Research

Development and Use of a Seedling Growth Retardation Assay to Quantify and Map Loci Underlying Variation in the Maize Basal Defense Response

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Author contributions: Y.W. and P.B.K. conceived the research and wrote the manuscript, Y.W. performed the experiments, and J.H. performed statistical analyses and advised on QTL analysis and edited the manuscript.

Accepted for publication 22 December 2020.

Funding

This work was funded, in part, by the Department of Energy Plant Feedstock Genomics for Bioenergy program grant number DE- SC0014116, and by the North Carolina State University and United States Department of Agriculture–Agricultural Research Service. Y. Wang's fellowship was funded by the China Scholarship Council Fellowship.

The author(s) declare no conflict of interest.

e-Xtra: Supplementary tables and one supplementary figure are available online.

Abstract

The pattern-triggered immune (PTI) response in plants is caused by the recognition of conserved microbe- or pathogen-associated molecular patterns (MAMPs) by plant pattern recognition receptors at the cell surface. The goal of this study was to develop a simple, robust assay to quantify the PTI response in maize and to determine whether it could be used to predict levels of disease resistance. Flg22, an epitope derived from bacterial flagellin, is a commonly studied MAMP. We developed a seedling growth retardation (SGR) assay by which we could measure growth retardation in maize seedlings exposed to the bacterial MAMP flg22. We observed variation across 21 maize inbred lines. We used 161 lines from a recombinant inbred line (RIL) population derived from a cross between the lines CML228 (a high responder) and B73 (a low responder) to map quantitative trait loci (QTL) for this response. We found heritable variation in the RIL population and identified flg22 response QTL on chromosomes 1, 2, and 8. We did not observe strong correlations between SGR traits and levels of flg22-induced reactive oxygen production or with other disease resistance or defense response traits we had previously measured in the same population. We discuss the implications of these findings.

Keywords: basal defense response, maize, QTL

Maize diseases and pests are estimated to cause global yield losses of 20 to 41% (Savary et al. 2019), with the greatest losses in tropical regions, where maize is often a staple crop. Genetically based disease resistance is the most effective method of controlling plant disease (Nelson et al. 2017). The plant immune system relies on the recognition of pathogen-derived molecules. One class of recognized molecules is known as microbe- or pathogen-associated molecular patterns (MAMPs): conserved molecules not specifically associated with pathogenesis; whereas others are considered effectors: molecules that are introduced into the host cell or apoplast which facilitate the pathogenesis process. MAMPs are usually recognized at the cell surface by transmembrane receptors called pattern recognition receptors (PRRs), resulting in a relatively low-level defense response known as pattern-triggered immunity (PTI). PTI is usually sufficient to

confer resistance to pathogens not specifically adapted to the host (Newman et al. 2013). The PTI response can include changes in ion flux across the plasma membrane (Jabs et al. 1997; Mithöfer et al. 2005), production of reactive oxygen species (ROS) (Lloyd et al. 2017, 2014) and nitric oxide (NO) (Rasul et al. 2012), callose deposition (Luna et al. 2011), modification of phytohormone concentrations (Furch et al. 2014), and induction or repression of plant defense-related genes (Valdés-López et al. 2011). PTI is thought to be a significant factor in nonhost resistance: the immunity of an entire plant species to an entire pathogen species (Lee et al. 2016; Li et al. 2005).

In pathogens adapted to a particular host, a subset of effectors may suppress host PTI responses, and so allow infection. At this point, in some cases, specialized resistance (R) proteins may detect the presence of specific effectors either through direct interaction or via the action of the effectors on another molecule that is monitored (or guarded) by the R protein (Bent and Mackey 2007). Detection of effectors triggers an effector-triggered immunity (ETI) defense response that includes many of the same responses as noted for PTI above and additionally often includes a hypersensitive response (HR), which is a rapid localized cell death response at the point of pathogen penetration (Hamdoun et al. 2013).

Although this widely used ETI and PTI dichotomy can be applied in many situations, there are other cases which do not fit neatly into one or the other category (Thomma et al. 2011). Cook et al. (2015) suggested that the plant immune system might be best understood as a system in which invasion patterns (IPs), pathogen- or host-derived molecules produced during invasion, are recognized by invasion pattern receptors (IPRs), and that there is a continuum of IPs between highly conserved IPs (MAMPs) and lineage-specific IPs with specific functions in pathogenesis (effectors). Likewise, the IPRs range from highly conserved transmembrane receptors (PRRs) to highly variable cytoplasmic R proteins. Ultimately, it is clear that, at one extreme of this continuum, a class of highly conserved microbial molecules exists which can be regarded as MAMPs that are recognized by highly conserved plant transmembrane receptors that can be regarded as PRRs.

All known plant PRRs can be classified as either receptor-like kinases (RLKs) or receptor-like proteins (RLPs) (Monaghan and Zipfel 2012). Both have extracellular and transmembrane domains, RLKs additionally possessing a cytoplasmic kinase domain. The PRR receptor domains themselves fall into several classes; leucine-rich repeat (LRR) extracellular domains are the most common and recognize proteinaceous MAMPs, lysin motifs (LysM) receptors recognize polysaccharides such as peptidoglycan (PGN) and chitin, and lectins recognize glycolipids. Several wall-associated kinases (WAKs), which have epidermal growth factor-like receptor domains and recognize plant cell-wall-derived oligogalacturonides, have been implicated in recognition during plant immunity and the damage-associated molecular pattern (DAMP) response (Hurni et al. 2015; Kohorn and Kohorn 2012; Kohorn et al. 2009; Zhang et al. 2020; Zuo et al. 2015).

After ligand perception by PRRs, downstream signaling is facilitated by interaction with coreceptors in receptor complexes. Of the coreceptors characterized thus far, the LRR-RLK BAK1, a member of the somatic embryogenesis receptor kinase family, is particularly noteworthy because it is required for the function of a large number of PRRs and at least 21 other defense-related membrane-localized proteins (Yasuda et al. 2017). Many plant species, including rice, tomato, and *Arabidopsis*, recognize a conserved 22-amino-acid domain of flagellin termed flg22, and trigger a defense response via the PRR FLS2 (Felix et al. 1999;

Zipfel et al. 2004), which itself is widely conserved across plant species. Alternative flg22 epitopes have been identified in various bacterial pathogens of *Arabidopsis*, rice, and grape which, in some cases, elicit differential levels of defense response (Clarke et al. 2013; Sun et al. 2006; Trdá et al. 2014; Wang et al. 2015).

Although the dogma would suggest otherwise, the response of different lines within the same plant species to the same MAMP has been shown to be quite variable. Extensive variation in the flg22 response has been documented between lines in tomato (Veluchamy et al. 2014), *Arabidopsis* (Vetter et al. 2012, 2016), *Brassica napus* (Lloyd et al. 2017, 2014), and soybean (Valdés-López et al. 2011). Similarly, the response to chitin is variable between lines in soybean and *B. napus* (Lloyd et al. 2017; Valdés-López et al. 2011) and the response to the MAMP epitope elf18 is variable between *Arabidopsis* lines (Vetter et al. 2016). We have also shown extensive variability in maize (Zhang et al. 2017) and sorghum (Kimball et al. 2019) for responses to both flg22 and chitin. In fact, although it has not been examined extensively, it seems likely that this type of variation is the rule rather than the exception. We and others have observed that the relative genotype ranking for PTI depends of what aspect of the response is being measured (Lloyd et al. 2017; Zhang et al. 2017). The main approaches to quantifying the MAMP response include measuring ROS production, mitogen-activated protein kinase phosphorylation, mRNA levels of specific MAMP-induced genes, lignin, callose and cell wall-bound phenols, seedling growth inhibition, peroxidase activity, and MAMP-induced resistance to pathogens (Lloyd et al. 2017, 2014; Mott et al. 2018; Nguyen et al. 2010; Zeidler et al. 2004; Zhang et al. 2017).

Although the large majority of the work on the molecular characterization of the plant recognition innate immune system has been performed in dicots, and in *Arabidopsis* in particular, some significant work has been performed in monocots, especially in rice. The rice LRR RLK OsFLS2, encoded by a homolog of *Arabidopsis* FLS2, recognizes flg22, triggers PTI (Takai et al. 2008), and complements FLS2 function in *Arabidopsis* (Takai et al. 2008). Chitin is recognized in rice mainly by a combination of the LysM RLP CEBiP (Kaku et al. 2006) and its coreceptor, the LysM RLK OsCERK1 (Shimizu et al. 2010). The LysM-RLPs OsLYP4 and OsLYP6 also interact with OsCERK1 and respond to the presence of both chitin and PGN (Liu et al. 2012).

The PTI response in maize has not been well characterized. Although none of the important host response genes have been identified, maize possesses close homologs of various rice PTI genes, including the homologs of OsCEBiP, OsCERK1, OsFLS2, OsBak1, OsLYP4, and OsLYP6, with homologies of 65.8, 44.5, 67.1, 89.5, 77.2, and 84.3%, respectively, at the amino acid level in the B73 genome (<https://www.maizegdb.org/>). We recently quantified responses in maize to two MAMPs, flg22 and chitooctaose (c8, a degradation product of chitin that is soluble in water) (Zhang et al. 2017). We initially used several methods to quantify PTI among diverse lines, including measurement of NO and ROS production and induction of transcription of several genes. We observed considerable variation in the relative line rankings based on these different traits. We subsequently examined the flg22- and c8-triggered response in a recombinant inbred line (RIL) population using a ROS assay (Samira et al. 2019). The flg22 and c8 ROS responses were highly correlated in this population and the main quantitative trait loci (QTL) for each response colocalized on chromosome 2 (Zhang et al. 2017). We also examined the correlation between PTI and quantitative disease resistance (QDR) to three diseases—southern leaf blight (SLB), gray leaf spot (GLS), and northern leaf blight (NLB)—in this RIL population. Moderate correlations were observed

between PTI traits and SLB resistance and no correlations were observed for the other two diseases (Zhang et al. 2017). In a similar study in sorghum, ROS responses elicited by flg22 and chitin were largely uncorrelated with levels of resistance to the fungal disease target leaf spot (Samira et al. 2020).

An alternative way of classifying plant disease resistance is as qualitative or quantitative. Qualitative disease resistance has been defined either phenotypically as complete resistance or genetically as based on single genes with very strong effects (Niks et al. 2015). In practice, both of these definitions usually apply and the underlying mechanism is usually, though not always (Büschges et al. 1997), based on ETI. Qualitative resistance is often not durable due to the ability of the pathogen to adapt to, overcome, or evade recognition (Parlevliet 2002) and is not available for every pathosystem. In contrast, QDR is defined either phenotypically as partial resistance or genetically as being under the control of multiple genes, each of which have relatively small effects (Poland et al. 2009). QDR is usually durable under field conditions (Cowger and Brown 2019; Kou and Wang 2010).

Over the last decade, it has become apparent that QDR is controlled by a range of genes associated with a variety of mechanisms. These include signaling, cell wall strengthening, transcriptional control, and metabolite transport (Nelson et al. 2017; Poland et al. 2011). Mechanisms associated with PTI have also been implicated in QDR. Several QDR genes are now known to be RLKs, implying that they may mediate their effects through PTI-like mechanisms (Cole and Diener 2013; Nelson et al. 2017). QDR in *Arabidopsis* to *Pseudomonas syringae* pv. *phaseolicola* was shown to be controlled, in part, by the segregation of the *FLS2* gene (Forsyth et al. 2010). Vetter et al. (2012) showed that the strength of flagellin perception is negatively correlated with susceptibility to *P. syringae* and Bhattarai et al. (2016) showed that resistance to bacterial spot in tomato is correlated with flg22 response. A striking protective effect of PTI against disease has been observed in a number of species, including sorghum (Tuleski et al. 2019), soy (Valdés-López et al. 2014), and *Arabidopsis* (Jacobs et al. 2011; Wan et al. 2008; Zipfel et al. 2004). However, little research has been undertaken to determine whether natural variation in the strength of the MAMP response contributes to variation in QDR, especially in crop plants.

We are interested in how variation in the strength of the PTI is related to general levels of QDR in maize and in plants in general. One consideration in addressing this question is what assay to use to quantify the MAMP response. As discussed above, we and others have shown that different assays can give quite different results that are not necessarily correlated with each other. Although some assays measure very specific outputs of the MAMP response, others measure traits that may better integrate the various aspects of PTI. For instance, a disease reduction assay measures the effect of inducing the MAMP response on levels of pathogen infection (Lloyd et al. 2014; Tuleski et al. 2019; Valdés-López et al. 2011). Another assay that measures the combined effects of various aspects of the response is a seedling growth retardation (SGR) assay. Assays of this type have been used in *Arabidopsis* and *Brassica* spp. to quantify the MAMP response (Lloyd et al. 2014; Vetter et al. 2016). The SGR assay is based on the observation that prolonged exposure of a germinating or growing seedling to a PTI elicitor often reduces seedling growth. This growth reduction is a manifestation of the growth–defense tradeoff that has been observed in many plant systems (Huot et al. 2014). The tradeoff is thought to occur because of the necessary reallocation of resources from growth to defense during periods when the plant perceives that it is under attack. It is mediated, at least in part, by hormone signaling pathways (Albrecht and Argueso 2017; Guo et al. 2018; Naseem

et al. 2015) and by ROS levels (Morales and Munné-Bosch 2016) and can be additionally observed at the transcriptional and translational levels (Li et al. 2016; Metegnier et al. 2017).

The goal of this study was to develop an SGR assay for maize that could be used to quantify PTI in a robust and relatively simple way. Furthermore, we wanted to identify genetic factors associated with variation in this response and determine whether variation in PTI as measured by this assay could be used as a proxy for general levels of QDR in maize so that this assay might be used by breeders to prescreen lines for disease resistance qualities.

MATERIALS AND METHODS

Plant materials

In all, 21 of 26 parental lines of the nested association mapping (NAM) population (McMullen et al. 2009), and 161 lines from an RIL population derived from a B73 × CML228 cross that constitute a subpopulation of the NAM population used in this study, are described in Supplementary Table S1.

SGR assay conditions

Maize seed was soaked in 1% H₂O₂ for 2 min and rinsed three times in sterile H₂O. Seed were germinated on brown germinating paper (size 38, 10 × 15 in.; Anchor Paper, St. Paul, MN, U.S.A.) for 4 days. Germinated seedlings were then wrapped in sponge (Jaece Identi-Plugs Foam Tube Plugs L800-B size B, cut into thin slices with a slit made in the center) and suspended at the top of 15-ml disposable Falcon tubes (Fisher catalog number 1495949B) with the tip of the emerging radicle dipped into the solution (Supplementary Fig. S1). Tubes were filled with water (control) or with various concentrations of flg22 Genscript (catalog number RP19986) solution (1, 5, 10, and 20 μM). All seedlings were grown in the growth chamber at 26°C with 16 h of light and 8 h of darkness. A typical SGR assay setup is shown in Supplementary Fig. S1.

Trait measurement

After 5 days of growth in the Falcon tubes, root length (RL; length of the longest root), root weight (RW), shoot length (SL; length of the longest shoot), shoot weight (SW), and fresh weight (FW) were measured. In order to measure these traits, the plant was removed from the tube and patted dry with paper towels. RL was measured with a ruler as the length of the longest root from the tip to the point where it joined the plant. SL was measured from the point where the upper root joined the stem to the tip of the longest leaf. To measure RW and SW, the remnants of the seed coat were removed and the plant was cut at the point where the upper root joined the plant, and the two parts were weighed separately. Weights were measured in grams and lengths in centimeters.

From these directly measured traits, we calculated derived trait indices to measure the response to flg22. The seedling growth inhibition (SGI) indices for each trait of each line were calculated as [(CK – flg22) × 100]/CK, where CK = average value of untreated seedlings and flg22 = average value of flg22-treated seedlings (Vetter et al. 2012). Therefore, positive SGI values indicated that the treatment reduced the trait values relative to the control, and vice versa. We also calculated CK – flg22. Although SGI was a measure of the proportional response to flg22, CK – flg22 was a measure of the absolute response.

Experimental design

For each replication in each experiment described, except for replication 3 of the RIL assessment, we measured five individual seedlings for the flg22 (treated) treatment and five seedlings for the water (control) condition. (For replication 3, we measured three individual seedlings). In each case, the treated and control plants for each line were placed in adjacent tubes in the same rack. Plants were grown in growth chambers in the North Carolina State University Phytotron. Trait values were the average of all of the seedlings measured for each line or treatment in each replication. For the RIL population, the experimental design was a split plot with three replications. Lines were the whole-plot factor and treatment was the subplot factor. For the RIL experiment, it was not feasible to process and take all of the measurements of all 1,630 seedlings at one time; thus, for each replication, the lines were randomized and divided into three sets. The three sets were set up in on three successive days and then processed and measured on three successive days 5 days later.

Statistical analyses

Descriptive analysis of traits (mean, range, standard deviation, and the coefficient of variation) were calculated using the descriptive Statistics model in SPSS 20.0 software (SPSS, Chicago, IL, U.S.A.). Split-plot analysis for each trait was conducted using the asreml-R package in R (R Core Team 2009). For each trait, we compared four models: model 1 = a full model fitting line, treatment, and line–treatment interaction as fixed effects; set–replication interaction and replication–line interaction (whole-plot error) as random effects; and separate error variances within each treatment; model 2 = a reduced model fitting only replication main effects instead of set–replication interactions; model 3 = a reduced model with a homogeneous error variance; and model 4 = a reduced model fitting replication main effects and a homogeneous error variance. Using the Bayesian Information Criterion, models 3 or 4 were best across all traits; therefore, we fit model 3 with homogeneous error variance and set–replication interaction for all traits to test the significance of fixed effects. *P* values for treatment, line, and treatment–line interaction effects were computed with Wald tests in asreml-R. Mixed linear models with fixed line and random set–replication effects were also fit for all traits within treatments, for the difference between control and treated experimental units, and for the scaled differences (SGI) using the lme4 package (Bates et al. 2015). *F* tests of line main effects within treatments or for difference or SGI were tested with the Kenward-Roger test in R package pbkrtest (Halekoh and Højsgaard 2014). Heritability of trait within treatment was also estimated using mixed models fit in lme4 with both replication and line modeled as random effects. Heritability on a line mean-basis was estimated as:

$$\hat{h}_{line-mean}^2 = \frac{\hat{\sigma}_{line}^2}{\hat{\sigma}_{line}^2 + \frac{\hat{\sigma}_{error}^2}{3}}$$

The average trait value of each inbred line across the three replications was used for correlation analysis in the “corrplot” package in R. Windows QTL Cartographer software v2.5 was used to perform QTL mapping using least square means of line values within treatments, together with genotypic values of 3,693 single-nucleotide polymorphism (SNP) markers. Composite interval mapping was performed with a walk speed of 1.0 centimorgans (cM), and 1,000 permutation tests were run to determine the threshold logarithm of odds at a significance level of 0.05 for each trait.

The CML228 × B73 mapping population used in this study is a subpopulation of the widely used maize NAM population (McMullen et al. 2009). The SNPs had all been previously scored in this population using a genotyping-by-sequencing approach and the scores are publicly available (<https://www.panzea.org/genotypes>).

RESULTS

MAMP response across different NAM parental lines

We developed a simple maize SGR assay in which maize seed was sterilized, washed, and germinated on germination paper for 4 days. The germinated seed was then suspended at the top of a 15-ml tube filled with a solution containing flg22, with the tip of the emerging radicle dipped into the solution (Supplementary Fig. S1). We used flg22 because it is a widely used and readily available MAMP. We had previously used both flg22 and chitin to elicit PTI in maize leaves and had found that the responses to both MAMPs were highly correlated, but that flg22 elicited a stronger response (Zhang et al. 2017).

RL, RW, SL, SW, and FW of 21 diverse lines were measured using this assay after seedlings were grown in water (control) and 20 μM flg22 (Supplementary Table S1). In most cases, the addition of flg22 led to reduced growth which corresponds to a positive SGI value (Fig. 1; Supplementary Table S1). However, RL, RW, SL, SW, and FW increased under flg22 treatment for several lines, notably Tzi8, M162W, CML69, CML333, B97, IL14H, and CML52.

QTL mapping

We had previously analyzed an RIL population derived from a B73 × CML228 cross for variation in ROS production induced by flg22 (Zhang et al. 2017). This population was a subpopulation of the widely used maize NAM population (McMullen et al. 2009). For the SGR assay, as for the ROS assay previously, B73 showed a relatively weak response and CML228 a relatively strong response to flg22 (Fig. 1; Supplementary Table S1). Therefore, we analyzed the same B73 × CML228 population for variation in growth retardation caused by flg22.

In order to optimize our assay conditions, we first assessed the growth response of B73 and CML228 to various concentrations

FIGURE 1

Responses of diverse lines to flg22. Seedling growth inhibition (SGI) indices comparing growth in water or 20 μM flg22 for root length (RL), root weight (RW), shoot length (SL), shoot weight (SW), and fresh weight (FW). SGI is a standardized growth inhibition value such that a positive value indicates growth inhibition by flg22, with a value of 1 indicating complete inhibition, 0 indicating no inhibition, and a negative value indicating growth enhancement. Standard errors are indicated.

of flg22 (1, 2, 10, and 20 μM). The responses of the two parents were most different at the 10- μM concentration (Figs. 2 and 3). Therefore, we used 10 μM flg22 in the following experiments.

We assessed the growth response of 161 lines from the B73 $\times$ CML228 RIL population to 10 μM flg22. Three full

replications were assessed. In replications 1 and 2, five seedlings were assessed per line for treated (flg22) and control (CK) conditions, whereas, in replication 3, three seedlings were used. We measured RL, RW, SW, SL, and FW for each seedling in each replication under flg22 and CK conditions. We also

FIGURE 2

Responses of B73 and CML228 to different concentrations of flg22. Seedlings were germinated in moist germination paper for 4 days and grown in water with 1, 2, 10, or 20 μM flg22 for 5 days.

FIGURE 3

Seedling growth inhibition of B73 and CML228 assessed under different concentrations flg22. Data are root length (RL), root weight (RW), shoot length (SL), shoot weight (SW), and fresh weight (FW) measured under control and flg22 conditions. Seedling growth inhibition (SGI) is a standardized growth inhibition value such that a positive value indicates growth inhibition, with a value of 1 indicating complete inhibition, 0 indicating no inhibition, and a negative value indicating growth enhancement. Standard errors are indicated.

calculated the following derived traits: RL(CK – flg22), RW(CK – flg22), SL(CK – flg22), SW(CK – flg22), FW(CK – flg22), RWSGI, RLSGI, SWSGI, SLSGI, and FWSGI (Supplementary Table S2). All measured traits were distributed in an approximately normal manner (Table 1; Fig. 4).

Line effects were significant for all of the directly measured traits at $P < 0.001$ (Table 1). For the derived trait CK – flg22, measuring the absolute magnitude of a line's response to treatment, line effects were significant ($P < 0.05$) only for RL and RW. Line effects were significant ($P < 0.05$) for the scaled response measure (SGI) for all traits except SL (Table 1). Similarly, heritabilities for the directly measured traits varied from 0.54 to 0.8 while heritabilities for the derived traits were lower, as would

be expected of traits calculated using two other trait values, varying from 0.00 to 0.26 for responses to treatment (CK – flg22) and from 0.02 to 0.29 for the scaled responses to treatment (SGI) (Table 1). Scaled responses to percentage of the untreated line value (SGI) had higher precision than the unscaled response measurements (CK – flg22). Replications were significantly correlated for each trait, Pearson correlation coefficients ranged from 0.26 to 0.62 ($P < 0.01$) (Supplementary Table S3). Treatment effects were highly significant ($P < 0.001$) for all directly measured traits and treatment–line interaction effects were significant ($P < 0.05$) for all traits except SL (Table 2). The significant treatment–line interactions for most traits are congruent with significant line variation for response to treatment

TABLE 1

Distribution of 161 B73 × CML228 recombinant inbred line means, P values of line F tests, and line mean heritabilities (h^2) within treatments or for response to treatments^a

Traits ^b	Mean	Standard deviation	Minimum	Maximum	Line P value	h^2
RLCK	9.946	1.196	6.250	11.967	<0.001	0.64
RWCK	0.184	0.039	0.093	0.316	<0.001	0.77
SLCK	6.338	0.762	4.250	8.280	<0.001	0.72
SWCK	0.203	0.038	0.098	0.352	<0.001	0.80
FWCK	0.387	0.067	0.229	0.668	<0.001	0.78
RLflg22	8.253	1.239	3.508	10.810	<0.001	0.54
RWflg22	0.152	0.039	0.064	0.292	<0.001	0.77
SLflg22	5.975	0.812	3.733	8.124	<0.001	0.75
SWflg22	0.183	0.038	0.099	0.352	<0.001	0.80
FWflg22	0.336	0.069	0.163	0.643	<0.001	0.79
RLSGI	0.152	0.136	–0.312	0.559	0.039	0.19
RWSGI	0.156	0.143	–0.452	0.549	0.019	0.25
SLSGI	0.054	0.061	–0.125	0.269	0.314	0.02
SWSGI	0.087	0.108	–0.381	0.451	0.010	0.24
FWSGI	0.124	0.108	–0.171	0.495	0.004	0.29
RL(CK – flg22)	1.693	1.235	–2.005	4.917	0.031	0.21
RW(CK – flg22)	0.031	0.024	–0.035	0.085	0.035	0.23
SL(CK – flg22)	0.363	0.387	–0.625	1.697	0.449	0.00
SW(CK – flg22)	0.019	0.020	–0.040	0.072	0.1091	0.14
FW(CK – flg22)	0.051	0.039	–0.051	0.141	0.150	0.26

^a Treatments = check (CK) or treatment (Flg22) and for response to treatments = CK – flg22 or scaled as percent of control (seedling growth inhibition [SGI]).

^b RL = root length; RW = root weight; SL = shoot length; SW = shoot weight; and FW = fresh weight.

FIGURE 4

Distribution of LSMEAN seedlings trait values under flg22 and H₂O (CK) conditions in the B73 × CML228 recombinant inbred line population. Traits are root length (RL), root weight (RW), shoot length (SL), shoot weight (SW), and fresh weight (FW). Mean = the population mean; 161 lines were evaluated.

observed for the same traits (Table 1). The significant correlations and line effects and relatively high heritabilities of the directly measured traits indicated that these traits were under substantial genetic control and that QTL analysis was appropriate.

TABLE 2

P values for treatment (i.e., flg22 treatment) and line main effects and treatment–line interaction for root length (RL), root weight (RW), shoot length (SL), shoot weight (SW), and fresh weight (FW)^a

Parameter	RL	RW	SL	SW	FW
Treatment	<0.001	<0.001	<0.001	<0.001	<0.001
Line	<0.001	<0.001	<0.001	<0.001	<0.001
Treatment × line	0.015	0.014	0.242	0.030	0.002

^a *P* values computed with Wald tests using data over all of the 161 B73 × CML228 recombinant inbred lines assessed.

We detected 33 QTL based on LSMEAN values of the measured and derived traits (Table 3). We divided the detected QTL into four types (Table 3). Type 1 were QTL pairs, where QTL for the same trait colocalized in both the control and the flg22 treatment conditions. We identified three pairs of type 1 QTL. Of these, two pairs (for FW and SW) colocalized on chromosome 1 and one for SL was on chromosome 8. Type 2 QTL were detected under control conditions but not under flg22 treatment. In all, 13 type 2 QTL were detected related to FW, RL, RW, SL, and SW on chromosomes 2, 3, 5, 6, and 9. Type 3 QTL were detected under flg22 treatment but not under the control conditions. Therefore, these QTL might be related to the flg22 response. Nine type 3 QTL for FW, RW, and SW were detected on chromosomes 2, 5, and 8. Type 4 QTL were for the derived traits which measured the response to flg22 (SGI and CK – flg22). Therefore, these QTL should be directly related to the flg22 MAMP response and were of the most interest. We

TABLE 3

Parameters of quantitative trait loci (QTL) associated with LSmeans of flg22 responses in the CML228 × B73 recombinant inbred line mapping population

Type ^a	Chr ^b	Trait ^c	Pos (cM) ^d	Physical position (bp) ^e	Support interval (cM) ^f	Physical interval (bp) ^g	LOD ^h	R ² (%) ⁱ	Add ^j
1	1	FWCK	64.8	49,568,510	62.8–67.6	47,204,119–52,878,657	4.92	10.43	0.022
1	1	FWflg22	61.6	45,588,501	57.4–63.6	39,638,275–48,421,942	4.42	8.86	0.021
1	1	SWCK	64.4	49,629,707	62.4–65.2	46,595,208–50,843,507	5.07	10.03	0.012
1	1	SWflg22	61.6	45,677,790	59.6–62.4	43,384,246–46,595,208	4.47	8.63	0.012
1	8	SLCK	77.6	150,035,371	75.2–80.8	146,993,200–154,091,599	5.12	10.16	–0.254
1	8	SLflg22	76	147,946,406	75.2–78.8	146,993,200–151,282,629	6.21	11.89	–0.295
2	2	RLCK	74	62,894,533	72.6–74.8	55,792,472–66,952,854	6.55	12.6	–0.43
2	5	SWCK	26.4	7,250,949	26–30	7,143,083–8,221,747	4.75	9.25	–0.012
2	3	FWCK	154.4	230,266,237	153.2–157.4	229,656,202–231,791,324	6.29	12.88	–0.025
2	3	SWCK	155.6	230,895,184	153.3–158.8	229,696,613–232,562,761	4.49	8.76	–0.012
2	5	SWCK	33.2	10,044,757	30–36.1	8,221,747–11,696,861	4.95	9.69	–0.012
2	6	RLCK	48.4	128,488,584	45.2–50.3	122,901,114–131,806,144	5.03	9.77	0.379
2	6	RWCK	20	94,032,377	16.4–23.6	90,429,704–97,635,051	3.94	7.78	0.011
2	9	RWCK	97.2	150,468,124	96.4–99	150,216,145–151,035,078	5.89	12.05	–0.014
2	9	FWCK	58.4	123,548,813	58–59.3	122,971,348–124,848,108	6.46	11.25	–0.025
2	9	FWCK	61.6	128,834,467	60.8–63.6	127,013,600–133,386,635	4.62	8.34	–0.021
2	9	RLCK	57.2	120,123,377	54.9–59.3	114,948,671–124,848,108	4.29	8.65	–0.353
2	9	RLCK	66	135,498,750	64.4–67.5	133,999,666–136,904,141	2.52	5.11	–0.317
2	9	SWCK	34.4	18,622,127	29.6–36.8	16,787,241–19,539,570	4.04	7.52	0.011
3	2	SWflg22	78.4	130,582,618	75.6–79.2	83,062,314–144,159,847	4.86	9.04	0.012
3	2	SWflg22	84.4	172,908,386	82.4–85.6	165,774,559–177,188,683	4.87	9.07	0.012
3	5	SWflg22	24.4	6,658,866	21.2–25.2	5,790,860–6,875,868	4.71	9.06	–0.012
3	8	FWflg22	76	147,997,030	74.8–79.6	146,574,796–152,263,734	5.79	11.94	–0.025
3	8	FWflg22	82	155,945,614	80–83.2	152,749,984–157,862,992	4.19	9.29	–0.022
3	8	RWflg22	76.8	148,948,481	74.8–79.8	146,574,796–152,509,010	6.21	13.12	–0.015
3	8	RWflg22	82	156,559,898	81.6–83.6	156,173,165–158,106,832	4.81	10.77	–0.013
3	8	SWflg22	73.6	144,818,905	71.4–74.8	141,599,772–146,574,796	4.66	9.07	–0.012
3	8	SWflg22	82.4	155,417,703	80–86.3	152,749,984–159,752,747	4.72	9.19	–0.012
4	1	RLSGI	125.2	214,872,289	122.8–127.2	211,997,482–217,267,962	4.05	9.09	–0.054
4	4	SLSGI	82	173,562,876	78.8–84.4	171,689,340–174,968,028	4.2	9.85	–0.024
4	8	FWSGI	70.8	139,670,921	69.6–71.2	135,567,392–141,038,764	4.53	10.23	0.036
4	8	RWSGI	70.8	139,818,165	70.1–71.2	137,682,117–141,038,764	5.26	12.79	0.053
4	8	RWSGI	76	147,893,942	75.6–78.2	147,411,605–150,546,801	4.05	10.03	0.047

^a Four types of QTL are noted: the type 1 are QTL detected under both control (CK) and flg22 conditions, type 2 are QTL identified under CK conditions, type 3 are QTL identified under flg22 conditions, and type 4 are QTL related to difference in the two conditions (seedling growth inhibition [SGI]).

^b Chromosome.

^c Traits are root length (RL), root weight (RW), shoot length (SL), shoot weight (SW), and fresh weight (FW) measured under CK and flg22 treatments or evaluated using the SGI index.

^d Genetic position of QTL peak.

^e Physical position of the QTL peak based on the B73 V4 genomic sequence.

^f Genetic positions defining the 2 logarithm of odds (LOD) support interval.

^g Physical positions defining the 2 LOD support interval.

^h LOD value at each QTL.

ⁱ R² (%) = percentage of phenotypic variance explained by QTL with additive effect.

^j Additive effect. For all traits, a positive value indicates alleles for increased trait values (weight or length for flg22 and CK, response to flg22 for SGI) are contributed by CML228, whereas a negative value indicates alleles for increased trait values (weight or length for flg22 and CK, response to flg22 for SGI) contributed by B73.

identified five type 4 QTL, all for SGI, on chromosomes 1, 4, and 8. Two of these, for FWSGI and RWSGI, colocalized on chromosome 8 with peaks at 70.8 cM and relatively high R^2 values of 10.2 and 12.8%, respectively. Another RWSGI QTL was located close by on chromosome 8, with a peak at 76 cM. This region is of particular interest because six type 3 QTL also mapped to this region from 73.6 to 82 cM. In each of these six cases, the QTL allele derived from CML228 contributed to decreased growth, or increased growth retardation, in response to flg22.

Correlation between flg22 responses and other disease and defense-related traits

Previous studies used the CML228 × B73 population to evaluate a number of traits related to disease resistance and the defense response. These include resistance to the fungal diseases SLB, GLS, and NLB, and traits related to the defense response such as HR severity, leaf flecking (light spotting in the leaf), and, most relevant to this study, ROS production induced by flg22 and c8 (Benson et al. 2015; Olukolu et al. 2016, 2014; Poland et al. 2011; Zhang et al. 2017). We analyzed the correlation of these traits with the LSMEAN values for the flg22 response traits from this study (Table 4). Of particular interest is the lack of correlation between the flg22 MAMP response traits measured in this study (SGI and CK – flg22) and the previously measured flg22 and c8-induced ROS production traits. The exceptions to this were weak correlations between RL SGI and RWSGI and RL(CK – flg22) and RW(CK – flg22) with flg22-induced ROS production (–0.18, –0.19, –0.22, and –0.19 respectively). The negative correlations indicated that a higher ROS response was weakly associated with a lower SGR response in these cases. The other noteworthy correlations were significant, though weak to moderate, correlations between most SGI and CK – flg22 traits and NLB resistance (–0.17 to –0.31). In these cases, a stronger SGR response was associated with higher NLB resistance.

DISCUSSION

The ultimate goal of this study was to develop a robust and simple way of measuring the basal defense response that might give breeders a means of identifying, at the seedling stage, germplasm with robust levels of disease resistance in the field. The signaling and mechanistic bases of the responses elicited by MAMPs derived from fungi, bacteria, or other classes of microbe are highly integrated and often converge (Saijo et al. 2018). For example, the BAK1 coreceptor interacts with receptors for bacterial-, fungal-, and oomycete-derived molecules as well as with DAMP receptors (Yasuda et al. 2017). In our previous study, we had noted that the strength of the ROS response elicited by the bacterial MAMP flg22 was highly correlated with the strength of the response elicited by the fungal MAMP chitin (Zhang et al. 2017). Therefore, we reasoned that this SGR assay might be a cheap and robust screen for resistance against a wide range of pathogens.

We noted variation among 21 lines for our flg22-mediated SGR assay. As discussed above, variation in the MAMP-induced defense response has been noted in a number of other species, including *B. napus*, soybean, *Arabidopsis*, and tomato. We have also noted such variation in our previous work on maize (Zhang et al. 2017) and sorghum (Kimball et al. 2019) using an assay measuring MAMP-induced ROS production (Samira et al. 2019). Unlike the ROS assay in which the MAMP is applied to the leaves, the SGR assay allowed us to identify responses in both directions (e.g., growth enhancement responses as well as growth retardation). We were surprised to note that 7 of 21 lines (Tzi8, M162W, CML69, CML333, MS71, IL14H, and CML52) showed primarily enhanced growth responses, almost as many as showed significant growth retardation (Fig. 1; Supplementary Table S1). Of the seven lines, four are tropical (Tzi8, CML69, CML333, and CML52); two are temperate, nonstiff stalk (MS71 and M162W); and one is a sweetcorn (IL14H). When the same lines were assessed using the ROS assay, three (CML69,

TABLE 4

Pearson correlation coefficients between seedling growth retardation traits induced by flg22 and hypersensitive response, disease resistance, genetic flecking, and reactive oxygen species (ROS) production induced by flg22 LSmean, and chitoctaoase (c8) traits measured in the same CML228 × B73 recombinant inbred line population^a

Trait ^b	HTR ($n = 155$) ^c	SLB ($n = 160$) ^d	GLS ($n = 120$) ^e	NLB ($n = 129$) ^f	Flecking ($n = 160$) ^g	flg22 ROS ($n = 158$) ^h	c8 ROS ($n = 154$) ⁱ
RLSGI	NS	NS	NS	–0.228**	NS	–0.184*	NS
RWSGI	NS	NS	NS	–0.302**	NS	–0.189*	NS
SLSGI	–0.158*	NS	NS	–0.174*	NS	NS	NS
SWSGI	–0.160*	NS	–0.186*	–0.252**	NS	NS	NS
FWSGI	–0.179*	NS	NS	–0.308**	NS	NS	NS
RL(CK – flg22)	–0.170*	NS	NS	–0.222*	NS	–0.220**	NS
RW(CK – flg22)	–0.159*	NS	NS	–0.279**	NS	–0.190*	NS
SL(CK – flg22)	NS	NS	NS	NS	NS	NS	NS
SW(CK – flg22)	NS	NS	–0.189*	–0.208*	NS	NS	NS
FW(CK – flg22)	–0.164*	NS	NS	–0.283**	NS	NS	NS

^a The number of comparisons (n) used to calculate the coefficient is noted in each case. Asterisks ** and * indicate significant at $P < 0.01$ and 0.05 , respectively, and NS = not significant. Traits were measured in the following ways: flecks = 0 (no flecking) to 10 (severe); hypersensitive response (HR) = 0 (severe) to 1 (mild); southern leaf blight (SLB) = 0 (resistant) to 9 (susceptible); northern leaf blight (NLB) = 0% (resistant) to 100% (susceptible); and gray leaf spot (GLS) = 0% (resistant) to 100% (susceptible). ROS production was measured in relative light units.

^b Traits are root length (RL), root weight (RW), shoot length (SL), shoot weight (SW), and fresh weight (FW) measured under control (CK) and flg22 treatments or evaluated using the seedling growth inhibition (SGI) index.

^c Height ratio trait (HTR), which is related to the strength of the HR. Data from Olukolu et al. (2014).

^d SLB resistance. Data from Kump et al. (2011).

^e GLS resistance. Data from Benson et al. (2015).

^f NLB resistance. Data from Poland et al. (2011).

^g Genetically conferred leaf flecking. Data from Olukolu et al. (2016).

^h LSmeans of the ROS response induced by flg22. Data from Zhang et al. (2017).

ⁱ LSmeans of the ROS response induced by c8. Data from Zhang et al. (2017).

M162W, and IL14H) demonstrated a relatively high response to flg22 (i.e., relatively high ROS production) while the others showed a low response (Zhang et al. 2017). We are not aware of any other examples of the defense response being associated with a growth enhancement, though few experiments of this type have been reported. It may be that lines that are insensitive to flg22 can use flg22 simply as a source of nutrition and, thus, may experience a moderate enhancement of growth if flg22 is present (Soper et al. 2011). This may be the explanation at least for the lines that showed very little response as measured by the ROS assay. It is possible that these lines are completely unable to recognize flg22 as an MAMP. Among 37 *Arabidopsis* genotypes assessed, all showed a growth retardation in a similar assay (Vetter et al. 2012).

We should also note that a number of lines showed a mixed response to flg22, with the SGI of some traits showing inhibition and some enhancement (Fig. 1). In these cases, most often RL was the outlying trait. For example, IL14H, MS71, B97, M162W, and CML69 all showed enhanced growth for all traits except RL. It is not clear why RL should give patterns of responses different than those of the other traits in some lines. For B73 and CML228, the two parents of the RIL population used in this study, the responses were fairly consistent across all of the traits.

The lack of correlation between the response to flg22 measured by the SGR and ROS assays is also of note (Table 4). In fact, where weak correlations were noted, they were in the opposite direction to what was expected. We had previously noted an apparent lack of correlation between PTI responses when measured by several different methods (ROS assay, NO production, and expression of four different genes) but these data were based on the analysis of only a handful of lines (Zhang et al. 2017). The data presented here, comparing more than 150 lines from the CML228 × B73 RIL population, confirms that the apparent strength of PTI may, in part, be a function of how the response is quantified.

We measured five traits in seedlings grown in flg22 solution or in water as a control; RL, RW, SL, SW, and total FW. Although these traits were generally correlated (Supplementary Table S4), they did show somewhat different patterns; therefore, all five traits were used for QTL analysis. Type 1 and type 2 QTL are effectively simply QTL for seedling growth. From that perspective, it is of interest to note that the RLCK QTL on chromosome 2 (74 cM) and chromosome 6 (20 cM) map to the same regions as consensus root length QTL identified in a meta-analysis by Hund et al. (2011). It appears that this approach may be useful for quantifying variation in early seedling growth characteristics.

Of more interest for our purposes are the type 3 and type 4 QTL. Type 3 QTL were identified in flg22 but not in control conditions, whereas type 4 were identified for the derived flg22 response trait SGI. Therefore, both of these types may be associated with the response to flg22. The most prominent genomic region identified by these classes of QTL was on chromosome 8 between 70.8 and 82.4 cM, encompassing six type 3 and three type 4 QTL. In this region, the CML228 allele conferred reduced growth in flg22 conditions and, therefore, increased flg22 response. Because this region is of the most interest to us, we investigated whether QTL for other related traits mapped there. GLS and SLB resistance QTL have been reported in this region in the full NAM population (Benson et al. 2015; Kump et al. 2011); however, in each case, the CML228 QTL allele was not found to confer a large effect relative to the B73 QTL allele. This corresponds with the lack of correlation between the SGR and disease phenotypes noted in Table 4. A type 3 SWflg22 QTL at 84.4 cM on chromosome 2 colocalized with the major QTL for

both chitin- and flg22-induced ROS production identified in our previous study (Zhang et al. 2017).

We developed the SGR assay as a way to measure the general MAMP response, reasoning that growth traits integrate the results of many of the more specific responses such as gene expression, ROS and NO production, and so on and, thus, may be a better metric to use to measure PTI. We were able to identify heritable variation in flg22-induced SGR and identify QTL associated with this variation but they seemed unrelated to the variation and QTL we had previously detected for MAMP-mediated ROS production in the same population. Furthermore, we did not detect a strong relationship between variation in this trait and other various disease-resistance and defense-response traits we had previously analyzed. Lack of correlation between PTI traits has been noted previously by us and others in a number of systems, including tomato, *Arabidopsis*, maize, and sorghum (Kimball et al. 2019; Lloyd et al. 2017; Roberts et al. 2019; Samira et al. 2020; Veluchamy et al. 2014; Vetter et al. 2016; Zhang et al. 2017). Because general PTI responses are often effective against fungal pathogens (Irieda et al. 2019), we had hoped to see some correlation; however, it may be that it simply is not possible to extrapolate results of a simple laboratory assay to the much more complex realities of plant-pathogen interactions in the field. For example, many resistance mechanisms likely have nothing to do with the recognition of or response to MAMPs and, in the cases where they do, MAMPs are likely presented to the plant in a much more localized fashion and at significantly lower concentrations. It is possible that SGR responses to different concentrations of flg22 or to different MAMPs might better correlate with resistance traits.

Although our ultimate goal to develop a robust and simple way of identifying, at the seedling stage, germplasm with robust levels of multiple disease resistance in the field was not achieved, we nevertheless developed an SGR assay and identified several genomic regions associated with variation in PTI response traits and with seedling growth. These data will inform future efforts toward the ultimate goal.

ACKNOWLEDGMENTS

We thank S. Sermons for technical assistance and J. Bergelson, R. Samira, and C. Zipfel for helpful discussion.

LITERATURE CITED

- Albrecht, T., and Argueso, C. T. 2017. Should I fight or should I grow now? The role of cytokinins in plant growth and immunity and in the growth-defence trade-off. *Ann. Bot.* 119:725-735.
- Bates, D., Mächler, M., Bolker, B. M., and Walker, S. C. 2015. Fitting linear mixed-effects models using lme4. *J. Stat. Softw.* 67:1-48.
- Benson, J. M., Poland, J. A., Benson, B. M., Stromberg, E. L., and Nelson, R. J. 2015. Resistance to gray leaf spot of maize: Genetic architecture and mechanisms elucidated through nested association mapping and near-isogenic line analysis. *PLoS Genet.* 11:e1005045.
- Bent, A. F., and Mackey, D. 2007. Elicitors, effectors, and *R* genes: The new paradigm and a lifetime supply of questions. *Annu. Rev. Phytopathol.* 45:399-436.
- Bhattarai, K., Louws, F. J., Williamson, J. D., and Panthee, D. R. 2016. Differential response of tomato genotypes to *Xanthomonas*-specific pathogen-associated molecular patterns and correlation with bacterial spot (*Xanthomonas perforans*) resistance. *Hortic. Res.* 3:16035.
- Büschges, R., Hollricher, K., Panstruga, R., Simons, G., Wolter, M., Frijters, A., van Daelen, R., van der Lee, T., Diergaarde, P., Groenendijk, J., Töpsch, S., Vos, P., Salamini, F., and Schulze-Lefert, P. 1997. The barley *Mlo* gene: A novel control element of plant pathogen resistance. *Cell* 88:695-705.
- Clarke, C. R., Chinchilla, D., Hind, S. R., Taguchi, F., Miki, R., Ichinose, Y., Martin, G. B., Leman, S., Felix, G., and Vinatzer, B. A. 2013. Allelic

- variation in two distinct *Pseudomonas syringae* flagellin epitopes modulates the strength of plant immune responses but not bacterial motility. *New Phytol.* 200:847-860.
- Cole, S. J., and Diener, A. C. 2013. Diversity in receptor-like kinase genes is a major determinant of quantitative resistance to *Fusarium oxysporum* f. sp. *matthioli*. *New Phytol.* 200:172-184.
- Cook, D. E., Mesarich, C. H., and Thomma, B. P. H. J. 2015. Understanding plant immunity as a surveillance system to detect invasion. *Annu. Rev. Phytopathol.* 53:541-563.
- Cowger, C., and Brown, J. K. M. 2019. Durability of quantitative resistance in crops: Greater than we know? *Annu. Rev. Phytopathol.* 57:253-277.
- Felix, G., Duran, J. D., Volko, S., and Boller, T. 1999. Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. *Plant J.* 18:265-276.
- Forsyth, A., Mansfield, J. W., Grabov, N., de Torres, M., Sinapidou, E., and Grant, M. R. 2010. Genetic dissection of basal resistance to *Pseudomonas syringae* pv. *phaseolicola* in accessions of *Arabidopsis*. *Mol. Plant-Microbe Interact.* 23:1545-1552.
- Furch, A. C. U., Zimmermann, M. R., Kogel, K. H., Reichelt, M., and Mithöfer, A. 2014. Direct and individual analysis of stress-related phytohormone dispersion in the vascular system of *Cucurbita maxima* after flagellin 22 treatment. *New Phytol.* 201:1176-1182.
- Guo, Q., Major, I. T., and Howe, G. A. 2018. Resolution of growth-defense conflict: Mechanistic insights from jasmonate signaling. *Curr. Opin. Plant Biol.* 44:72-81.
- Halekoh, U., and Højsgaard, S. 2014. A Kenward-Roger approximation and parametric bootstrap methods for tests in linear mixed models-the R package pbrtest. *J. Stat. Softw.* 59:1-32.
- Hamdoun, S., Liu, Z., Gill, M., Yao, N., and Lu, H. 2013. Dynamics of defense responses and cell fate change during *Arabidopsis-Pseudomonas syringae* interactions. *PLoS One* 8:e83219.
- Hund, A., Reimer, R., and Messmer, R. 2011. A consensus map of QTLs controlling the root length of maize. *Plant Soil* 344:143-158.
- Huot, B., Yao, J., Montgomery, B. L., and He, S. Y. 2014. Growth-defense tradeoffs in plants: A balancing act to optimize fitness. *Mol. Plant* 7: 1267-1287.
- Hurni, S., Scheuermann, D., Krattinger, S. G., Kessel, B., Wicker, T., Herren, G., Fitze, M. N., Breen, J., Prestler, T., Ouzunova, M., and Keller, B. 2015. The maize disease resistance gene *Hm1* against northern corn leaf blight encodes a wall-associated receptor-like kinase. *Proc. Natl. Acad. Sci. U.S.A.* 112:8780-8785.
- Irieda, H., Inoue, Y., Mori, M., Yamada, K., Oshikawa, Y., Saitoh, H., Uemura, A., Terauchi, R., Kitakura, S., Kosaka, A., Singkaravanit-Ogawa, S., and Takano, Y. 2019. Conserved fungal effector suppresses PAMP-triggered immunity by targeting plant immune kinases. *Proc. Natl. Acad. Sci. U.S.A.* 116:496-505.
- Jabs, T., Tschöpe, M., Colling, C., Hahlbrock, K., and Scheel, D. 1997. Elicitor-stimulated ion fluxes and O₂ from the oxidative burst are essential components in triggering defense gene activation and phytoalexin synthesis in parsley. *Proc. Natl. Acad. Sci. U.S.A.* 94:4800-4805.
- Jacobs, S., Zechmann, B., Molitor, A., Trujillo, M., Petutschig, E., Likpa, V., Kogel, K.-H., and Schäfer, P. 2011. Broad-spectrum suppression of innate immunity is required for colonization of *Arabidopsis* roots by the fungus *Piriformospora indica*. *Plant Physiol.* 156:726-740.
- Kaku, H., Nishizawa, Y., Ishii-Minami, N., Akimoto-Tomiya, C., Dohmae, N., Takio, K., Minami, E., and Shibuya, N. 2006. Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor. *Proc. Natl. Acad. Sci. U.S.A.* 103:11086-11091.
- Kimball, J., Cui, Y., Chen, D., Brown, P., Rooney, W., Stacey, G., and Balint-Kurti, P. J. 2019. Identification of QTL for target leaf spot resistance in *Sorghum bicolor* and investigation of relationships between disease resistance and variation in the MAMP response. *Sci. Rep.* 9:18285.
- Kohorn, B. D., Johansen, S., Shishido, A., Todorova, T., Martinez, R., Defeo, E., and Obregon, P. 2009. Pectin activation of MAP kinase and gene expression is WAK2 dependent. *Plant J.* 60:974-982.
- Kohorn, B. D., and Kohorn, S. L. 2012. The cell wall-associated kinases, WAKs, as pectin receptors. *Front. Plant Sci.* 3:88.
- Kou, Y., and Wang, S. 2010. Broad-spectrum and durability: Understanding of quantitative disease resistance. *Curr. Opin. Plant Biol.* 13:181-185.
- Kump, K. L., Bradbury, P. J., Wissner, R. J., Buckler, E. S., Belcher, A. R., Oropeza-Rosas, M. A., Zwonitzer, J. C., Kresovich, S., McMullen, M. K., Ware, D., Balint-Kurti, P. J., and Holland, J. B. 2011. Genome-wide association study of quantitative resistance to southern leaf blight in the maize nested association mapping population. *Nat. Genet.* 43:163-168.
- Lee, S., Whitaker, V. M., and Hutton, S. F. 2016. Mini review: Potential applications of non-host resistance for crop improvement. *Front. Plant Sci.* 7:997.
- Li, B., Meng, X., Shan, L., and He, P. 2016. Transcriptional regulation of pattern-triggered immunity in plants. *Cell Host Microbe* 19:641-650.
- Li, X., Lin, H., Zhang, W., Zou, Y., Zhang, J., Tang, X., and Zhou, J.-M. 2005. Flagellin induces innate immunity in nonhost interactions that is suppressed by *Pseudomonas syringae* effectors. *Proc. Natl. Acad. Sci. U.S.A.* 102:12990-12995.
- Liu, B., Li, J.-F., Ao, Y., Qu, J., Li, Z., Su, J., Zhang, Y., Liu, J., Feng, D., Qi, K., He, Y., Wang, J., and Wang, H.-B. 2012. Lysin motif-containing proteins LYP4 and LYP6 play dual roles in peptidoglycan and chitin perception in rice innate immunity. *Plant Cell* 24:3406-3419.
- Lloyd, S. R., Ridout, C. J., and Schoonbeek, H. 2017. Methods to quantify PAMP-triggered oxidative burst, MAP kinase phosphorylation, gene expression, and lignification in Brassicas. Pages 325-335 in: *Plant Pattern Recognition Receptors*. L. Shan and P. He, eds. *Methods in Molecular Biology*, Vol. 1578. Humana Press, New York, NY, U.S.A. doi:10.1007/978-1-4939-6859-6_28
- Lloyd, S. R., Schoonbeek, H., Trick, M., Zipfel, C., and Ridout, C. J. 2014. Methods to study PAMP-triggered immunity in *Brassica* species. *Mol. Plant-Microbe Interact.* 27:286-295.
- Luna, E., Pastor, V., Robert, J., Flors, V., Mauch-Mani, B., and Ton, J. 2011. Callose deposition: A multifaceted plant defense response. *Mol. Plant-Microbe Interact.* 24:183-193.
- McMullen, M. D., Kresovich, S., Villeda, H. S., Bradbury, P., Li, H., Sun, Q., Flint-Garcia, S., Thornsberry, J., Acharya, C., Bottoms, C., Brown, P., Browne, C., Eller, M., Guill, K., Harjes, C., Kroon, D., Lepak, N., Mitchell, S. E., Peterson, B., Pressoir, G., Romero, S., Rosas, M. O., Salvo, S., Yates, H., Hanson, M., Jones, E., Smith, S., Glaubitz, J. C., Goodman, M., Ware, D., Holland, J. B., and Buckler, E. S. 2009. Genetic properties of the maize nested association mapping population. *Science* 325:737-740.
- Meteignier, L.-V., El-Oirdi, M., Cohen, M., Barff, T., Matteau, D., Lucier, J.-F., Rodrigue, S., Jacque, P.-E., Yoshioka, K., and Moffett, P. 2017. Translatome analysis of an NB-LRR immune response identifies important contributors to plant immunity in *Arabidopsis*. *J. Exp. Bot.* 68:2333-2344.
- Mithöfer, A., Ebel, J., and Felle, H. H. 2005. Cation fluxes cause plasma membrane depolarization involved in β -glucan elicitor-signaling in soybean roots. *Mol. Plant-Microbe Interact.* 18:983-990.
- Monaghan, J., and Zipfel, C. 2012. Plant pattern recognition receptor complexes at the plasma membrane. *Curr. Opin. Plant Biol.* 15:349-357.
- Morales, M., and Munné-Bosch, S. 2016. Oxidative stress: A master regulator of plant trade-offs? *Trends Plant Sci.* 21:996-999.
- Mott, G. A., Desveaux, D., and Guttman, D. S. 2018. A high-sensitivity, microtiter-based plate assay for plant pattern-triggered immunity. *Mol. Plant-Microbe Interact.* 31:499-504.
- Naseem, M., Kaldorf, M., and Dandekar, T. 2015. The nexus between growth and defence signalling: Auxin and cytokinin modulate plant immune response pathways. *J. Exp. Bot.* 66:4885-4896.
- Nelson, R., Wiesner-Hanks, T., Wissner, R., and Balint-Kurti, P. 2017. Navigating complexity to breed disease-resistant crops. *Nat. Rev. Genet.* 19:21-33.
- Newman, M.-A., Sundelin, T., Nielsen, J. T., and Erbs, G. 2013. MAMP (microbe-associated molecular pattern) triggered immunity in plants. *Front. Plant Sci.* 4:139.
- Nguyen, H. P., Chakravarthy, S., Velásquez, A. C., McLane, H. L., Zeng, L., Nakayashiki, H., Park, D.-H., Collmer, A., and Martin, G. B. 2010. Methods to study PAMP-triggered immunity using tomato and *Nicotiana benthamiana*. *Mol. Plant-Microbe Interact.* 23:991-999.
- Niks, R. E., Qi, X., and Marcel, T. C. 2015. Quantitative resistance to biotrophic filamentous plant pathogens: Concepts, misconceptions, and mechanisms. *Annu. Rev. Phytopathol.* 53:445-470.
- Olukolu, B. A., Bian, Y., De Vries, B., Tracy, W. F., Wissner, R. J., Holland, J. B., and Balint-Kurti, P. J. 2016. The genetics of leaf flecking in maize and its relationship to plant defense and disease resistance. *Plant Physiol.* 172:1787-1803.
- Olukolu, B. A., Wang, G.-F., Vontimitta, V., Venkata, B. P., Marla, S., Ji, J., Gachomo, E., Chu, K., Negeri, A., Benson, J., Nelson, R., Bradbury, P., Nielsen, D., Holland, J. B., Balint-Kurti, P. J., and Johal, G. 2014. A genome-wide association study of the maize hypersensitive defense response identifies genes that cluster in related pathways. *PLoS Genet.* 10: e1004562.
- Parlevliet, J.-E. 2002. Durability of resistance against fungal, bacterial and viral pathogens; present situation. *Euphytica* 124:147-156.
- Poland, J. A., Bradbury, P. J., Buckler, E. S., and Nelson, R. J. 2011. Genome-wide nested association mapping of quantitative resistance to northern leaf blight in maize. *Proc. Natl. Acad. Sci. U.S.A.* 108:6893-6898.
- Poland, J. A., Balint-Kurti, P. J., Wissner, R. J., Pratt, R. C., and Nelson, R. J. 2009. Shades of gray: The world of quantitative disease resistance. *Trends Plant Sci.* 14:21-29.
- Rasul, S., Dubreuil-Maurizi, C., Lamotte, O., Koen, E., Poinssot, B., Alcaraz, G., Wendehenne, D., and Jeandroz, S. 2012. Nitric oxide production mediates oligogalacturonide-triggered immunity and resistance to *Botrytis cinerea* in *Arabidopsis thaliana*. *Plant Cell Environ.* 35:1483-1499.

- R Core Team. 2009. R: A Language and Environment for Statistical Computing. <http://www.r-project.org>
- Roberts, R., Mainiero, S., Powell, A. F., Liu, A. E., Shi, K., Hind, S. R., Strickler, S. R., Collmer, A., and Martin, G. B. 2019. Natural variation for unusual host responses and flagellin-mediated immunity against *Pseudomonas syringae* in genetically diverse tomato accessions. *New Phytol.* 223:447-461.
- Saijo, Y., Loo, E. P., and Yasuda, S. 2018. Pattern recognition receptors and signaling in plant-microbe interactions. *Plant J.* 93:592-613.
- Samira, R., Kimball, J., Samayoa, L. F., Holland, J. B., Jamann, T. M., Brown, P. J., Stacey, G., and Balint-Kurti, P. J. 2020. Genome-wide association analysis of the strength of the MAMP-elicited defense response and resistance to target leaf spot in sorghum. *Sci. Rep.* 10:20817.
- Samira, R., Zhang, X., Kimball, J., Cui, Y., Stacey, G., and Balint-Kurti, P. 2019. Quantifying MAMP-induced production of reactive oxygen species in sorghum and maize. *Bio-Protoc.* 9:e3304.
- 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.
- Shimizu, T., Nakano, T., Takamizawa, D., Desaki, Y., Ishii-Minami, N., Nishizawa, Y., Minami, E., Okada, K., Yamane, H., Kaku, H., and Shibuya, N. 2010. Two LysM receptor molecules, CEBIP and OsCERK1, cooperatively regulate chitin elicitor signaling in rice. *Plant J.* 64:204-214.
- Soper, F. M., Paungfoo-Lonhienne, C., Brackin, R., Rentsch, D., Schmidt, S., and Robinson, N. 2011. *Arabidopsis* and *Lobelia anceps* access small peptides as a nitrogen source for growth. *Funct. Plant Biol.* 38:788-796.
- Sun, W., Dunning, F. M., Pfund, C., Weingarten, R., and Bent, A. F. 2006. Within-species flagellin polymorphism in *Xanthomonas campestris* pv. *campestris* and its impact on elicitation of *Arabidopsis* *FLAGELLIN SENSING2*-dependent defenses. *Plant Cell* 18:764-779.
- Takai, R., Isogai, A., Takayama, S., and Che, F.-S. S. 2008. Analysis of flagellin perception mediated by flg22 receptor OsFLS2 in rice. *Mol. Plant-Microbe Interact.* 21:1635-1642.
- Thomma, B. P. H. J., Nürnberger, T., and Joosten, M. H. A. J. 2011. Of PAMPs and effectors: The blurred PTI-ETI dichotomy. *Plant Cell* 23:4-15.
- Trdá, L., Fernandez, O., Boutrot, F., Héloir, M.-C. M., Kelloniemi, J., Daire, X., Adrian, M., Clément, C., Zipfel, C., Dorey, S., and Poinssot, B. 2014. The grapevine flagellin receptor VvFLS2 differentially recognizes flagellin-derived epitopes from the endophytic growth-promoting bacterium *Burkholderia phytofirmans* and plant pathogenic bacteria. *New Phytol.* 201:1371-1384.
- Tuleski, T. R., Kimball, J., do Amaral, F. P., Pereira, T. P., Tadra-Sfeir, M. Z., Pedrosa, F. de O., de Souza, E. M., Balint-Kurti, P., Monteiro, R. A., and Stacey, G. 2019. *Herbaspirillum rubrisubalbicans* as a phytopathogenic model to study the immune system of Sorghum bicolor. *Mol. Plant-Microbe Interact.* 33:235-246.
- Valdés-López, O., Khan, S. M., Schmitz, R. J., Cui, S., Qiu, J., Joshi, T., Xu, D., Diers, B., Ecker, J. R., and Stacey, G. 2014. Genotypic variation of gene expression during the soybean innate immunity response. *Plant Genet. Resour.* 12:S27-S30.
- Valdés-López, O., Thibivilliers, S., Qiu, J., Xu, W. W., Nguyen, T. H. N., Libault, M., Le, B. H., Goldberg, R. B., Hill, C. B., Hartman, G. L., Biers, B., and Stacey, G. 2011. Identification of quantitative trait loci controlling gene expression during the innate immunity response of soybean. *Plant Physiol.* 157:1975-1986.
- Veluchamy, S., Hind, S. R., Dunham, D. M., Martin, G. B., and Panthee, D. R. 2014. Natural Variation for responsiveness to flg22, flgII-28, and csp22 and *Pseudomonas syringae* pv. *tomato* in heirloom tomatoes. *PLoS One* 9:e106119.
- Vetter, M., Karasov, T. L., and Bergelson, J. 2016. Differentiation between Vetter, M. M., Kronholm, I., He, F., Häweker, H., Reymond, M., Bergelson, J., Robatzek, S., and de Meaux, J. 2012. Flagellin perception varies quantitatively in *Arabidopsis thaliana* and its relatives. *Mol. Biol. Evol.* 29:1655-1667.
- Wan, J., Zhang, X.-C., Neece, D., Ramonell, K. M., Clough, S., Kim, S.-y., Stacey, M. G., and Stacey, G. 2008. A LysM receptor-like kinase plays a critical role in chitin signaling and fungal resistance in *Arabidopsis*. *Plant Cell* 20:471-481.
- Wang, S., Sun, Z., Wang, H., Liu, L., Lu, F., Yang, J., Zhang, M., Zhang, S., Guo, Z., Bent, A. F., and Sun, W. 2015. Rice OsFLS2-mediated perception of bacterial flagellins is evaded by *Xanthomonas oryzae* pvs. *oryzae* and *oryzicola*. *Mol. Plant* 8:1024-1037.
- Yasuda, S., Okada, K., and Saijo, Y. 2017. A look at plant immunity through the window of the multitasking coreceptor BAK1. *Curr. Opin. Plant Biol.* 38:10-18.
- Zeidler, D., Zähringer, U., Gerber, I., Dubery, I., Hartung, T., Bors, W., Hutzler, P., and Durner, J. 2004. Innate immunity in *Arabidopsis thaliana*: Lipopolysaccharides activate nitric oxide synthase (NOS) and induce defense genes. *Proc. Natl. Acad. Sci. U.S.A.* 101:15811-15816.
- Zhang, N., Pombo, M. A., Rosli, H. G., and Martin, G. B. 2020. Tomato wall-associated kinase SIWak1 depends on Fls2/Fls3 to promote apoplastic immune responses to *Pseudomonas syringae*. *Plant Physiol.* 183:1869-1882.
- Zhang, X., Valdés-López, O., Arellano, C., Stacey, G., and Balint-Kurti, P. 2017. Genetic dissection of the maize (*Zea mays* L.) MAMP response. *Theor. Appl. Genet.* 130:1155-1168.
- 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.
- Zuo, W., Chao, Q., Zhang, N., Ye, J., Tan, G., Li, B. I., B., Xing, Y., Zhang, B., Liu, H., Fengler, K. A., Zhao, J., Zhao, X., Chen, Y., Lai, J., Yan, J., and Xu, M. 2015. A maize wall-associated kinase confers quantitative resistance to head smut. *Nat. Genet.* 47:151-157.