Breakthrough Report

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

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Short Title:
Defense signaling in wheat protoplasts

Abstract

Plant resistance (*R*) and pathogen avirulence (*Avr*) gene interactions play key roles in crop resistance to pathogens. Efficient molecular screening tools for crop species are required to define interactions that trigger host resistance and to understand the virulence mechanisms of pathogenic fungi. *Avr* identification supports genome-based pathogen surveillance to enhance pre-emptive breeding of disease resistant cereal crops. To this end, 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 and is applicable to all wheat cultivars tested so far. It is suitable for multiplexed *Avr* screening, with interactions tested in pools of up to ten *Avr* candidates. Our assay is based on newly identified Avr/R-induced marker genes which we used to create promoter-luciferase reporter constructs. We combined these reporter constructs with a dual-color ratiometric reporter system that normalizes read-outs accounting for experimental variability and Avr/R-induced cell death. 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 genomic 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 *R* genes is a major way 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 correspoding *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. 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 and *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. We repeated the experimental setup while omitting the reporter plasmid and collected protoplasts at 4 hpt for

RNA sequencing (Figure 2 A, Treatment groups table 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 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/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 use 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 each of these plasmids along with internal normalization plasmid *UBI-pRedf* and *UBI-AvrSr50* into protoplasts isolated from cv. GaboSr50 and Gabo. 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. We observed similar specific reporter activation for all three promoters downstream of *AvrSr35* in wheat cv. C6969 (*Triticum aestivum*) carrying *Sr35* (Figure 3), which was introgressed from *Triticum monococcum* (McIntosh et al., 1984). These results indicate that our new ratiometric reporter system is able to detect specific defense activation downstream of two Avr/R interactions when the *R* gene is expressed endogenously.

We next addressed whether the induction of D2, D14, and D15 promoters is specific to AvrSr50/Sr50 and AvrSr35/Sr35 interactions. We extended our analysis to the recently published *AvrSr27/Sr27* (Upadhyaya et al., 2021) gene combination to test whether the *pD2, pD14* and *pD15* can serve as reporters of *Avr/R*-mediated response more broadly. We used protoplasts isolated from cv. Coorong, which carries *Sr27* (Upadhyaya et al., 2021), and transfected them with a plasmid that expresses *AvrSr27* under the control of the *UBI* promoter. We compared the induction of all three defense reporters in cv. Coorong protoplasts transfected with *AvrSr50* and cv. Fielder protoplasts, which does not carry *Sr27,* transfected with *UBI-AvrSr27*. We included *UBI-pRedf* as internal normalization control. We observed that all *pD2, pD14,* and *pD15* have high levels of normalized luminescence in cv. Coorong expressing *AvrSr27* compared to the controls (Figure 3). These results support D2, D14, and D15 as commonly applicable defense reporters 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.

The new 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. C69.69) with their corresponding *Avr* construct *(AvrSr50, AvrSr27* and *AvrSr35*, respectively). The cultivars Clement, Compair, HeinesPeko, (Dracatos et al., 2016) and Kenya W1483 (McIntosh et al., 1995) were tested with overexpression of *AvrSr50/Sr50* combination. The cultivars Avocet (*Yr8)* and Fielder were tested with *AvrSr27/Sr27* and *AvrSr35/Sr35* combinations, respectively. We include a negative control in all cultivars by expressing the *R* gene together with an un-recognized *Avr* (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 control. 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 all wheat cultivars tested.

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

To explore multiplexed screening of *Avr* candidates using our protoplast assay, we transfected a pool of ten *Avr* candidates, including *AvrSr50*, *AvrSr27*, *AvrSr35*, and seven other wheat rust candidate effector genes used in our studies, into protoplasts isolated from cv. GaboSr50 and cv. Gabo. The same *Avr* pool excluding the *AvrSr50* was used as a negative control, and *AvrSr50* alone was used 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, both *AvrSr50* alone and the pooled mix containing *AvrSr50* induced a high level of normalized luminescence of p*D14* when compared to the pooled mix without *AvrSr50* and all other treatment groups including controls in cv. Gabo protoplasts (Figure 5A). 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 mixture with no statistical

difference between the two treatment groups (Figure 5A). In contrast, there was a clear Avr-unspecific reduction in red luminescence induced by both multiplexed mixtures in both cv. Gabo and cv. GaboSr50 protoplast (Figure 5B). This indicates that the ratiometric dual-color luciferase system combined with *pDefense* reporter is highly sensitive in detecting Avr/R interactions and more specific than general luciferase based cell death based assays. Overall, these results showed 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. In addition, we also reduce variability of luminescence measurement by adapting a ratiometric dual-color luciferase reporter system (Sarrion-Perdigones et al., 2019). 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. 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 in 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 and leading to cell death. Similarly, in *Arabidopsis*, an activated NRG1.1 protein, known as downstream signaling component of plant *R* proteins, compose a Ca^{2+} permeable non-selective cation channel enabled by oligomerization, which control cell Ca^{2+} influx and induce 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.

We acknowledge future improvement will further extend the applicability of our positive defense reporter as described here. Similar to the previous wheat protoplast cell-death based assays (Saur et al., 2019), the assay we developed uses a large amount of DNA material for transfection to

obtain adequate luminescence signal induced by target Avr/R interactions. For example, in our transfections, we typically use 10-15 µg of plasmid for each target, which requires a large amount of pure plasmid DNA extraction. This could be time-consuming and expensive for preparing a large list of *Avr* candidates for screening. Moreover, we observed a larger quantity of DNA having a negative effect on the transfection efficiency when we transformed ten *Avr* candidates in one transfection. Future experiments will address the sensitivity of our positive defense reporters and the amount of plasmid required for robust responses and multiplexed avirulence effector screening. It is likely that further optimization as reported recently (Arndell et al., 2023) will allow us to reduce required plasmid amounts. Our study is complementary to a recent report that describes highly multiplexed avirulence effector recognition screening using cell death as a negative enrichment method. 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 ^{o}C 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°C 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.

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. A template spreadsheet 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, K1483, Avocet 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.*

Positive selection/pool screening

Protoplasts were prepared from GaboSr50 wheat leaves and transfected as above. Replicates which contained the *Avr* pools used 10 µg of each plasmid mixed prior to transfection, while replicates without pooled *Avrs* contained at total of 10 µg of *UBI-AvrSr50*. Measurements used the ratiometric reporters and measurements, with *pDefense14-ELuc* as reporter.

Data availability

All supplemental data is available on Zenodo (DOI: 10.5281/zenodo.7880015, link: https://doi.org/10.5281/zenodo.7880015. All plasmids generated will be made available from Addgene, with plasmid maps deposited on Zenodo (DOI: 10.5281/zenodo.7844465, link: https://doi.org/10.5281/zenodo.7844465). 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.

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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., and E. C. 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.

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Figure Legends

Figure 1: Cell death and differential gene expression in *Avr/R* interactions. **A-B**, Luciferase activity was measured at different hours of post-transfection (hpt) in **(A)** cv. Fielder protoplasts expressing *luciferase* alone, and *luciferase* with either *Sr35, AvrSr35,* or *Sr35* plus *AvrSr35* and in **(B)** 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 indicated 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)** shows principal component analysis (PCA) of the RNAseq analysis; all 36 samples **(B)**, cv. Fielder **(C)** and cv. Gabo/GaboSr50 **(D)**. (**E)** 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 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: Positive pDefense reporters detect defense signaling triggered by three distinct *Avr/R* pairs in wheat protoplasts expressing the *R* gene endogenously. Each panel of each row shows the normalized luminescence of the positive defense reporter pDefense14 **(A)**, pDefense15 **(B)**, or pDefense2 **(C)**. From left to right, protoplasts from wheat cultivars C6969 carrying *Sr35*, GaboSr50 carrying *Sr50*, and Coorong carrying *Sr27* were transfected with *AvrSr35*, *AvrSr50* and *AvrSr27* 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.

Figure 4: The positive defense reporter *pDefence14* is broadly applicable to all tested wheat cultivars. Each panel of each row shows the normalized luminescence of the positive defense reporter *pDefense14*. Row **(A)**: Protoplast isolated from three wheat cultivars carrying a described *R* gene, were transfected with its cognate *Avr* or controls. Rows **(B-C)**: Protoplasts isolated from various wheat cultivars were transfected with both *R* and *Avr* constructs. The cultivars Clement, Compair, HeinesPeko, and Kenya W1483 were tested with *AvrSr50/Sr50*. Avocet and Fielder were tested with *AvrSr50/Sr50* and *AvrSr27/Sr27*, respectively. An *avr* (unrecognised by R protein) was used as a control and *UBI-pRedf* was used for normalization of all experiments.

Figure 5: 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.* A pool of ten *Avr* candidates, with and without *AvrSr50* or *AvrSr50* alone was transfected into protoplasts isolated from cultivars GaboSr50 (left column) and Gabo (right column). One-way analysis of variance (ANOVA) and post hoc Tukey's HSD test was used to assess differences among normalized luminescence or red

luminescence of treatment groups. Treatments marked with differing lowercase letters show statistically significant difference (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.

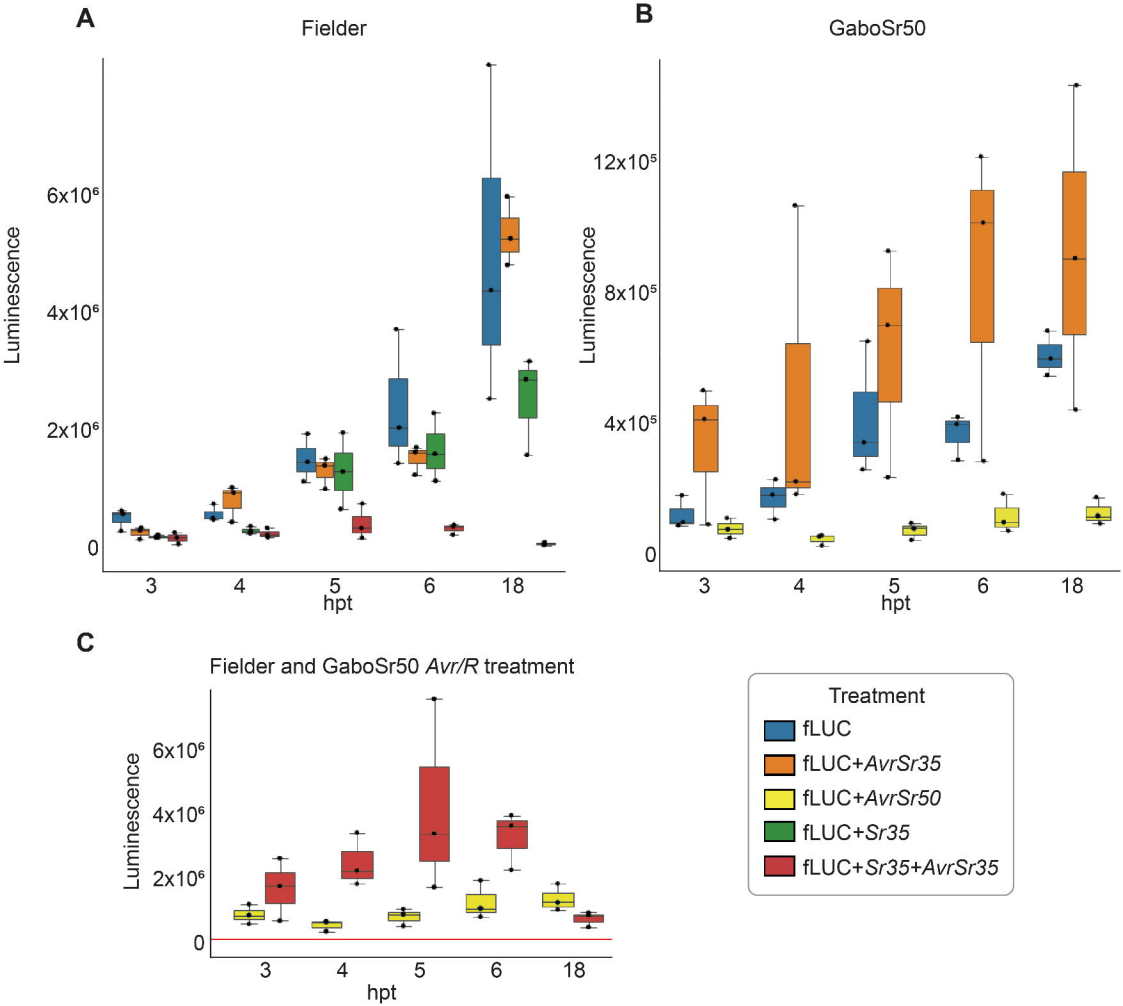
Supplemental Material S4: GO terms analysis Query tables and g:profiler results tables

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, 4 and 5.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Fielder
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2x10⁶
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3
4
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6
18
hpt
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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
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Treatment
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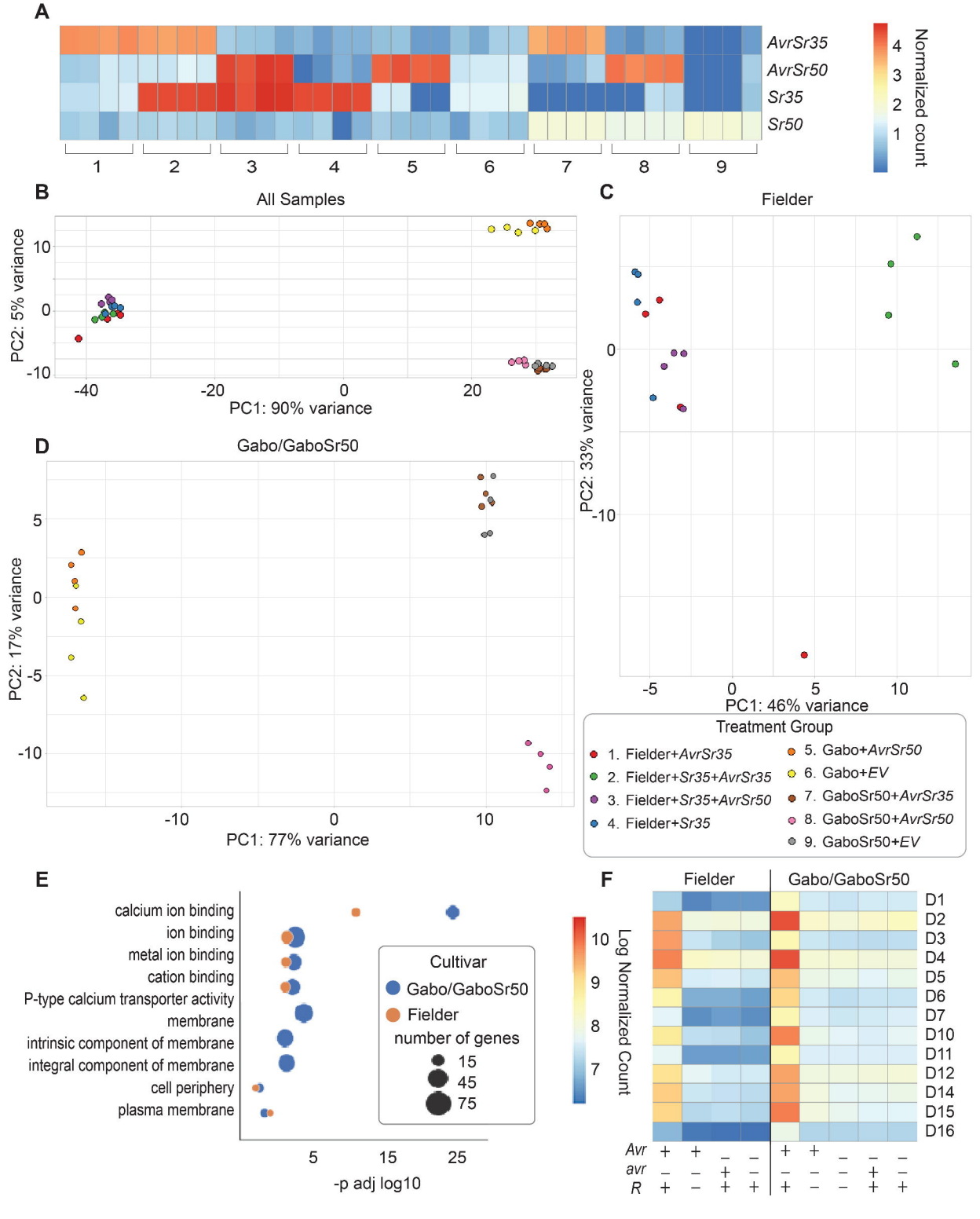
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AvrSr35
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Sr50
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2
3
4
5
6
7
8
9
Normalized count
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All Samples
PC2: 5% variance
PC1: 90% variance
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
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D5
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Avr
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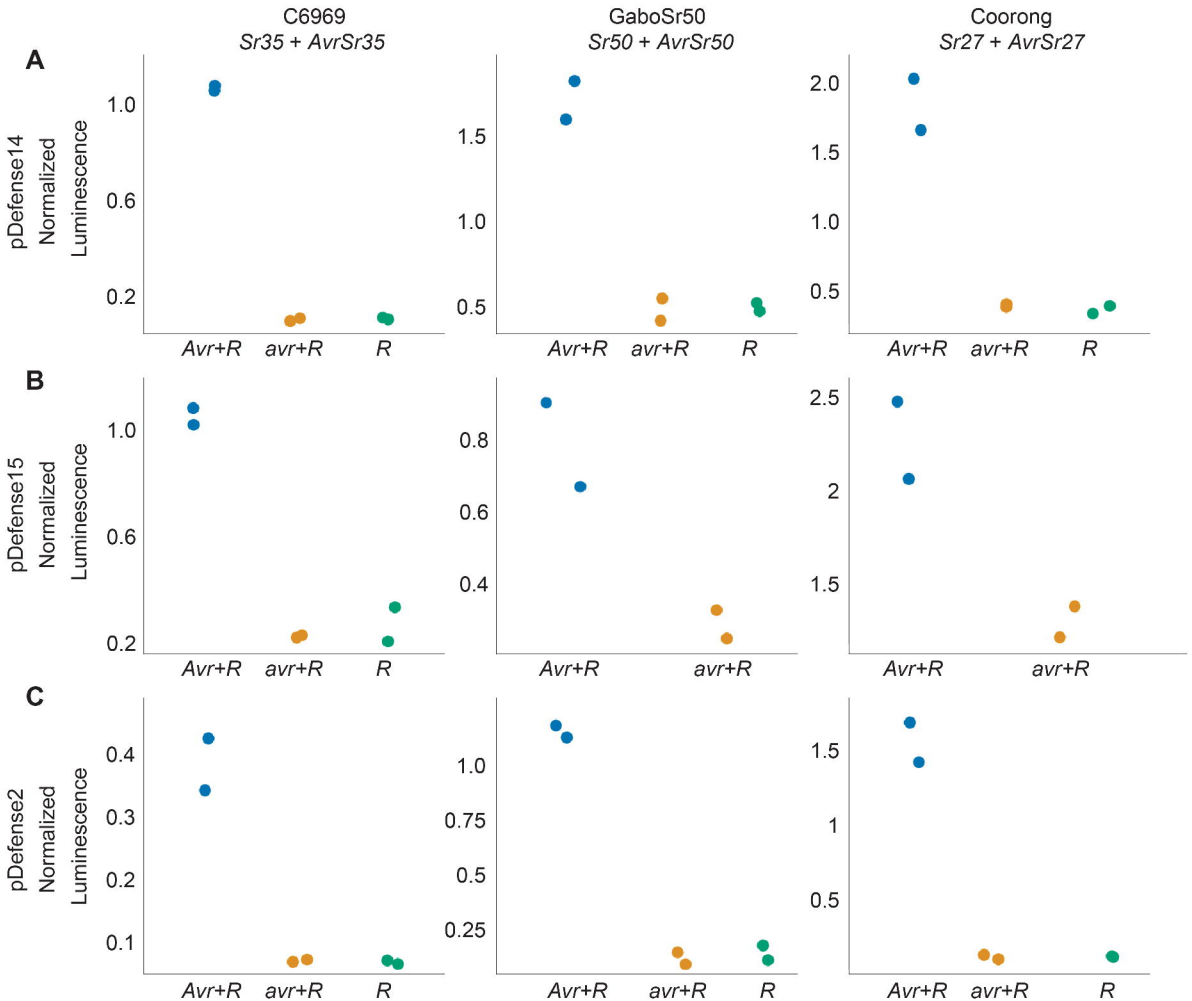
C6969
Sr35 + AvrSr35
GaboSr50
Sr50 + AvrSr50
Coorong
Sr27 + AvrSr27
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B
C
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pDefense2 Normalized Luminescence
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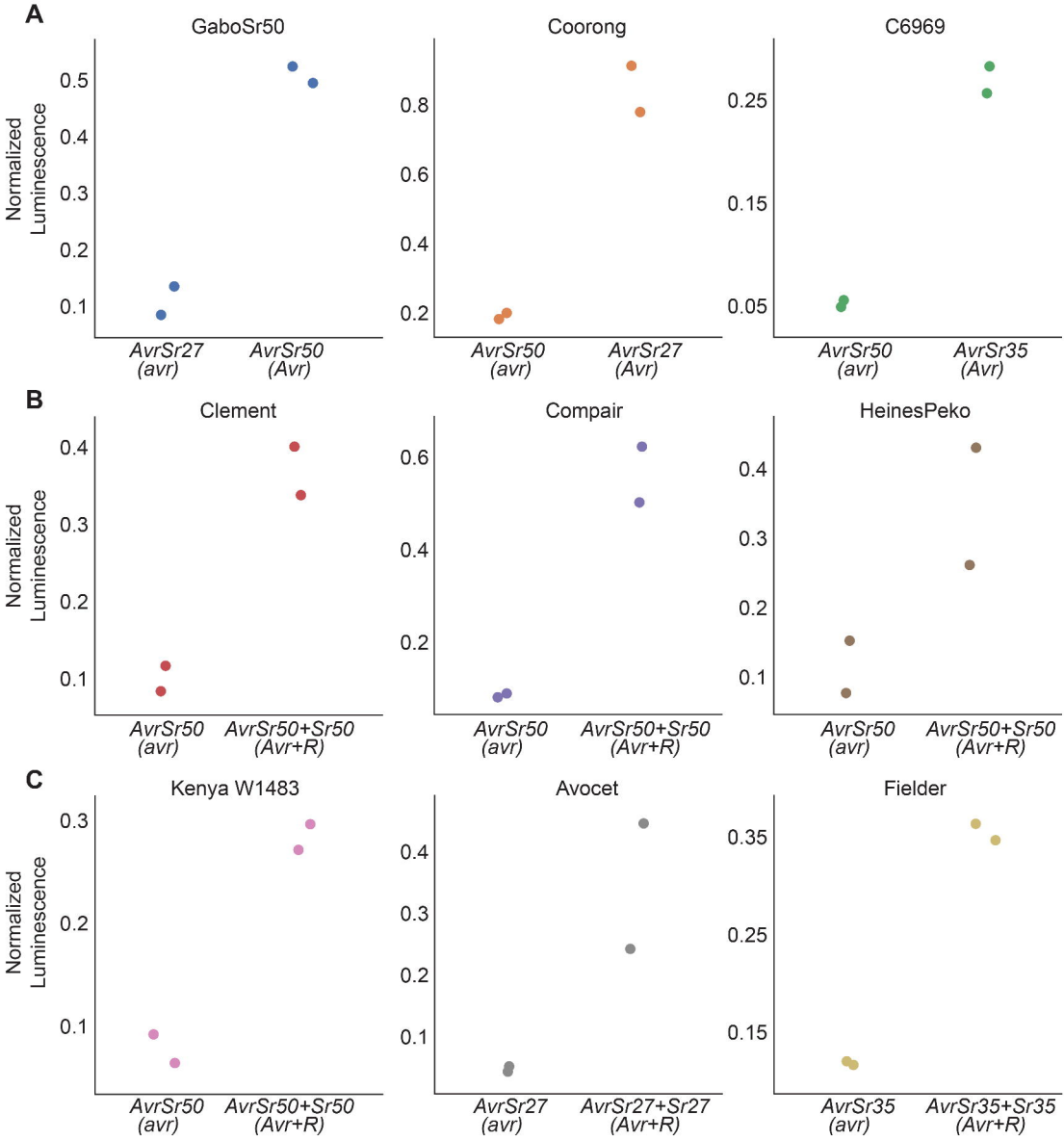
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Coorong
C6969
Normalized Luminescence
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0.2
0.25
0.15
0.05
AvrSr27 (avr)
AvrSr50 (Avr)
AvrSr50 (avr)
AvrSr27 (Avr)
AvrSr50 (avr)
AvrSr35 (Avr)
B
Clement
Compair
HeinesPeko
0.4
0.3
0.2
0.1
0.6
0.4
0.2
0.4
0.3
0.2
0.1
AvrSr50 (avr)
AvrSr50+Sr50 (Avr+R)
AvrSr50 (avr)
AvrSr50+Sr50 (Avr+R)
AvrSr50 (avr)
AvrSr50+Sr50 (Avr+R)
C
Kenya W1483
Avocet
Fielder
0.3
0.2
0.1
0.4
0.3
0.2
0.1
0.35
0.25
0.15
AvrSr50 (avr)
AvrSr50+Sr50 (Avr+R)
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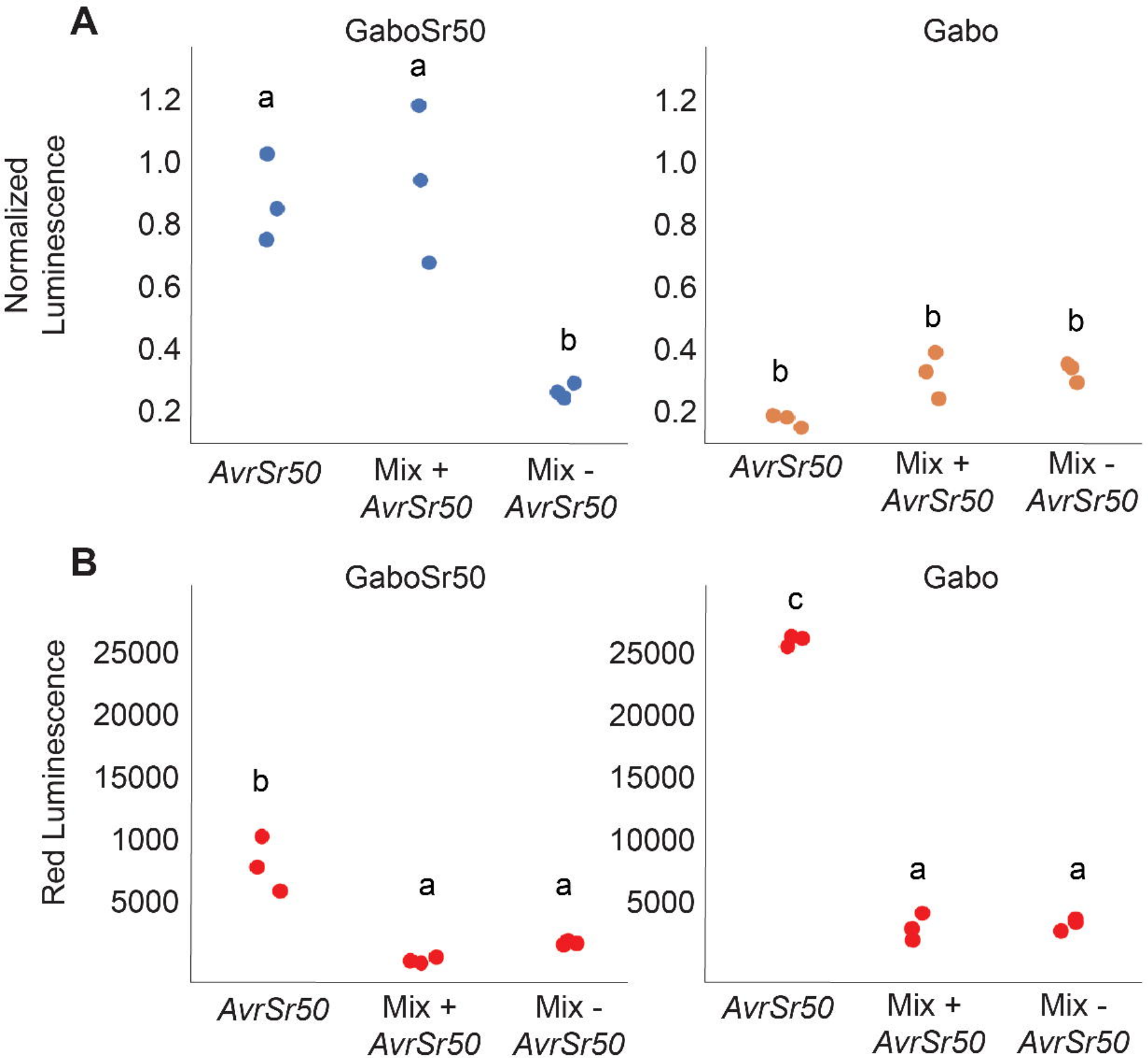
AvrSr35 (avr)
AvrSr35+Sr35 (Avr+R)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Gabo
Normalized Luminescence
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b
b
b
b
AvrSr50
Mix + AvrSr50
Mix - AvrSr50
B
GaboSr50
Gabo
Red Luminescence
25000
20000
15000
1000
5000
10000
b
a
a
c
a
a
AvrSr50
Mix + AvrSr50
Mix - AvrSr50