multiplexed effector libraries (n>600) of *Pgt* were co-transfected into cv. Fielder protoplasts overexpressing specific *Sr* genes from plasmids. The author used the overexpression of YFP (Yellow Fluorescent Protein) as a cell viability marker to separate live protoplast from dead protoplast via flow cytometry. This allowed for negative enrichment analysis of expressed effectors when comparing live protoplast before and after flow cytometry via RNAseq analysis. The author could identify new matching *Avr* genes for two out of five *Sr* genes including *AvrSr13/Sr13* and *AvrSr22/Sr22* but not for *Sr21*, *Sr26*, and *Sr61*. Such large scale avirulence effector screening approaches will be much more efficient when combined with our positive reporter assays that will allow researchers to first identify avirulence effector pools that are recognized by a specific wheat cultivar. In addition, further improvements of the reported negative enrichment screen will likely enable screening in wheat cultivars expressing *R* genes endogenously similar to our assay. Hence the combination of both multiplexed avirulence effector screening approaches promises to usher in a new area of avirulence gene identification of economically important pathogens of wheat and other crop species.

Methods

Protoplast Isolation

Protoplast isolation and plant growth protocol are described here: https://dx.doi.org/10.17504/protocols.io.q26g7r3zkvwz/v1. Wheat plants of cv. Fielder, Gabo and GaboSr50 were grown for 7-9 days in a growth cabinet with 150 µmol/m^2/s light intensity and at 21 ^oC with 16 hr day length. Protoplasts were isolated by making a shallow cut across the leaf epidermis and peeling back the epidermal layer to expose the mesophyll layer beneath. The cut segments were placed, peeled side down in 0.6 M Mannitol for 10 mins, then placed into a 7 cm diameter petri dish containing filtered enzyme solution (MES-pH5.7 (20 mM), Mannitol (0.6 M), KCI (10 mM), Cellulase (Yakult R-10) 1.5% w/v, Macerozyme (Yakult R-10) 0.75% w/v, $CaCl_2$(10 mM). BSA 0.1% w/v). The dish was wrapped in aluminum foil to shield it from light and placed on an orbital shaker, horizontally rotating at 60 RPM, for three hours. Protoplasts were collected by gentle centrifugation at 100 x g for 3 mins in a 30 mL round-bottomed tube, then the enzyme solution was removed and the pellet was washed in W5 solution (MES-pH5.7 (2 mM), KCI (5 mM), $CaCl_2$ (125 mM), NaCl (154 mM). 5 µL of concentrated protoplasts were removed and used to quantify cells and check quality using a hemocytometer and light microscope. Protoplasts were washed again with W5, this time left on ice for 45 minutes to settle. W5 solution was removed, and protoplasts were resuspended to 300,000-350,000 protoplasts/mL using MMG (MES-pH5.7 (4 mM), Mannitol (0.4 mM), $MgCl_2$ (15 mM)). Protoplasts were used immediately for transfection.

Protoplast transfection

High concentration (1 µg/µL) plasmid DNA was extracted via maxi-prep (Promega SV Wizard). Lower quality plasmid DNA (e.g. from miniprep kits) typically resulted in low transfection rates. The *Avr*, *R* and *luciferase* genes were expressed under control of maize ubiquitin promoter (*UBI*), and 10 µg of each *Avr* and *R* plasmid and 20 µg of *luciferase* reporter plasmid were used for each replicate. Due to high concentration and purity, plasmids were heated at 65^oC for 10 min before use to reduce viscosity and ensure consistent measurement and delivery into cells. The required plasmid DNA was added to 14 mL round-bottom culture tubes (Thermofisher, #150268). 300 uL of protoplasts and 300 µL of PEG solution (PEG 4000 (40% w/v), Mannitol (0.2 M), $CaCl_2$ (100 mM)) was then added and mixed immediately by gently flicking/rotating the tube three times. Samples were incubated for 15 minutes, and the reaction halted with 900 µL W5. Protoplasts were then collected by 100 x g centrifugation for 2 min, resuspended in 500 µL

modified W5 (W5 + 1 mM glucose) and transferred to a transparent, sterile 12 well culture plate (Costar, #3513) whose wells were coated with 10% BSA to prevent sticking. Incubation was carried out in light conditions, approximately 100 µmol/m^2/s. Wide bore or cut tips were used throughout for handling protoplasts.

Protoplast Quantification

After the required incubation time (3, 4, 5, 6, and 18-hours for time course experiment, 4-18 hrs for *pDefense-ELuc* reporters) protoplasts were prepared for luminescence measurement. 200 µL of 1x cell lysis buffer (Promega #E3971) was added and samples vortexed and incubated for 15 min. Samples were centrifuged again to collect cell debris and 50 µL of lysate was added to an opaque, flat-bottom, white, solid polystyrene 96 well plate (e.g. Corning® 96 well NBS™ Microplate #CLS3600). Plate was read for total luminescence (Tecan infinite 200Pro, 1000 ms integration 0 ms settle) without addition of substrate, then 50 µL Steady-Glo (Promega #E2520) Luciferin substrate was added, and the plate was read again.

Protoplast RNAseq sampling

Protoplasts were prepared as described above, using 315,00 protoplasts in 900 µL MMG per replicate, with 4 replicates for each treatment group The *UBI-fLUC* reporter was excluded. Additional replicates of each condition included the reporter to confirm cell-death after 16hrs (Data not shown). At 4 hrs post transfection the protoplasts were pelleted, snap-frozen, and stored at -80 until RNA isolation. Samples from each cultivar were prepared and processed in batches. Qiagen RNeasy Plant Mini Kit was used as directed, (#74904) omitting the first mechanical cell lysis step as freezing and addition of RLT buffer was sufficient to lyse cells. Qiagen RNase-Free DNase Set (#79254) was used as directed. RNA was checked for absence of DNA contamination by PCR and gel electrophoresis and quantitated using Qubit and Nanodrop. Total RNA Illumina sequencing was performed on the NovaSeq platform, in a 2x150bp paired-end configuration, with poly A selection & strand specific library preparation for the 36 samples. The resulting sequence data is deposited in the NCBI Sequence Read Archive (SRA) under BioProject number PRJNA957082.

RNAseq Analysis

Transcript abundance was quantified with kallisto (Bray et al., 2016) using the Ensembl *Triticum aestivum* transcriptome (IWGSC, INSDC Assembly GCA_900519105.1, Jul 2018) plus cDNA sequences for *AvrSr50, Sr50, AvrSr35* and *Sr35* as reference. DESeq2 (Love et al., 2014) was used to test for differential expression. We defined upregulation at a 4-fold change or greater. GaboSr50 with AvrSr50 was compared against the four control treatment groups; Gabo_AvrSr50, Gabo_EV, GaboSr50_AvrSr35 and GaboSr50_EV. Then, the intersect of the upregulated genes was taken, for a total of 272 genes. For Fielder, Fielder_Sr35_AvrSr35 was compared to the 3 control treatment groups; Fielder_AvrSr35, FielderSr35_AvrSr50, Fielder_Sr35. Again, taking the intersect of the upregulated genes returned 98 upregulated genes. 66 genes were commonly upregulated between both lists (Zenodo Supplemental Data S5). For GO terms analysis, we repeated the comparisons above with lower upregulation of 2-fold change and used the gProfiler webtool and Ensembl *Triticum aestivum* annotation, running the g:GOst program (Raudvere et al., 2019). The recommended g:SCS multiple testing correction algorithm was used.

pDefense Promoter Selection

Expression in log fold change and transcripts per million (TPM) was visualized for the 66 commonly upregulated genes, present in both 2-fold upregulated lists used for GO analysis. Thirteen genes were selected based on their overall expression and upregulation in *Avr/R* treatment groups. The entire gene and region were compared in Geneious and 600-800 bp upstream of the 13 genes was selected, including any annotated 5'UTR. These promoter sequences then were synthesized with 5'and 3' 4bp MoClo promoter overhangs flanked by BsaI recognition sites for Golden Gate cloning as promoter+5'UTR standard parts (Weber et al., 2011). Any BpiI and BsaI enzyme recognition sites within the promoter sequence were removed prior to synthesis. Promoters that could not be synthesized were cloned by PCR, using primers (Zenodo Supplemental Data S6) with BpiI recognition sites and 4bp overhang to facilitate assembly into universal level 0 vector *pAGM9121* (Addgene Plasmid #51833). Any BsaI recognition sites were removed as per Grutzner & Marillionnet, (2020).

pDefense Plasmid Constructs

Plasmids were designed in Geneious. The plasmid constructs were designed according to the MoClo syntax, assembled by Golden Gate cloning and verified by whole plasmid sequencing.

Supplemental Data (Zenodo Supplemental Data S6) and Plasmid maps Zenodo (DOI: 10.5281/zenodo.7844465, link: https://zenodo.org/record/7844465) are available, with all generated plasmids deposited on Addgene.

pWDV1 vector

The wheat dwarf virus genomic sequence from the isolate Enkoping1 (GenBank: AJ311031.1) was modified to remove the V1 and V2 genes which encode the viral coat and transfer proteins, respectively. In addition, one and two BsaI type II restriction sites were mutated to enable downstream golden gate cloning steps. All mutations were in the Rep and RepA open reading frames and were silent, with the exception that the mutation generated at amino acid position 263 for RepA results in a Serine to Threonine substitution at amino acid position 235 of the Rep protein which is encoded by an alternately spliced transcript from the same Rep/RepA open reading frame. Two BsaI sites were inserted between the Long and short intergenic regions in place of the removed V1 and V2 genes. The BsaI sites were designed to generate a GGAG overhang downstream of the long intergenic region and a CGCT overhang upstream of the short intergenic region to facilitate level 1 golden gate assemblies as described previously (Engler et al., 2014). BsmBI sites were also included at either end of the modified viral genome sequence to facilitate golden gate cloning into the pYTK001 vector (Lee et al., 2015) which was chosen for its small size, ease of cloning and to facilitate selection and plasmid replication in bacteria. The modified wheat dwarf virus sequence, called WDV_1, was synthesised as a ‘gene’ fragment by twist bioscience and directly incorporated into the pYTK001 vector by golden gate cloning using BsmBI. We sequence verified plasmids via whole plasmid sequencing. We named the resulting plasmid *pWDV1*. We used *pWDV1* for subsequent cloning of genes by golden gate cloning using the BsaI entry site.

Ratiometric Assay

All isolation and transfection steps were as above aside from the substitution of the fLUC reporter for the reporter (*UBI-pDefense)* and normalization (*UBI-pRedf*) plasmids. These require the same substrate for activation, but express luciferase with different emission spectra, read by specific green and red filters in the luminescence plate reader.A *UBI-ELuc* construct was also generated to calibrate normalization and deconvolution calculations, necessary to resolve the overlap in emission spectra of the two luciferase reporters (González-Grandío et al., 2021).

These calibration experiments were performed separately three times and with three replicates per measurement, according to the instructions for Promega Chroma-Glo assay (Chroma-Glo(TM) Luciferase Assay System Technical Manual, TM062). The plate reader was programmed to measure total luciferase, then with green and red filters, with 1000 ms integration time and 0 ms settle time, measured well-wise. Template spreadsheets for performing calculations and a Python script for automating deconvolution and plotting are recorded on protocols.io (https://dx.doi.org/10.17504/protocols.io.q26g7r3zkvwz/v1).

Cultivar testing

Protoplasts were isolated from nine different wheat cultivars (GaboSr50, Coorong, C6969, Clement, Compair, HeinesPeko, KenyaW1483, AvocetYr8 and Fielder), using the method described above. All were grown in the same conditions as above for 8 days. Where the cultivar carried an *R* gene (GaboSr50, Coorong, C6969) the recognized *Avr* (*AvrSr50*, *AvrSr27* and *AvrSr35* respectively) was transfected alone, with an avr (unrecognized by R protein) as control. In the other six cultivars both the *Avr* and *R* gene were co-transfected. Normalized luminescence was calculated from the ratio of deconvoluted *pDefense-Eluc* to *UBI-pRedf.*

Multiplex screening

Protoplasts were prepared from cv. GaboSr50 and cv. Gabo wheat leaves and transfected as above. Measurements used the ratiometric reporters and measurements, with pDefense14-ELuc as reporter and UBI-pRedf for normalisation. Avr pools excluding the AvrSr50 acted as negative controls, and 1 µg of pWDV1-AvrSr50 alone acted as a positive control. Data processing and statistics was carried out as above.

Data availability

All supplemental data is available on Zenodo (DOI: 10.5281/zenodo.7844446., link: https://doi.org/10.5281/zenodo.7844446 All plasmids generated will be made available from Addgene, with plasmid maps deposited on Zenodo (DOI: 10.5281/zenodo.7844464, link: https://doi.org/10.5281/zenodo.7844464). Code used to perform RNAseq analysis is deposited on Github (https://github.com/ritatam/wheat-protoplast-RNAseq-DESeq2) and all RNAseq data is available on NCBI Sequence Read Archive (SRA) under BioProject number PRJNA957082.

Acknowledgements

We thank Prof. Robert Park for providing seeds of cv. C6969, A/Prof. Sambasivam Periyannan for providing seeds of cv. Fielder, cv. Gabo, cv. GaboSr50, and cv. Coorong, Dr. Michael Ayliffe and Dr. Peter Dodds for providing *pUBI-AvrSr35*, *pUBI-Sr35*, *pUBI-AvrSr50*, and *pUBI-Sr50*. B.S. acknowledges the financial support by the Australian Research Council via a Future Fellowship (FT180100024). This work was supported by computational resources provided by the Australian Government through the National Computational Infrastructure (NCI) under the ANU Merit Allocation Scheme.

Author Contributions

S. W., B. D., J. P. R., and B. S. conceived the project. S. W., B. D., R. T., L. M., N. H., E. C., and J. G. performed the experiments, S. W., B. D., and B. S. wrote the manuscript. R. T., E. C., and J. P. R. provided extensive feedback on the manuscript. B. S. secured funding. All authors read and approved the manuscript.

References

Aarts, N., Metz, M., Holub, E., Staskawicz, B.J., Daniels, M.J., and Parker, J.E. (1998). Different requirements for EDS1 and NDR1 by disease resistance genes define at least two R gene-mediated signaling pathways in Arabidopsis. Proceedings of the National Academy of Sciences **95**: 10306–10311.

Arndell, T. et al. (2023). Pooled effector library screening in protoplasts rapidly identifies novel Avr genes.: 2023.04.28.538616.

Bi, G. et al. (2021). The ZAR1 resistosome is a calcium-permeable channel triggering plant immune signaling. Cell **184**: 3528-3541.e12.

Boudsocq, M., Willmann, M.R., McCormack, M., Lee, H., Shan, L., He, P., Bush, J., Cheng, S.-H., and Sheen, J. (2010). Differential innate immune signalling via Ca2+ sensor protein kinases. Nature **464**: 418–422.

Bourras, S. et al. (2019). The AvrPm3-Pm3 effector-NLR interactions control both race-specific resistance and host-specificity of cereal mildews on wheat. Nat Commun **10**: 2292.

Bouton, C., King, R.C., Chen, H., Azhakanandam, K., Bieri, S., Hammond-Kosack, K.E., and Kanyuka, K. (2018). Foxtail mosaic virus: A Viral Vector for Protein Expression in Cereals. Plant Physiology **177**: 1352–1367.

Bray, N.L., Pimentel, H., Melsted, P., and Pachter, L. (2016). Near-optimal probabilistic RNA-seq quantification. Nat Biotechnol **34**: 525–527.

Century, K.S., Holub, E.B., and Staskawicz, B.J. (1995). NDR1, a locus of Arabidopsis thaliana that is required for disease resistance to both a bacterial and a fungal pathogen. Proceedings of the National Academy of Sciences **92**: 6597–6601.

Chen, J. et al. (2017). Loss of *AvrSr50* by somatic exchange in stem rust leads to virulence for *Sr50* resistance in wheat. Science **358**: 1607–1610.

Christensen, A.H., Sharrock, R.A., and Quail, P.H. (1992). Maize polyubiquitin genes: structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. Plant Mol Biol **18**: 675–689.

Coppinger, P., Repetti, P.P., Day, B., Dahlbeck, D., Mehlert, A., and Staskawicz, B.J. (2004). Overexpression of the plasma membrane-localized NDR1 protein results in enhanced bacterial disease resistance in Arabidopsis thaliana. The Plant Journal **40**: 225–237.

Dagvadorj, B., Outram, M.A., Williams, S.J., and Solomon, P.S. (2022). The necrotrophic effector ToxA from Parastagonospora nodorum interacts with wheat NHL proteins to facilitate Tsn1-mediated necrosis. The Plant Journal **110**: 407–418.

Dodds, P.N. and Rathjen, J.P. (2010). Plant immunity: towards an integrated view of plant–pathogen interactions. Nat Rev Genet **11**: 539–548.

Dracatos, P.M., Zhang, P., Park, R.F., McIntosh, R.A., and Wellings, C.R. (2016). Complementary resistance genes in wheat selection “Avocet R” confer resistance to stripe rust. Theor Appl Genet **129**: 65–76.

Engler, C., Youles, M., Gruetzner, R., Ehnert, T.-M., Werner, S., Jones, J.D.G., Patron, N.J., and Marillonnet, S. (2014). A Golden Gate Modular Cloning Toolbox for Plants. ACS Synth. Biol. **3**: 839–843.

Förderer, A. et al. (2022). A wheat resistosome defines common principles of immune receptor channels. Nature **610**: 532–539.

Gil-Humanes, J., Wang, Y., Liang, Z., Shan, Q., Ozuna, C.V., Sánchez-León, S., Baltes, N.J., Starker, C., Barro, F., Gao, C., and Voytas, D.F. (2017). High-efficiency gene targeting in hexaploid wheat using DNA replicons and CRISPR/Cas9. The Plant Journal **89**: 1251–1262.

González-Grandío, E., Demirer, G.S., Ma, W., Brady, S., and Landry, M.P. (2021). A Ratiometric Dual Color Luciferase Reporter for Fast Characterization of Transcriptional Regulatory Elements in Plants. ACS Synth. Biol. **10**: 2763–2766.

Hewitt, T. et al. (2021). A highly differentiated region of wheat chromosome 7AL encodes a Pm1a immune receptor that recognizes its corresponding AvrPm1a effector from Blumeria graminis. New Phytol **229**: 2812–2826.

Jacob, P. et al. (2021). Plant "helper" immune receptors are Ca^{2+} -permeable nonselective cation channels. Science **373**: 420–425.

Jensen, C. and Saunders, D.G.O. (2023). Magnaporthe oryzae pathotype Triticum (MoT) can act as a heterologous expression system for fungal effectors with high transcript abundance in wheat. Sci Rep **13**: 108.

Laflamme, B., Dillon, M.M., Martel, A., Almeida, R.N.D., Desveaux, D., and Guttman, D.S. (2020). The pan-genome effector-triggered immunity landscape of a host-pathogen interaction. Science **367**: 763–768.

Lee, M.E., DeLoache, W.C., Cervantes, B., and Dueber, J.E. (2015). A Highly Characterized Yeast Toolkit for Modular, Multipart Assembly. ACS Synth Biol **4**: 975–986.

Lee, W.-S., Hammond-Kosack, K.E., and Kanyuka, K. (2012). Barley Stripe Mosaic Virus-Mediated Tools for Investigating Gene Function in Cereal Plants and Their Pathogens: Virus-Induced Gene Silencing, Host-Mediated Gene Silencing, and Virus-Mediated Overexpression of Heterologous Protein. Plant Physiology **160**: 582–590.

Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biology **15**: 550.

Luo, M. et al. (2021). A five-transgene cassette confers broad-spectrum resistance to a fungal rust pathogen in wheat. Nat Biotechnol **39**: 561–566.

Mago, R. et al. (2015). The wheat Sr50 gene reveals rich diversity at a cereal disease resistance locus. Nat Plants **1**: 15186.

McIntosh, R., Dyck, P., The, T., Cusick, J., and Milne, D. (1984). Cytogenetical studies in wheat XIII. Sr35 - a third gene from Triticum monococcum for resistance to Puccinia graminis tritici. Zeitschrift für Pflazenzüchtung **92**: 1–14.

McIntosh, R., Wellings, C., and Park, R. (1995). Wheat Rusts: An Atlas of Resistance Genes. In (CSIRO Publishing).

Meile, L., Croll, D., Brunner, P.C., Plissonneau, C., Hartmann, F.E., McDonald, B.A., and Sánchez-Vallet, A. (2018). A fungal avirulence factor encoded in a highly plastic genomic region triggers partial resistance to septoria tritici blotch. New Phytologist **219**: 1048–1061.

Müller, M.C. et al. (2022). Ancient variation of the AvrPm17 gene in powdery mildew limits the effectiveness of the introgressed rye Pm17 resistance gene in wheat. Proceedings of the National Academy of Sciences **119**: e2108808119.

Ortiz, D., Chen, J., Outram, M.A., Saur, I.M.L., Upadhyaya, N.M., Mago, R., Ericsson, D.J., Cesari, S., Chen, C., Williams, S.J., and Dodds, P.N. (2022). The stem rust effector protein AvrSr50 escapes Sr50 recognition by a substitution in a single surface-exposed residue. New Phytol **234**: 592–606.

Praz, C.R. et al. (2017). AvrPm2 encodes an RNase-like avirulence effector which is conserved in the two different specialized forms of wheat and rye powdery mildew fungus. New Phytol **213**: 1301–1314.

Raudvere, U., Kolberg, L., Kuzmin, I., Arak, T., Adler, P., Peterson, H., and Vilo, J. (2019). g:Profiler: a web server for functional enrichment analysis and conversions of gene lists (2019 update). Nucleic Acids Research **47**: W191–W198.

Saintenac, C., Zhang, W., Salcedo, A., Rouse, M.N., Trick, H.N., Akhunov, E., and Dubcovsky, J. (2013). Identification of wheat gene Sr35 that confers resistance to Ug99 stem rust race group. Science **341**: 783–786.

Salcedo, A. et al. (2017). Variation in the *AvrSr35* gene determines *Sr35* resistance against wheat stem rust race Ug99. Science **358**: 1604–1606.

Sarrion-Perdigones, A., Chang, L., Gonzalez, Y., Gallego-Flores, T., Young, D.W., and Venken, K.J.T. (2019). Examining multiple cellular pathways at once using multiplex hextuple luciferase assaying. Nat Commun **10**: 5710.

Saur, I.M.L., Bauer, S., Lu, X., and Schulze-Lefert, P. (2019). A cell death assay in barley and wheat protoplasts for identification and validation of matching pathogen AVR effector and plant NLR immune receptors. Plant Methods **15**: 118.

Savary, S., Willocquet, L., Pethybridge, S.J., Esker, P., McRoberts, N., and Nelson, A. (2019). The global burden of pathogens and pests on major food crops. Nat Ecol Evol **3**: 430–439.

Selote, D., Shine, M.B., Robin, G.P., and Kachroo, A. (2014). Soybean NDR1-like proteins bind pathogen effectors and regulate resistance signaling. New Phytologist **202**: 485–498.

Upadhyaya, N.M. et al. (2021). Genomics accelerated isolation of a new stem rust avirulence gene–wheat resistance gene pair. Nat. Plants **7**: 1220–1228.

Weber, E., Gruetzner, R., Werner, S., Engler, C., and Marillonnet, S. (2011). Assembly of Designer TAL Effectors by Golden Gate Cloning. PLOS ONE **6**: e19722.

Wulff, B.B. and Krattinger, S.G. (2022). The long road to engineering durable disease resistance in wheat. Current Opinion in Biotechnology **73**: 270–275.

Zheng, M.S., Takahashi, H., Miyazaki, A., Hamamoto, H., Shah, J., Yamaguchi, I., and Kusano, T. (2004). Up-regulation of Arabidopsis thaliana NHL10 in the hypersensitive response to Cucumber mosaic virus infection and in senescing leaves is controlled by signalling pathways that differ in salicylate involvement. Planta **218**: 740–750.

Zhong, Z. et al. (2017). A small secreted protein in Zymoseptoria tritici is responsible for avirulence on wheat cultivars carrying the Stb6 resistance gene. New Phytologist **214**: 619–631.

Zipfel, C., Robatzek, S., Navarro, L., Oakeley, E.J., Jones, J.D.G., Felix, G., and Boller, T. (2004). Bacterial disease resistance in Arabidopsis through flagellin perception. Nature **428**: 764–767.

Figure Legends

Figure 1: Rapid onset of cell-death downstream of *Avr/R* interactions in protoplasts. **A-C**, Luciferase activity was measured at defined timepoints in hours post-transfection (hpt) in wheat protoplasts. **(A)** Shows boxplots of cv. Fielder protoplasts expressing *luciferase* alone, and *luciferase* with either *Sr35*, *AvrSr35*, or *Sr35* plus *AvrSr35*. **(B)** shows boxplots of cv. GaboSr50 protoplasts expressing *luciferase* alone, and *luciferase* with either *AvrSr35* or *AvrSr50*. **(C)** shows the luminescence of the Avr/R interaction treatment groups from cv. Fielder and cv. GaboSr50 alone when compared to the no plasmid transfection control replicates (red line) which is the average of nine replicate across the six timepoints.

Figure 2: Co-expression of *Avr/R* gene pairs triggers defense signaling in wheat protoplasts. **(A)** Heat map shows expression of *Avr* and *R* genes by normalized gene count. Each column represents a replicate and the treatment group is indicated by a number as shown in the legend. *R* or *Avr* genes are expressed in their respective treatment groups only. This includes endogenously expressed *Sr50* in cv. GaboSr50. **(B-D)** show principal component analysis (PCA) of the RNAseq analysis; all 36 samples **(B)**, cv. Fielder **(C)** and cv. Gabo/GaboSr50 **(D)**. (**E)** shows the GO terms of genes that are upregulated 2 fold in *Avr/R* treatment groups. A full list of upregulated genes with overrepresented GO terms is recorded in supplemental data (Zenodo Supplemental Data S4). **(F)** shows a heatmap of the expression of thirteen candidate reporter genes (D1-7, D10-12, D14-16) that are commonly upregulated by *Sr50/AvrSr50* and *Sr35/AvrSr35* in cv. GaboSr50 or cv. Fielder, respectively. Each square represents the mean of four replicates for treatment group.

Figure 3: *pDefense14* is a positive defense reporter in wheat protoplasts. (**A**) Wheat protoplasts transfected with *pDefense14* and *UBI-pRedF* constructs show an increase in normalized luminescence in the *Avr/R* treatment, compared to treatments with *R* or *avr* only and without *R* or *Avr*. Panel **(B)** shows corresponding non-normalized red-filtered luminescence for the treatments in panel A. Treatments marked with common letters were not significantly different (P >0.05) assessed with one-way analysis of variance (ANOVA) and post hoc Tukey's HSD test.

Figure 4: pWDV1 plasmid reduces amount of *Avr* plasmid required to detect defense signaling using *pDefense* reporter. *AvrSr50* was cloned into *pWDV1* (*pWDV1-AvrSr50*) and non-viral plasmid backbone (*pICH4772-UBI-AvrSr50)* and transfected in increasing amounts (0.1, 0.5, 1, 5 and 15 µg of plasmid DNA) alongside *pDefense14* and *UBI-pRedf.* Reporters remained in the non-replicative plasmid. Controls in cv. GaboSr50 were transfected with *pWDV1-AvrSr35* (1 and 15 µg) and cv. Gabo transfected with *pWDV1-AvrSr50* (1 µg) or *pWDV1-AvrSr35* (1 µg). One-way analysis of variance (ANOVA) and post hoc Tukey's HSD test was used to assess differences within normalized luminescence (A) and red luminescence (B) groups. Treatments marked with a common letter were not significantly different (P >0.05).

Figure 5: Positive pDefense reporter detect defense signaling triggered by three distinct *Avr/R* pairs in wheat protoplasts expressing the *R* gene endogenously. From left to right, protoplasts from wheat cultivars Coorong carrying *Sr27*, C6969 carrying *Sr35* and GaboSr50 carrying *Sr50* were transfected with *UBI-pDefense14* and *AvrSr27*, *AvrSr35* and *AvrSr50* constructs, respectively, and each wheat cv. was transfected with *avr* (unrecognized *Avr*) construct and empty vector (EV) as indicated in the column headers and x-axis labels. *UBI-pRedf* was used for normalization of all experiments. One-way analysis of variance (ANOVA) and post hoc Tukey's HSD test was used to assess differences among normalized luminescence for each cultivar. Treatments marked with a common letter were not significantly different (P >0.05).

Figure 6: The positive defense reporter *pDefence14* is broadly applicable to all tested wheat cultivars. Each panel shows the normalized luminescence of the positive defense reporter *pDefense14*. Protoplasts isolated from various wheat cultivars were transfected with both *R* and *Avr* constructs, aside from cultivars GaboSr50, C6969 and Coorong which carry *Sr50, Sr35* and

Sr27 respectively. The cultivars Corack, Clement, Kenya W1483, Avocet Yr8 and Compair, tested with *AvrSr50/Sr50* and Heines Peko was tested with *AvrSr27/Sr27.*An *avr* (unrecognised by R protein) was used as a control and *UBI-pRedf* was used for normalization of all experiments. One-way analysis of variance (ANOVA) and post hoc Tukey's HSD test was used to assess differences among means of normalized luminescence measurements for each cultivar. Treatments marked with common letters were not significantly different (P >0.05).

Figure 7: The positive defense reporter enables multiplexed *Avr* screening. Each panel in **(A)** shows the normalized luminescence of the positive defense reporter *pDefense14*. Each panel in (**B**) shows the red luminescence of *UBI-pRedf.* Pools exemplifying ten or fifty *Avr* candidates were transfected into protoplasts isolated from cv. GaboSr50 (left) and cv. Gabo (right). The pools include 1 µg of *pWDV1-AvrSr50*, and 4.5 µg of each *pWDV1-AvrSr35* and *pWDV1-AvrSr27* in the pool of ten, or 24.5 µg of each *pWDV1-AvrSr35* and *pWDV1-AvrSr27* in the pool of fifty so that one µg of *Avr* plasmid represents one Avr candidate. The same *Avr* pools excluding the *AvrSr50* are used as negative controls, and 1 µg of *pWDV1-AvrSr50* alone for positive control. All replicates were co-transfected with *pD14* reporter *UBI-pRedf* for normalization. One-way analysis of variance (ANOVA) and post hoc Tukey's HSD test was used to assess differences among means of normalized luminescence and red luminescence measurements for each treatment group. Treatments marked with common letters were not significantly different (P >0.05).

Supplemental Figure 1: Preliminary successful screening of *pDefense2, -14* and *-15* defense reporters. Normalized luminescence for *pDefense14,* (panel **A**)*, pDefense 15* (panel **B**) and *pDefense2* (panel **C**), in three wheat cultivars. Left to right, cv. C6969 which carries *Sr35*, cv. GaboSr50 which carries *Sr50* and cv. Coorong, which carries *Sr27*. Avr+R treatments were transfected with plasmid which expressed a recognized Avr, avr+R treatments expressed a non-recognized avr and R alone expressed an empty vector. Reporters were normalized against *UBI-pRedf* for each replicate. The three reporters produced an increase in normalized luminescence in treatments with Avr/R interaction, across the three Avr/R pairs tested.

Supplemental Figure 2: *pDefense* reporter can detect lower purity *Avr* plasmid. In **(A)** both maxi prepped (left) and mini prepped (right) *AvrSr35* plasmid was co-transfected with *R* and *avr*

plasmids, in protoplasts from the cultivar Fielder. **(B)** shows corresponding non-normalized red-filtered luminescence. One-way analysis of variance (ANOVA) and post hoc Tukey's HSD test was used to assess differences among means of normalized and red-filtered luminescence measurements for each treatment. Treatments marked with common letters were not significantly different (P >0.05).**Supplemental Material Legends**

Supplemental Material S1: Luminescence data for Figure 1, protoplast time course experiment.

Supplemental Material S2: List of samples and treatment groups for RNAseq experiment.

Supplemental Material S3: Lists of Gabo/GaboSr50, Fielder and all cultivar genes upregulated by 2-fold.

Supplemental Material S4: GO terms analysis Query tables and g:profiler results tables

Supplemental Material S5: Lists of Gabo/GaboSr50, Fielder and all cultivar genes upregulated by 4-fold.

Supplemental Material S6: List of primers used.

Supplemental Material S7: List of plasmids used.

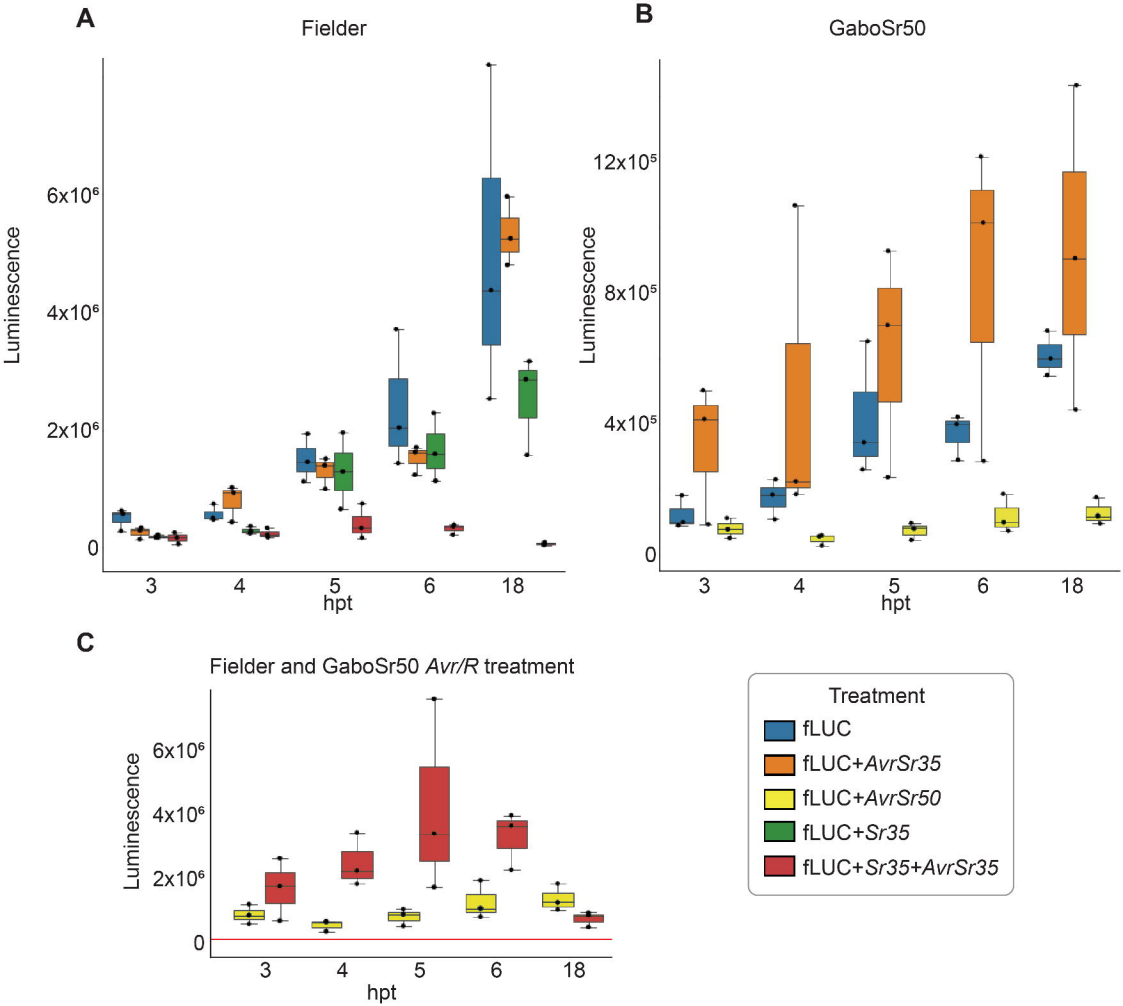
Supplemental Material S8: Luminescence data used for generating Figures 3 -7.

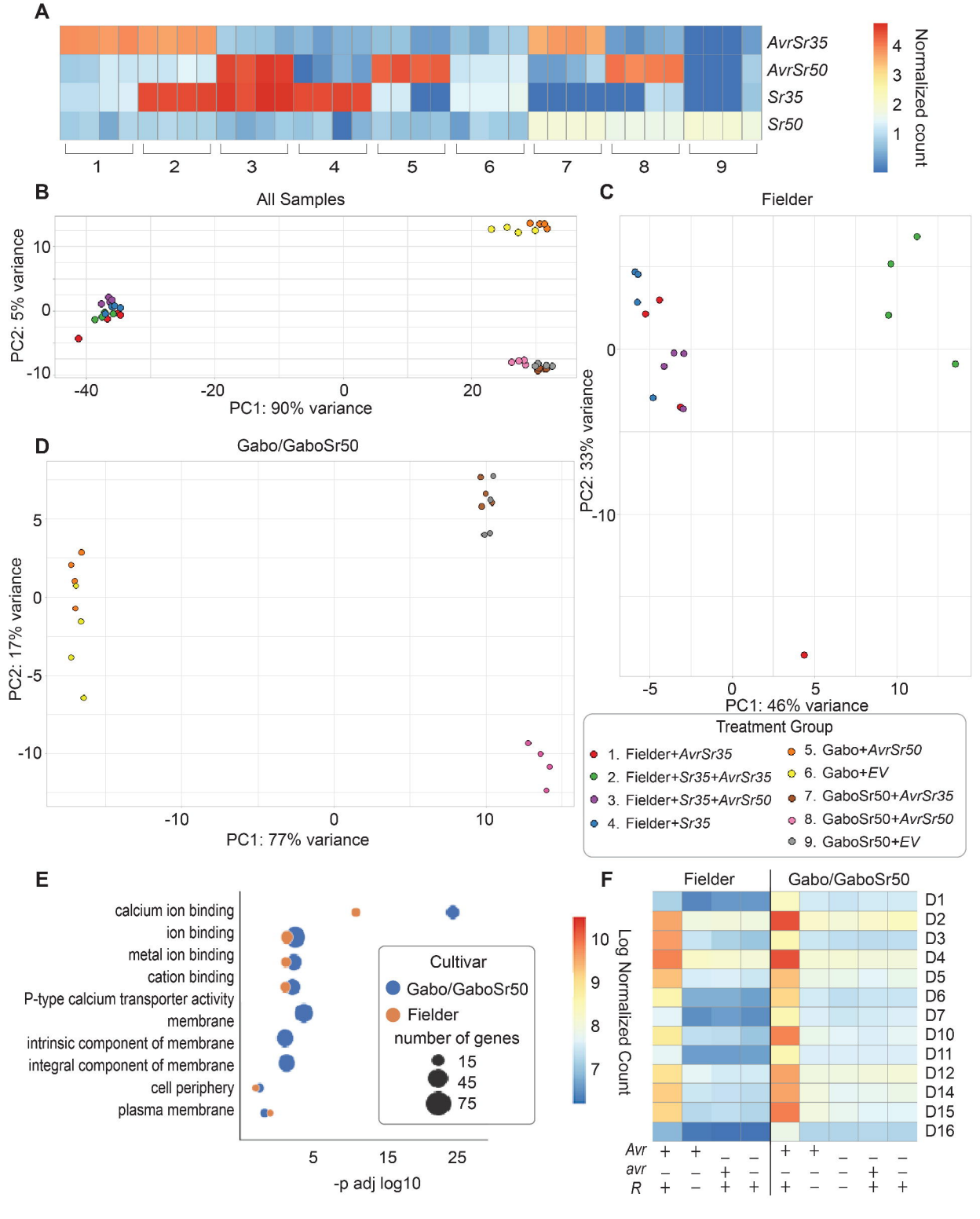
Supplemental Material S9: Statistical output for Figures 3-7.

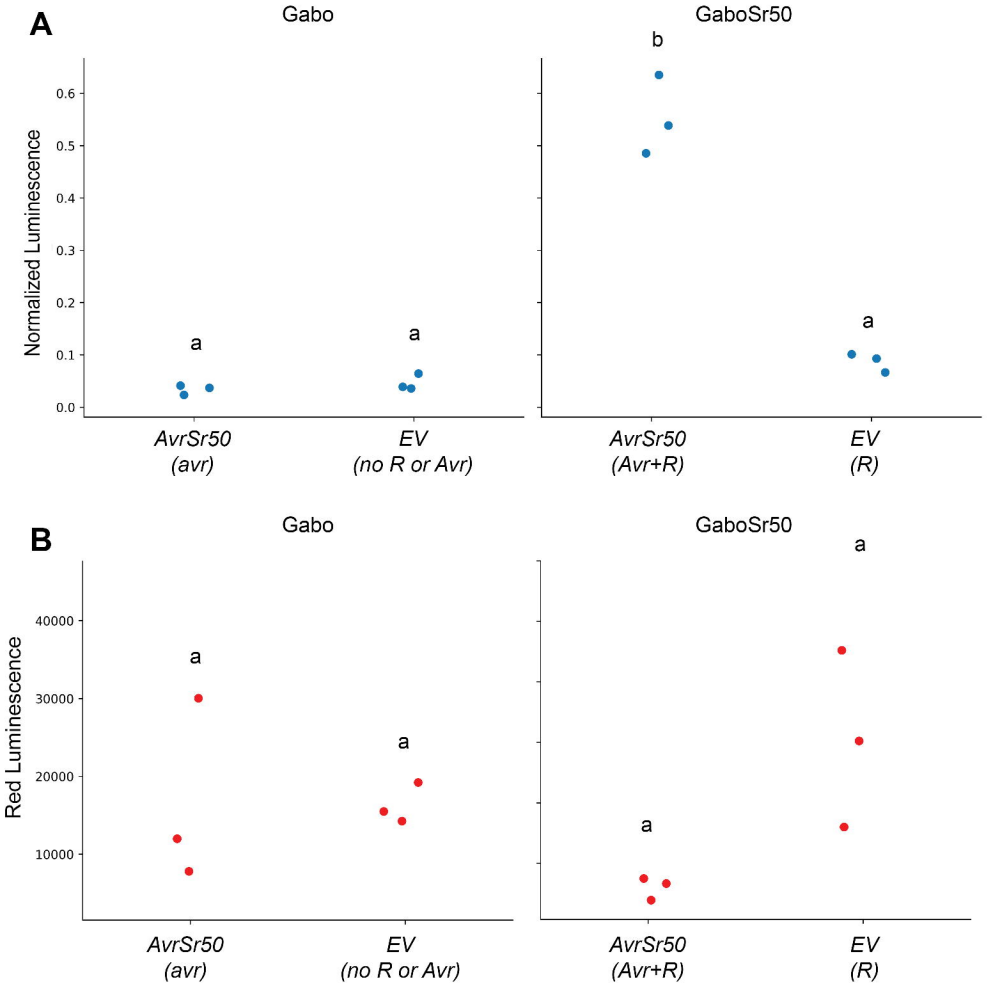
Supplemental Material S10: Data and statistical output for supplemental figures 1 and 2.

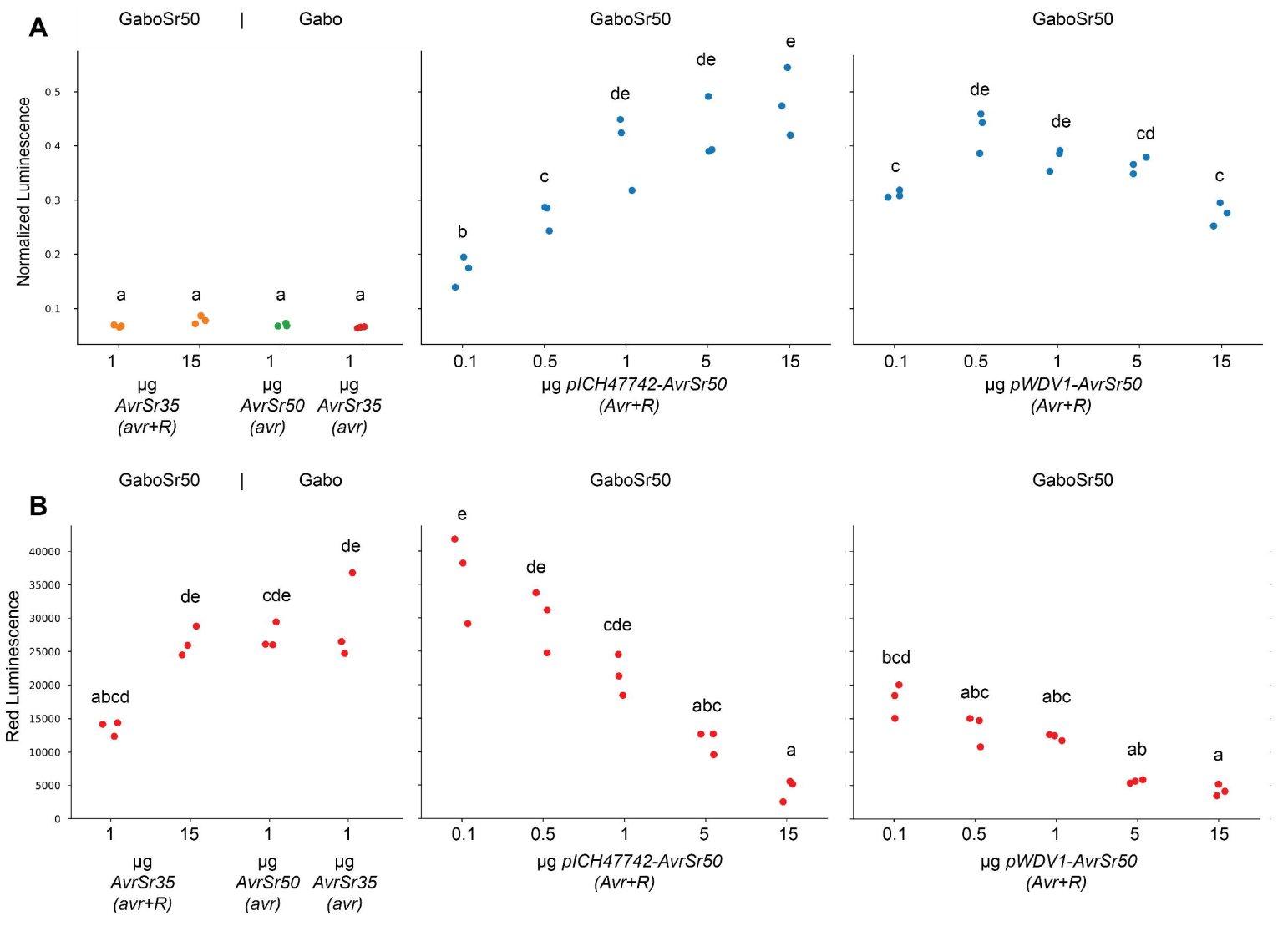
754

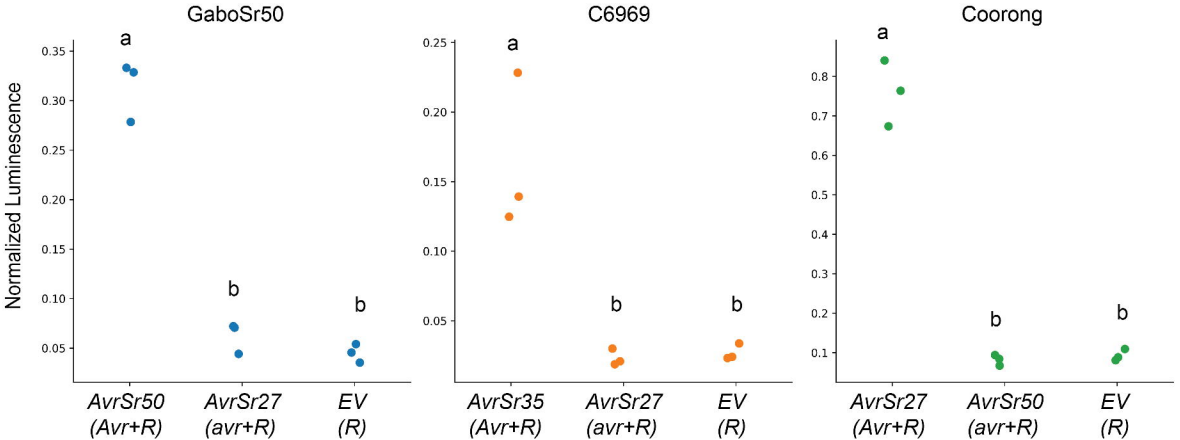
755

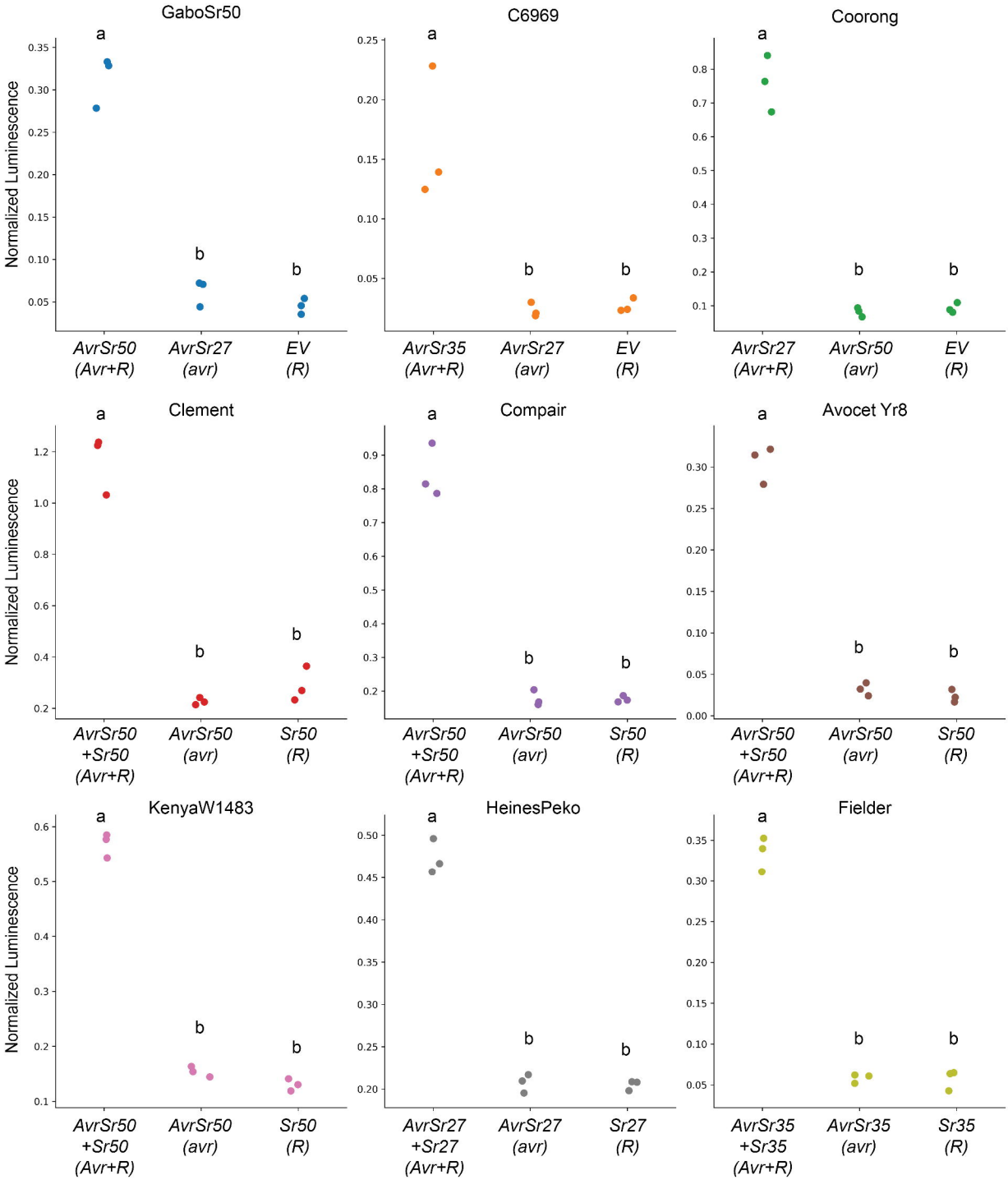

A
Fielder
Luminescence
6x10⁶
4x10⁶
2x10⁶
0
3
4
5
6
18
hpt
B
GaboSr50
Luminescence
12x10⁵
8x10⁵
4x10⁵
0
3
4
5
6
18
hpt
C
Fielder and GaboSr50 Avr/R treatment
Luminescence
6x10⁶
4x10⁶
2x10⁶
0
3
4
5
6
18
hpt
Treatment
fLUC
fLUC+AvrSr35
fLUC+AvrSr50
fLUC+Sr35
fLUC+Sr35+AvrSr35

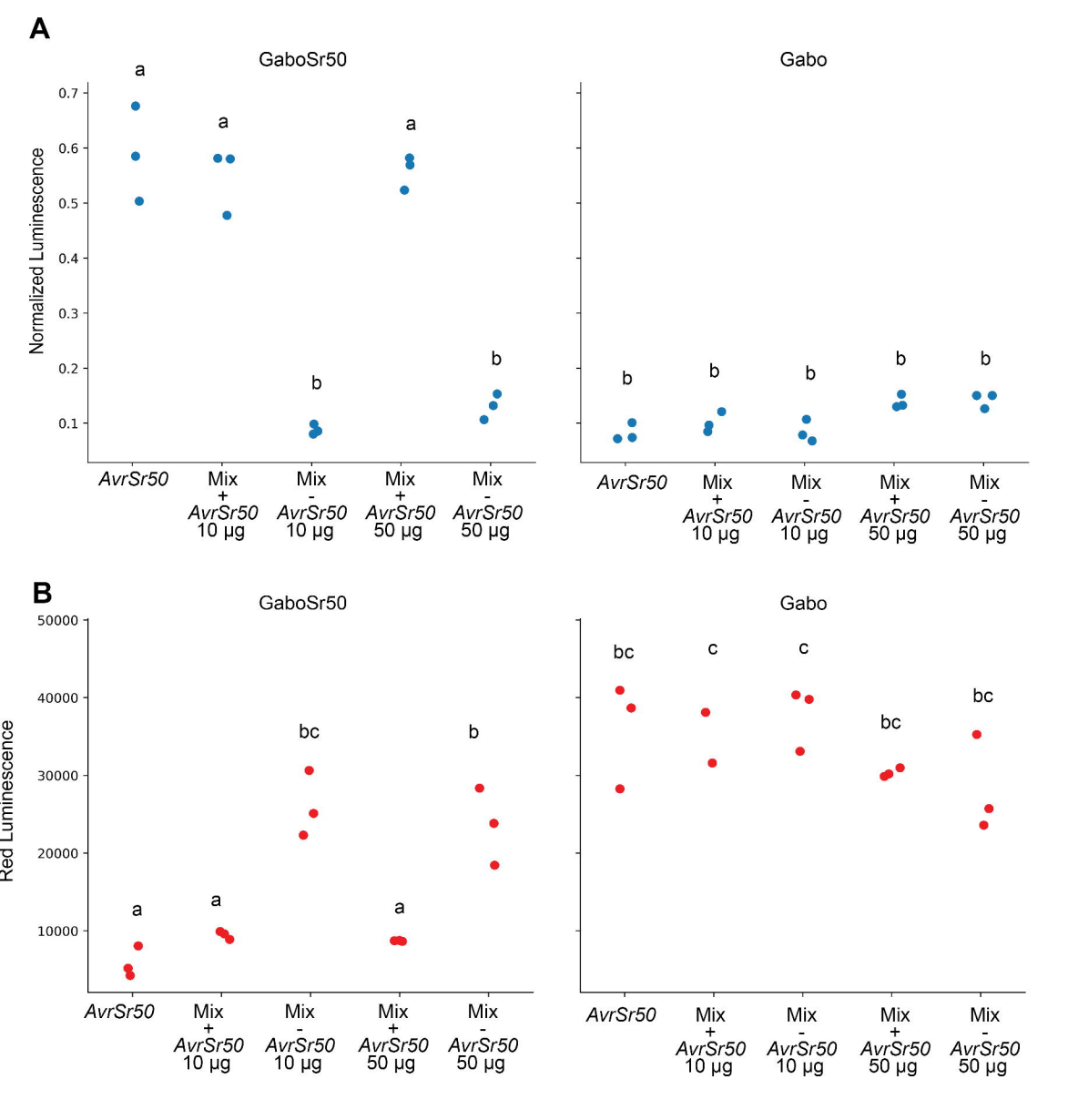

A
AvrSr35
AvrSr50
Sr35
Sr50
1
2
3
4
5
6
7
8
9
Normalized count
B
All Samples
PC2: 5% variance
PC1: 90% variance
C
Fielder
PC2: 33% variance
PC1: 46% variance
D
Gabo/GaboSr50
PC2: 17% variance
PC1: 77% variance
Treatment Group
1. Fielder+AvrSr35
2. Fielder+Sr35+AvrSr35
3. Fielder+Sr35+AvrSr50
4. Fielder+Sr35
5. Gabo+AvrSr50
6. Gabo+EV
7. GaboSr50+AvrSr35
8. GaboSr50+AvrSr50
9. GaboSr50+EV
E
calcium ion binding
ion binding
metal ion binding
cation binding
P-type calcium transporter activity
membrane
intrinsic component of membrane
integral component of membrane
cell periphery
plasma membrane
-p adj log10
Cultivar
Gabo/GaboSr50
Fielder
number of genes
15
45
75
F
Fielder
Gabo/GaboSr50
Log Normalized Count
D1
D2
D3
D4
D5
D6
D7
D10
D11
D12
D14
D15
D16
Avr
avr
R


A
Gabo
GaboSr50
Normalized Luminescence
0.0
0.1
0.2
0.3
0.4
0.5
0.6
a
a
b
a
AvrSr50
(avr)
EV
(no R or Avr)
AvrSr50
(Avr+R)
EV
(R)
B
Gabo
GaboSr50
Red Luminescence
10000
20000
30000
40000
a
a
a
a
AvrSr50
(avr)
EV
(no R or Avr)
AvrSr50
(Avr+R)
EV
(R)


A
GaboSr50 | Gabo
GaboSr50
GaboSr50
Normalized Luminescence
0.5
0.4
0.3
0.2
0.1
a
a
a
a
b
c
de
de
e
c
de
de
cd
c
1
15
1
1
0.1
0.5
1
5
15
0.1
0.5
1
5
15
µg AvrSr35 (avr+R)
µg AvrSr50 (avr)
µg AvrSr35 (avr)
µg pICH47742-AvrSr50 (Avr+R)
µg pWDV1-AvrSr50 (Avr+R)
B
GaboSr50 | Gabo
GaboSr50
GaboSr50
Red Luminescence
40000
35000
30000
25000
20000
15000
10000
5000
0
abcd
de
cde
de
e
de
cde
abc
a
bcd
abc
abc
ab
a
1
15
1
1
0.1
0.5
1
5
15
0.1
0.5
1
5
15
µg AvrSr35 (avr+R)
µg AvrSr50 (avr)
µg AvrSr35 (avr)
µg pICH47742-AvrSr50 (Avr+R)
µg pWDV1-AvrSr50 (Avr+R)


GaboSr50
C6969
Coorong
Normalized Luminescence
a
b
b
a
b
b
a
b
b
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.25
0.20
0.15
0.10
0.05
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
AvrSr50 (Avr+R)
AvrSr27 (avr+R)
EV (R)
AvrSr35 (Avr+R)
AvrSr27 (avr+R)
EV (R)
AvrSr27 (Avr+R)
AvrSr50 (avr+R)
EV (R)


GaboSr50
C6969
Coorong
Clement
Compair
Avocet Yr8
KenyaW1483
HeinesPeko
Fielder
Normalized Luminescence
a
b
AvrSr50 (Avr+R)
AvrSr27 (avr)
EV (R)
AvrSr35 (Avr+R)
AvrSr27 (avr)
EV (R)
AvrSr27 (Avr+R)
AvrSr50 (avr)
EV (R)
AvrSr50 +Sr50 (Avr+R)
AvrSr50 (avr)
Sr50 (R)
AvrSr27 +Sr27 (Avr+R)
AvrSr27 (avr)
Sr27 (R)
AvrSr35 +Sr35 (Avr+R)
AvrSr35 (avr)
Sr35 (R)


A
GaboSr50
Gabo
Normalized Luminescence
0.7
0.6
0.5
0.4
0.3
0.2
0.1
a
a
a
b
b
b
b
b
b
b
AvrSr50
Mix + AvrSr50 10 µg
Mix - AvrSr50 10 µg
Mix + AvrSr50 50 µg
Mix - AvrSr50 50 µg
B
GaboSr50
Gabo
Red Luminescence
50000
40000
30000
20000
10000
a
a
a
bc
b
bc
c
c
bc
bc
AvrSr50
Mix + AvrSr50 10 µg
Mix - AvrSr50 10 µg
Mix + AvrSr50 50 µg
Mix - AvrSr50 50 µg