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# The impact of pRAP vectors on plant genetic transformation and pathogenesis studies including an analysis of *BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1)*-mediated resistance

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## ABSTRACT

Crop improvement can be facilitated through efficient gene transfer, leading to pRAP plasmid development. Comparative hairy root transformation results from 24 previously published articles examining 29,756 roots show a 70% transformation efficiency. Average gene overexpression was 11.24-fold and –3.84-fold in RNAi roots. New studies show *Glycine max BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1)* overexpression leads to a 67% decrease in *Heterodera glycines* parasitism while *BAK1-1 RNAi* led to a 4.8-fold increase in parasitism. The results show pathogen associated molecular pattern triggered immunity (PTI) functions in the *G. max-H. glycines* pathosystem during defense. Consequently, the pRAP vectors have applicability for studying basic biology and defense in other agricultural plants including *Manihot esculenta* (cassava), *Zea mays* (maize), *Oryza sativa* (rice), *Triticum aestivum* (wheat), *Sorghum bicolor* (sorghum), *Brassica rapa* (rape seed), *Solanum tuberosum* (potato), *Solanum lycopersicum* (tomato), *Elaeis guineensis* (oil palm), *Saccharum officinalis* (sugarcane) and *Beta vulgaris* (sugar beet) since each have BAK1 homologs.

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## Introduction

For decades, genes have been studied through mutant analyses, allowing for an understanding of their function. The ability to examine the function of those genes, more recently, has been facilitated through molecular approaches that allow for the manipulation of their expression so it occurs in ways that normally may not happen. The altered expression includes overexpression/heterologous expression/ectopic expression or the suppression of expression through procedures that resemble a hypomorphic null mutant (RNA interference [RNAi]) (Jefferson et al. 1987; Wang et al. 1988; Fire et al. 1998; Klink and Wolniak 2000). These procedures each have their value. However, being able to accomplish the sought-after expression in the biological system under study can be met with challenges due to the specific model (De Saeger et al. 2020).

For molecular genetic analyses of plants, typically, the most simplistic means of gene transfer is desired. Early methods of genetic transformation used in plant studies included various forms of biolistic propulsion, lipotransfection or agents including *Agrobacterium tumefaciens* or its root-inducing relative *A. rhizogenes* due to their natural ability to shuttle genes horizontally through their plasmid DNA (Chilton et al. 1977; Otten et al. 1981; Zambryski et al. 1983; Tepfer 1984). Each method has its advantages and disadvantages. However, plant transformation procedures involving plasmids may only function under specific conditions or in a certain range of host tissue. This problem forces the development or re-engineering of plasmids to allow a greater range of

genetic manipulation than originally sought. The availability of such plasmids can be of great importance to the genetic analysis of dicot and monocot crops of significant worldwide cultivation and those important to U.S. agriculture (Karimi et al. 2002; Tilman et al. 2011; Ray et al. 2019). These plants include, but are not limited to *G. max*, *G. hirsutum*, *M. esculenta*, *Z. mays*, *O. sativa*, *T. aestivum*, *S. bicolor*, *B. rapa*, *S. tuberosum*, *S. lycopersicum*, *E. guineensis*, *S. officinalis* and *B. vulgaris* (Tilman et al. 2011; Ray et al. 2019).

The study presented here provides the DNA sequences and vector maps for the pRAP15 overexpression/heterologous expression/ectopic expression and pRAP17 RNAi plasmids. The pRAP15 plasmid has been developed to facilitate the expression/heterologous expression/ectopic expression of plant genes, while also being used to express non-plant genes (Matsye et al. 2012). The pRAP17 plasmid has been developed for RNAi of host or pathogen genes, differing from previous studies (Klink et al. 2009; Matsye et al. 2011; Ranjan et al. 2011). The expression of pathogen genes, in particular those derived from parasitic nematodes, were thought to function as ingested RNA and would be able to activate an RNAi effect to decrease its cognate RNA abundance in the pathogen (Timmons and Fire 1998; Urwin et al. 2002). The DNA sequence information and genetic maps should be helpful in the design or creation of plasmids with similar features for use in the desired specific application. Data from published experiments spanning over 10 years of research performed in different lab settings and previously unpublished data is analyzed and summarized to

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highlight the utility of these plasmids for other crop species when requiring specific types of analysis that are not currently tractable or easy to perform. These studies serve as a backdrop to a new analysis of a *G. max* homolog of the *Arabidopsis thaliana* membrane pathogen recognition receptor (PRR) co-receptor *BRI1-ASSOCIATED RECEPTOR KINASE 1* (*BAK1*) homolog that in *A. thaliana* functions in pathogen associated molecular pattern triggered immunity (PTI) (Jones and Dangl 2006). The new study provides further insight on the use of the pRAP15 and pRAP17 plasmids examining the *G. max* PTI homolog *BAK1-1*. In combination with prior observations made for the effector triggered immunity (ETI) gene *NONRACE-SPECIFIC DISEASE RESISTANCE 1* (*NDR1*), the results presented here reveal membrane-spanning receptors for PTI and ETI each have defense roles in the *G. max*-*H. glycines* pathosystem. The strength of effect that *BAK1-1* expression has on *H. glycines* parasitism, in relation to prior observations made for the *G. max* *BOTRYTIS INDUCED KINASE 1* (*BIK1-6*), indicates that more than one co-receptor likely functions through *BIK1-6* (Pant et al. 2014).

## Materials and methods

### Plasmid preparation

The pRAP15 and pRAP17 plasmids have been developed from the p\*7WG2D overexpression and p\*7GWIWG2(II) RNAi vectors (Karimi et al. 2002, 2007; Klink et al. 2009; Matsye et al. 2012). The pRAP15 overexpression/heterologous expression/ectopic expression plasmid and pRAP17 RNAi plasmids have been obtained from bacterial stocks originally prepared in the *E. coli* *ccdB* survival cells selected on LB-tetracycline (5 µg/ml) plates at 37°C according to the manufacturer's instructions (Invitrogen). A single colony was picked and grown on LB-kanamycin 50 µg/ml liquid culture overnight at 37°C. The plasmid prep was prepared using the Wizard® miniprep according to the manufacturer's instructions (Promega). Aliquots from these preparations were used for DNA sequencing.

### Plasmid library preparation and WGS sequencing

Whole-genome sequencing was performed (Omega Bioservices). DNA concentration was measured using the QuantiFluor dsDNA System on a Quantus Fluorometer (Promega). A Kapa Biosystems HyperPrep kit (Kapa Biosystems) was used for whole-genome library construction. Briefly, 1 µg of genomic DNA was fragmented using a Bioruptor sonicator (Diagenode). DNA fragment ends were repaired, 3' adenylated, and ligated to adapters. The resulting adapter-ligated libraries were PCR-amplified, Illumina indexes added, and pooled for multiplexed sequencing on an Illumina Miseq platform (Illumina) using the pair-end 300 bp run format. The DNA sequences relating to the pRAP15 (PRJNA734620) and pRAP17 (PRJNA734623) plasmids are available in Genbank.

### Generation of pRAP15 and pRAP17 plasmid maps

Paired end reads were merged and duplicates removed. Reads were then *de-novo* assembled into contigs. The largest contig was annotated using Geneious version 2021.0 using the plasmid features database (<https://www.geneious.com>).

The similarity percent, when searching the plasmid features database, was set to 80%. The figwort mosaic virus (FMV) genome, p\*7WG2D and p\*7GWIWG2(II) plasmid sequences were added to Geneious to facilitate the analysis (Bhattacharyya et al. 2002; Karimi et al. 2002, 2007).

### Data analysis

Data from 24 previously published experiments performed in different laboratories have been used in the comparative analyses (Klink et al. 2009, 2017; Ibrahim et al. 2011; Matsye et al. 2012; Matthews et al. 2013, 2014; Youssef et al. 2013a, 2013b; Pant et al. 2014, 2015a, 2015b; Woo et al. 2014; Maldonado et al. 2014a, 2014b; Sharma et al. 2016, 2020; McNeece et al. 2017, 2019; Lawaju et al. 2018, 2020; Austin et al. 2019; Niraula et al. 2020a, 2020b, 2021). The data has been obtained from the appropriately cited works or unpublished data that was associated with the references. The data has been compiled, analyzed and summarized using excel. There are 143 published genes that have been analyzed using the pRAP15 plasmid that had a statistically significant effect in the respective plant-pathosystem examined here. Other genes that have not resulted in statistically significant effects on plant pathogen success ( $[p \geq 0.05]$ , Student's *t*-test) are not reported here even though they had been expressed to statistically significant levels ( $[p < 0.05]$ , Student's *t*-test) (Yuan et al. 2006). There are 79 published genes that have been analyzed using the pRAP17 RNAi plasmid that had a statistically significant effect in the respective plant-pathosystem examined here ( $[p < 0.05]$ , Student's *t*-test). Genetic analyses that employed pRAP17, but, however, did not result in a statistically significant effect on pathogen success ( $[p \geq 0.05]$ , Student's *t*-test) while having its target gene expression impacted to a statistically significant level ( $[p < 0.05]$ , Student's *t*-test), are not reported here (Yuan et al. 2006). Details of the plant transformation process are available in the cited works. Furthermore, a detailed plant transformation protocol is available (Matthews and Youssef 2016). The annotations of the studied *G. max* genes span the earlier 2010 genome annotation (Wm82.a1.v1.1) and later 2015 annotation (Wm82.a2.v1) for accuracy under the specific set conditions.

### *G. max* BAK1-1 analyses

The *G. max* BAK1-1 (Glyma.15G051600) protein identification has been performed using the *A. thaliana* BAK1 (AT4G33430) protein sequence. The *A. thaliana* BAK1 protein sequence was used to BlastP query the *G. max* proteome housed at Phytozome using their default parameters (Goodstein et al. 2012). The cloning, maintenance of *H. glycines* stocks, generation of transgenic plants and demonstration of relative transcript abundance has been performed according to Lawaju et al. (2020). The *H. glycines* infection of *G. max* and enumeration of the effect the transgene has on parasitism has been performed through the calculation of the female index (FI) (Golden et al. 1970). The procedure was followed according to Lawaju et al. (2020) for transgenic analyses by enumerating the hardened female carcass containing the eggs (cysts) and then calculating the FI where the  $FI = (N_x/N_s) \times 100$  (Golden et al. 1970).  $N_x$  is the pRAP(15/17)- gene of interest (*BAK1-1*-OE/RNAi)-transformed (experimental) root cyst count.  $N_s$  is the pRAP(15/17)-*ccdB* (control) root cyst count. The FI is

calculated as cysts per whole root system (wr) grown within 100 cc of soil and cysts per gram (pg) of root system. The wr analysis only considers the ability of *H. glycines* to parasitize *G. max* roots. The pg analysis is standardized by weighing the roots to consider the effect the transgene expression has on root mass in relation to *H. glycines* parasitism and may be a more accurate means to determine the effect a particular gene has on *H. glycines* parasitism (McNeece et al. 2019). Statistical analysis of significance for the FI analysis have been performed using the Mann–Whitney–Wilcoxon (MWW) Rank Sum Test which is a nonparametric test of the null hypothesis not requiring the assumption of normal distributions (Mann and Whitney 1947; McNeece et al. 2019) ( $p < 0.05$ ). The RT-qPCR analyses used to determine the relative change in gene expression that is caused by the genetic engineering event is calculated using  $2^{-\Delta\Delta CT}$  (Livak and Schmittgen 2001). The calculation of significance used the Student's *t*-test (Yuan et al. 2006). Three independent biological replicates with each replicate having 12–15 roots have been used in the analysis. PCR and RT-qPCR primers used in the analysis are provided (Supplemental Table 1).

### BAK1 gene identification

The *G. max* BAK1-1 protein sequence was used to further query the proteome of *G. max* as well as the proteomes of *M. esculenta*, *Z. mays*, *O. sativa*, *T. aestivum*, *H. vulgare*, *S. bicolor*, *B. rapa*, *S. tuberosum*, *S. lycopersicum* and *G. hirsutum* which are housed at Phytozome (<https://phytozome.jgi.doe.gov>) under default settings (Goodstein et al. 2012). The proteome sequences for *E. guineensis* (<http://gbrowse.mpob.gov.my>); *S. officinalis* (<https://sugarcane-genome.cirad.fr/>) and *B. vulgaris* (<https://bvseq.boku.ac.at/>) were also mined using the *G. max* BAK1-1 protein sequence under their default settings to identify their BAK1-1 paralogs (Singh et al. 2013; Dohm et al. 2014; Garsmeur et al. 2018; Ong et al. 2020).

## Results

### Plasmid sequencing and generation of plasmid maps

The pRAP15 and pRAP17 DNA backbones have been derived from p\*7WG2D and p\*7GWIWG2(II), respectively, and subsequently modified for the uses described here within (Bhattacharyya et al. 2002; Karimi et al. 2002, 2007; Klink et al. 2009; Matsye et al. 2012). The pRAP15 plasmid sequence has been generated by using 1,580,698 total paired end reads. The paired end reads were merged with the duplicates removed, resulting in 109,372 total reads that were used in the *de-novo* assembly. The largest contig was formed by the assembly of 75,790 reads, leading to the assembly of the 14,758 bp pRAP15 plasmid (Figure 1). The pRAP17 plasmid has been created by using 1,574,592 total paired end reads. The paired end reads were merged with the duplicates removed, resulting in 105,985 total reads that were used in the *de-novo* assembly. The largest contig was formed by the assembly of 59,774 reads, leading to the production of the 15,596 bp pRAP17 plasmid (Figure 2). The general expected features of the pRAP15 and pRAP17 plasmids, based off the original p\*7WG2D and p\*7GWIWG2(II) backbones, respectively, are that they are Gateway® compatible for the efficient directional cloning of genes at the attR sites (Karimi et al.

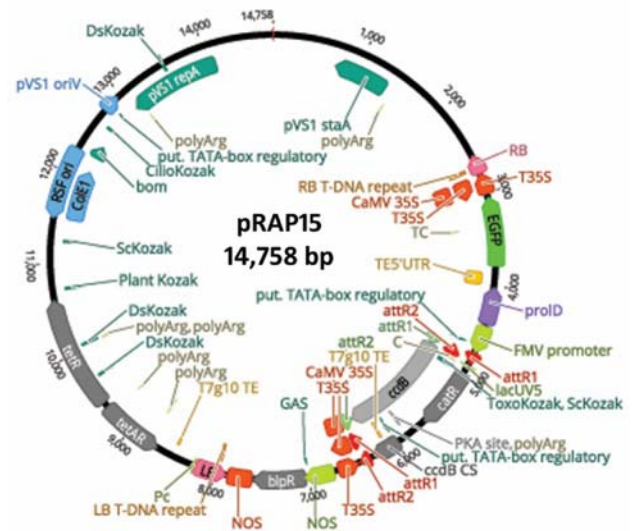


Figure 1. The pRAP15 plasmid map.

2002, 2007; Curtis and Grossniklaus 2003; Klink et al. 2009; Matsye et al. 2012). Maintenance of the original un-engineered pRAP15 and pRAP17 plasmids (lacking the insertion of a transgene) is accomplished due to the presence of a chloramphenicol Cm(r)-*ccdB* lethality gene (Tam and Kline 1989; Bernard and Couturier 1991; Salmon et al. 1994). The pRAP15 and pRAP17 plasmids contain an enhanced green fluorescent protein (eGFP) gene driven by the *roID* promoter and terminated by t35S translational terminator for effective visual reporting in plant tissue (White et al. 1985; Elmayan and Tepfer 1995; Haseloff et al. 1997). Plant selection can also be done chemically with the Basta® selectable marker encoded by the *bar* gene which confers resistance to the herbicide bialphos, useful for tissue culture (Thompson et al. 1987; Rathore et al. 1993; Karimi et al. 2002, 2007). The *bar* gene is driven by the nopaline synthase promoter and terminated by the nopaline synthase terminator.

### Genetic elements added to generate pRAP15 and pRAP17

Through analysis using p\*7WG2D and p\*7GWIWG2(II), it has become learned that gene expression became negatively affected during infection by various pathogens under study

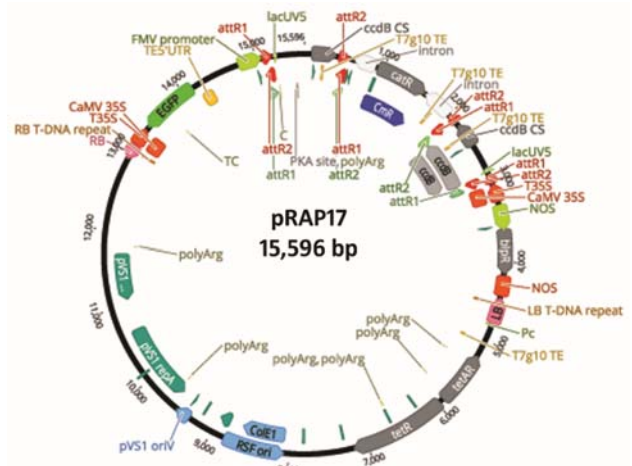


Figure 2. The pRAP17 plasmid map.

(Klink et al. 2009; Matsye et al. 2012; Matthews et al. 2013, 2014). The p\*7WG2D and p\*7GWIWG2(II) plasmids were then used to generate the pRAP15 and pRAP17 plasmids, respectively, through the addition of the subsequently described genetic elements (Bhattacharyya et al. 2002; Karimi et al. 2002, 2007; Klink et al. 2009; Matsye et al. 2012). The genetic elements that have been added include the tetracycline resistance gene (TetR) inserted outside of the left and right border, facilitating selection in *E. coli* or, importantly, *A. rhizogenes* or other bacteria. The pRAP15 and pRAP17 plasmids also have added to them the figwort mosaic virus (FMV) sub-genomic transcript (Sgt) promoter, consisting of a 301-bp FMV-Sgt promoter fragment [sequence -270 to +31 from the transcription start site (TSS)] (Bhattacharyya et al. 2002). The promoter allows effective transgene expression in the presence of various pathogens throughout infection which will be discussed in the next section (Klink et al. 2009; Matsye et al. 2012; Pant et al. 2015b; Sharma et al. 2016; Lawaju et al. 2018; Niraula et al. 2020a). Consequently, the expression of eGFP, driven by *rolD* and the targeted gene driven by FMV-Sgt should not interfere with each other since they rely on different promoters. As will be described later, this desired condition has been consistently observed since tissue exhibiting eGFP fluorescence also exhibits the expected altered expression of the targeted gene. Experiments have also been presented using pRAP15 to overexpress, heterologously express or ectopically express transgenes in *G. max*, *Gossypium hirsutum* (upland cotton) and *Allium cepa* (onion) (Matsye et al. 2012; Youssef et al. 2013a, 2013b; Matthews et al. 2014; Pant et al. 2015b; McNeece et al. 2017; Niraula et al. 2020a).

### Studies where pRAP vectors have been employed

The pRAP series of plasmids were originally developed for use in understanding plant cell biology and/or the cellular interactions occurring between *G. max* and *H. glycines* (Klink et al. 2009; Matsye et al. 2012). This objective would be met through genetic engineering of candidate *G. max* defense genes for overexpression or suppressed gene expression of pathogen genes through host-mediated expression of parasite genes (Klink and Wolniak 2000; Klink et al. 2009; Ibrahim et al. 2011; Matsye et al. 2012; Matthews et al. 2013). The pRAP15 and pRAP17 plasmids have since been used for different studies, including the analysis of whole plant gene families (McNeece et al. 2019; Lawaju et al. 2020). An important aspect of the utility of the plasmids is whether they can be used to efficiently transform plant tissue.

Transformation efficiency for *G. max* using the pRAP vectors have not been extensively studied, although reports of 40% efficiency for pRAP17 and 60% efficiency for pRAP15 have been described (Youssef et al. 2013b). Analyses of transformation data from 12 different studies that previously did not analyze transformation efficiency and used different *G. max* genotypes are presented (Table 1).

### Expression of transgenes

Many of the overexpressed *G. max* genes that have been studied were identified through laser microdissection of plant root cells known as syncytia that were directly parasitized by *H. glycines*, but undergoing a resistant reaction (Klink et al. 2007; Matsye et al. 2011). The genes were cloned and used in transgenic studies to generate resistance (Matsye et al. 2012; Pant et al. 2014). The method was subsequently adapted for the heterologous expression of plant defense genes of *A. thaliana* in *G. max* as overexpression constructs using pRAP15 or additional *H. glycines* genes as RNAi cassettes in pRAP17 (Ibrahim et al. 2011; Youssef et al. 2013b). Some of the earlier studies have reported the expression of red fluorescent protein (RFP) and  $\beta$ -glucuronidase to facilitate visual reporting (Matsye et al. 2012; Pant et al. 2014). Since those studies, additional experiments have been performed to examine the details of the cellular interactions occurring between *G. max* and other pathogens using overexpressed and/or RNAi of soybean genes and examining their effect on *Meloidogyne incognita* (root knot nematode [RKN]) and the ascomycete *Macrophomina phaseolina* (charcoal rot) (McNeece et al. 2017; Lawaju et al. 2018). The heterologous expression of *A. thaliana* genes has been done to examine their effect on *M. incognita* parasitism (Youssef et al. 2013a). Similar experiments have been performed in *G. hirsutum* to examine their effect on *M. incognita* (Pant et al. 2015b; McNeece et al. 2017; Niraula et al. 2020a). The work summarized here spans 24 publications, incorporating transgenic research performed in *G. max* and *G. hirsutum* in examining the overexpression of *G. max* genes in *G. max*, ectopic expression of *A. thaliana* genes in *G. max* and heterologous expression (ectopic expression) of *G. max* genes in *G. hirsutum*, RNAi of *G. max*, *H. glycines* and *M. incognita* genes in *G. max* (Klink et al. 2009, 2017; Ibrahim et al. 2011; Matsye et al. 2012; Matthews et al. 2013, 2014; Youssef et al. 2013a, 2013b; Pant et al. 2014, 2015a, 2015b; Woo et al. 2014; Maldonado et al. 2014a, 2014b; Sharma et al. 2016, 2020; McNeece et al. 2017, 2019; Lawaju et al. 2018, 2020; Austin

**Table 1.** Efficiency of plant transformation as revealed by eGFP expression in several *G. max* genotypes.

Plasmid	Genotype	Transgenic event	Number of plants			
			Transformed (eGFP)	Not transformed (no eGFP)	Total	Percent
pRAP15	Williams 82/PI 518671	control-OE	3,987	1,673	5,660	70.44
pRAP15	Williams 82/PI 518671	OE	6,163	2,851	9,033	68.23
pRAP17	Peking/PI 548402	control-RNAi	3,115	1,379	4,494	69.31
pRAP17	Peking/PI 548402	RNAi	6,270	2,815	9,096	68.93
pRAP17	Williams 82/PI 518671	control-RNAi	107	41	148	72.3
pRAP17	Williams 82/PI 518671	RNAi	418	174	592	70.6
pRAP17	DT97-4290/PI 642055	control-RNAi	60	60	120	50
pRAP17	DT97-4290/PI 642055	RNAi	329	284	613	53.67
TOTAL	n/a	n/a	20,449	9,277	29,756	68.72

Note: Transformation efficiency data previously not reported. The studies from which the transformation efficiency was calculated include Klink et al. (2009); Matsye et al. (2012); Pant et al. (2014); Pant et al. (2015a); Sharma et al. (2016); Aljaafri et al. (2017); McNeece et al. (2017); Lawaju et al. (2018); McNeece et al. (2019); Sharma et al. (2020); Lawaju et al. (2020) and Niraula et al. (2020b). The expected gene expression of the target gene was confirmed by RT-qPCR in the respective references. The eGFP reporter expression was also confirmed by PCR from cDNA made from RNA in the respective references.

**Table 2.** Performance of pRAP15 and pRAP17 vectors in functional genetic analyses on pathogens.

Plasmid	Experiment	Gene source	Host	Target pathogen	Gene count	High %	Low%	Average	StDv	outcome
pRAP15	OE	<i>G. max</i>	<i>G. max</i>	<i>H. glycines</i>	104	87	29	63.6	14	suppress pathogen
pRAP17	RNAi	<i>G. max</i>	<i>G. max</i>	<i>H. glycines</i>	85	2,205	141	425.7	371.7	facilitate pathogen
pRAP17	RNAi	<i>H. glycines</i>	<i>G. max</i>	<i>H. glycines</i>	4	93	81	87.5	5	suppress pathogen
pRAP17	RNAi	<i>M. incognita</i>	<i>G. max</i>	<i>M. incognita</i>	2	95	92	93.5	2.1	suppress pathogen
pRAP17	RNAi	<i>A. thaliana</i>	<i>G. max</i>	<i>H. glycines</i>	4	57	44	50	5.3	suppress pathogen
pRAP15	OE	<i>A. thaliana</i>	<i>G. max</i>	<i>M. incognita</i>	2	77	53	65	17	suppress pathogen
pRAP15	OE	<i>A. thaliana</i>	<i>G. max</i>	<i>H. glycines</i>	12	68	37	53.1	9.8	suppress pathogen
pRAP15	OE	<i>G. max</i>	<i>G. hirsutum</i>	<i>M. incognita</i>	4	97	66	83.3	13.5	suppress pathogen
pRAP15	OE	<i>G. max</i>	<i>G. max</i>	<i>M. phaseolina</i>	4	35	30	32.3	2.6	suppress pathogen
pRAP17	RNAi	<i>G. max</i>	<i>G. max</i>	<i>M. phaseolina</i>	4	142	140	141	0.8	facilitate pathogen

et al. 2019; Niraula et al. 2020a, 2020b, 2021) (Table 2). Not examined here is the expression of the phytoene desaturase 3 (PDS3) gene (NM\_117498.3) in *A. thaliana* as an RNAi construct using pRAP17 which led to the development of seedlings deficient in chlorophyll maintenance (Youssef et al. 2013a, 2013b). What has been learned from these experiments is that a wide range of genes from different plant sources can be used to perform various types of analyses in different plant pathosystems. The negative effect on pathogen success can be quite high, such as an 90.6% reduction obtained for the expression of the *G. max* *BOTRYTIS INDUCED KINASE 1* (*BIK1*) (Glyma.14G068700) in *G. max* in relation to *H. glycines* parasitism (Pant et al. 2014). In some cases, the negative effect is observed at different stages of the pathogen's life cycle. For example, the heterologous expression of the *G. max* hemicellulose-modifying xyloglucan endotransglycosylase/hydrolase *XTH43* (Glyma.17G065100) in *G. hirsutum* has a dramatic effect on *M. incognita* (97% reduction), but only later in its development at its J2 stage (Niraula et al. 2020a). The negative effect of host (*G. max*)-expressed RNAi constructs of pathogen genes, along with the *M. incognita* mitochondrial stress-70 protein precursor (*BI773411*) can result in as high as a 94% reduction of the target pathogen *M. incognita* (Ibrahim et al. 2011). Data has been compiled from the 24 publications and analyzed here to obtain the overall expected impact that gene expression has (Table 2).

### The pRAP15 and pRAP17 plasmids effectively alter gene expression in the expected manner

The important complimentary data to these functional genetic experiments that have assayed pathogen success is the level of effect on gene expression that can be expected to be obtained with pRAP15 and pRAP17. The pRAP15 plasmid has been used to overexpress targeted *G. max* genes that function in different ways. For example, experiments have targeted the *G. max* vesicle transport apparatus gene alpha soluble NSF attachment protein ( $\alpha$ -SNAP) (Glyma.18G022500), part of the major *H. glycines* resistance locus, *rhg1*, the membrane-anchored receptor-like cytoplasmic kinase *BIK1-6*, the hormone biosynthesis *NONEXPRESSOR OF PR1* (*NPR1-2*) (Glyma.09G020800), an ascorbate peroxidase (Glyma.12G073100) and *XTH43*, among others (Matsye et al. 2012; Matthews et al. 2013; Pant et al. 2014). RNA expression data derived from 84 different overexpressed genes obtained by RT-qPCR range from a low of an increase in relative transcript abundance of 1.78-fold for *NONRACE-SPECIFIC DISEASE RESISTANCE1* (*NDRI-1*) (Glyma.12G214100) to 293.8-fold for *LESION SIMULATING DISEASE1* (*LSD1*)

(Glyma.08G129400), averaging an increase in relative transcript abundance of 11.24-fold,  $p < 0.05$  for each individual gene (Student's *t*-test) (Table 3). High relative levels of expression of defense genes in overexpression lines have also been observed in *G. max* roots ectopically expressing *AtPAD4* in comparison to control roots that would lack its expression, complicating analyses of fold change (Youssef et al. 2013a). Standard deviation is not presented here since these are averages obtained from different genes that are likely under different forms of regulation within plant tissue (McNeece et al. 2019). RNA expression for 68 different genes targeted for RNAi have been examined by RT-qPCR, ranging from  $-19.48$ -fold for *MAPK13-1* (Glyma.12G073700) to  $-1.31$ -fold for the *G. max* homolog of mammalian uncoordinated 18 (*MUNC18*) (Glyma.17G135500) and averaging a decrease in relative transcript abundance of  $-3.84$ -fold,  $p < 0.05$  for each individual gene (Student's *t*-test) (Table 3). Standard deviation is not presented since these are averages obtained from different genes that are likely to be under different forms of regulation within plant tissue (McNeece et al. 2019). Plant genes targeted for overexpression and RNAi maintain their increased or decreased relative transcript abundances throughout the engineered defense or susceptibility processes, respectively (Matsye et al. 2012; Pant et al. 2014; Sharma et al. 2016).

### An analysis of a membrane receptor functioning in PTI

Very little is known about the role of PRR co-receptors functioning in PTI in the *G. max*-*H. glycines* pathosystem. In the analysis presented here, *G. max* *BAK1-1* expression in syncytia undergoing a defense response is presented (Table 4, Supplemental Table 2). Analyses of transgenic vs total plants (transgenic and nontransgenic) have been performed, revealing a high level of transgenic roots obtained in the experiments (Figure 3). The relative change in transcript abundance of *BAK1-1* in overexpression and RNAi lines is statistically significant ( $p < 0.05$ , Student's *t*-test) (Figure 4). To examine the effect that *BAK1-1* expression has on *H. glycines* parasitism, *H. glycines* cyst counts have been performed, leading to the calculation of a FI. The analysis began by infestation of the soil in pots containing the transgenic pRAP15 and pRAP17 controls along with the *BAK1-1*-OE and *BAK1-1*-RNAi plants with

**Table 3.** Level of effect the transgene has on the targeted *G. max* gene. \* statistically significant,  $p < 0.05$ .

Plant expression	Expression outcome	Gene count	Average*	High*	Low*
Overexpression	Induced	82	11.24	293.8	1.78
RNAi	Suppressed	68	-3.84	-19.48	-1.31

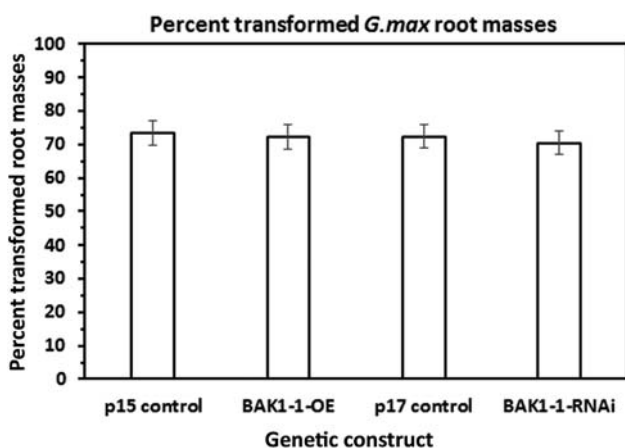
**Table 4.** Expression of the *G. max* BAK1 gene family in syncytia undergoing a defense response. n/a, not applicable since the gene had no probe set fabricated on the Affymetrix microarray. M, expression measured ( $p < 0.05$ ). NM, expression not measured ( $p \geq 0.05$ ). \* Used in the transgenic analysis.

Accession	Gene name	Affymetrix probe	Expression (dpi)		
			0	3	6
Glyma.15G051600.1	BAK1-1	Gma.3203.1.S1_at	M	M	M
Glyma.08G180800.1	BAK1-2	GmaAffx.10091.1.S1_at	M	M	M
Glyma.05G119500.1	BAK1-3	no probe	n/a	n/a	n/a
Glyma.02G076100.1	BAK1-4	GmaAffx.48636.1.S1_at	NM	NM	NM
Glyma.05G119600.1	BAK1-5	GmaAffx.201.1.S1_at	NM	NM	NM
Glyma.08G074500.1	BAK1-6	no probe	n/a	n/a	n/a
Glyma.20G173000.1	BAK1-7	no probe	n/a	n/a	n/a
Glyma.10G218800.1	BAK1-8	Gma.10649.2.S1_at	NM	NM	NM
Glyma.19G039100.1	BAK1-9	GmaAffx.24064.2.S1_s_at	NM	NM	NM
Glyma.13G050200.1	BAK1-10	GmaAffx.24064.1.S1_at	NM	NM	NM
Glyma.18G278200.1	BAK1-11	no probe	n/a	n/a	n/a
Glyma.11G240900.1	BAK1-12	no probe	n/a	n/a	n/a
Glyma.01G071700.1	BAK1-13	no probe	n/a	n/a	n/a
Glyma.02G036500.1	BAK1-14	GmaAffx.53227.1.S1_at	NM	NM	M
Glyma.01G028700.1	BAK1-15	GmaAffx.75246.1.S1_at	NM	NM	NM
Glyma.05G177500.1	BAK1-16	no probe	n/a	n/a	n/a
Glyma.08G255300.1	BAK1-17	no probe	n/a	n/a	n/a
Glyma.18G016400.1	BAK1-18	GmaAffx.13860.1.S1_at	NM	NM	NM
Glyma.08G134600.1	BAK1-19	GmaAffx.74562.1.S1_at	NM	NM	NM

2,000 J2 *H. glycines* and allowing infection and parasitism to occur over its 30-day life cycle. The cyst count in the pRAP15 control roots in the *H. glycines*-susceptible *G. max*<sub>[Williams 82/PI 518671]</sub> genotype is  $217.72 \pm 11.19$  per root system while it is  $48.29 \pm 2.37$  cysts per gram of root. The cyst count in the pRAP17 control roots in the *H. glycines*-susceptible *G. max*<sub>[Peking/PI 548402]</sub> genotype is  $33.01 \pm 4.13$  per root system while it is  $12.97 \pm 1.54$  cysts per gram of root. Using these values as a base line, the FI of the *BAK1-1*-OE and *BAK1-1*-RNAi-expressing roots have been determined ( $p < 0.05$ , MWW) (Figure 5). The similarity between the cysts per whole root (wr) and cysts per gram (pg) of root tissue demonstrates the expression of the transgene has negligible effect on root development. To confirm this observation, analyses have been performed on wet root weights with the percent difference calculated showing that the roots are the same, statistically ( $p \geq 0.05$ , MWW) (Figure 6).

### Altered *BAK1-1* expression changes the relative transcript abundance of signaling genes

The defense response that *G. max* has toward *H. glycines* parasitism involves *BIK1-6* whose overexpression increases

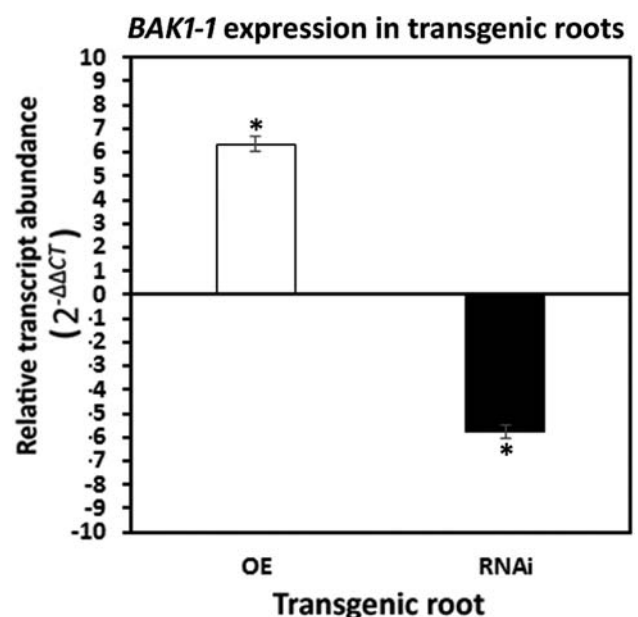


**Figure 3.** Percent transformed *G. max* root masses. Analyses of transgenic vs total plants (transgenic and nontransgenic) for *BAK1-1*-OE, *BAK1-1*-RNAi and their pRAP15 (p15) and pRAP17 (p17) controls, respectively. The percent are the average of three independent replicates ( $p < 0.05$ , MWW).

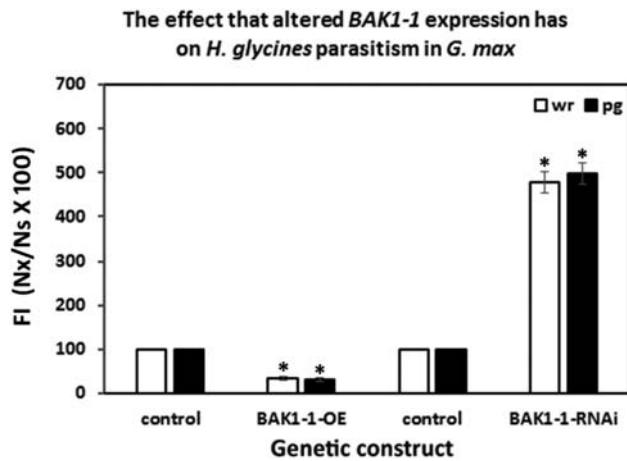
the relative transcript abundance for mitogen activated protein kinase 3 (MAPK3) MAPK3-1 and MAPK3-2 while its RNAi decreases their relative transcript abundance (McNeece et al. 2019). An RT-qPCR analyses of *BAK1-1*-OE lines shows a statistically significant increase in the relative transcript abundance for MAPK3-1 and MAPK3-2 ( $p < 0.05$ , Student's *t*-test). In contrast, *BAK1-1*-RNAi results in a concomitant statistically significant decrease in the relative transcript abundance of MAPK3-1 and MAPK3-2 ( $p < 0.05$ , Student's *t*-test) (Figure 7).

### A bioinformatics-based identification of *BAK1* homologs in important agricultural crops

Studies have demonstrated the importance of various agricultural crops to U.S. and world agriculture and how climate change affects them (Tilman et al. 2011; Ray et al. 2019). A number of these crops are understudied as compared to



**Figure 4.** The role that the *G. max* *BAK1-1* transgene construct has on its relative transcript abundance in overexpression and RNAi roots (\* statistically significant,  $p < 0.05$ , Student's *t*-test).

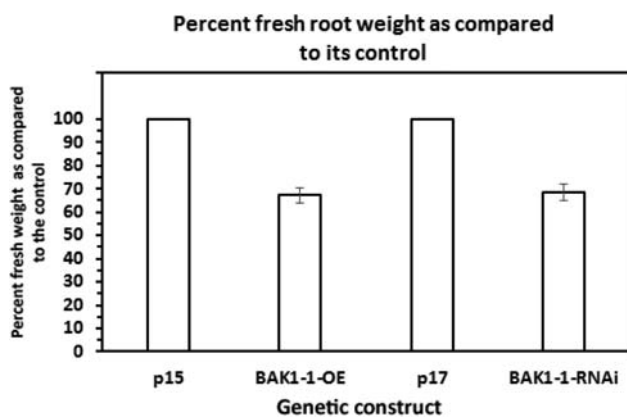


**Figure 5.** The effect *G. max* BAK1-1 expression has on *H. glycines* parasitism. The overexpression studies were performed in the *H. glycines*-susceptible genotype *G. max*<sub>[Williams 82/PI 518671]</sub> (\* statistically significant,  $p < 0.05$ , MWW). The RNAi studies were performed in the *H. glycines*-resistant genotype *G. max*<sub>[Williams 82/PI 518671]</sub> ( $p < 0.05$ , MWW). wr, analysis of *H. glycines* cysts per whole root; pg, analysis of *H. glycines* cysts per gram of root tissue.

model organisms. The identification of the *G. max* BAK1-1 performing an important role in its defense to *H. glycines* makes it likely that BAK1 homologs exist and perform important roles in growth, development and disease resistance in other important agricultural crops. An analysis using the *G. max* BAK1-1 protein sequence has been used in BlastP searches under default settings, leading to the identification of additional BAK1 homologs in *G. max* as well as *G. hirsutum*, *M. esculenta*, *Z. mays*, *O. sativa*, *T. aestivum*, *S. bicolor*, *B. rapa*, *S. tuberosum*, *S. lycopersicum*, *E. guineensis*, *S. officinalis* and *B. vulgaris* (Table 5, Supplemental Table 3). The *E. guineensis* BAK1 paralogs were cut off at values below  $1E-47$  due to concerns as to whether the additionally identified sequences were truly BAK1 paralogs.

## Discussion

The pRAP series of plasmid vectors have been generated to facilitate the engineering in of genes from different sources into plants (Klink et al. 2009; Matsye et al. 2012). The experiments originally targeted plant parasitic nematodes to suppress parasitism and develop much needed resistance to this devastating pathogen (Klink et al. 2009; Matsye et al. 2012). Parasitic nematodes were targeted because they are greatly



**Figure 6.** Root mass analysis. Analyses of 3 independent biological replicates each with 12–16 plants. Fresh weight of the transgenic root mass was measured. Statistical analysis employed MWW,  $p < 0.05$  cutoff.

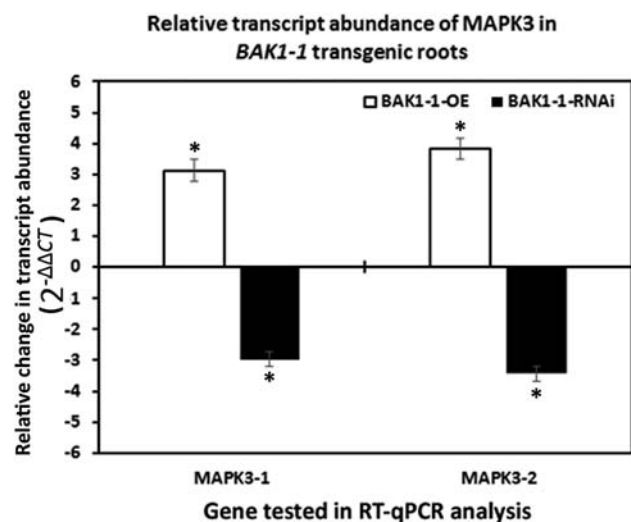
understudied pathogens as compared to other disease-causing agents including bacteria, fungi and viruses and there was an urgent need to do so (Jones and Dangl 2006; Klink and Matthews 2009; Wrather and Koenning 2009). The analysis presented here, including the new experiments examining *G. max* BAK1-1, further demonstrate the utility of pRAP15 and pRAP17 in studying plant defense processes, while highlighting the importance of the major signal transduction branch involving PRRs and PTI in the studied pathosystem.

## The utility of the pRAP15 and pRAP17 sequences

The pRAP15 and pRAP17 sequences are useful for different applications. Firstly, the plasmid DNA sequences make possible the generation of PCR primers for the determination of genetic elements that have been engineered into the *ccdB* sites. This information is critically important for the pRAP17 plasmid whereby the genes are ligated in as inverted repeats. The plasmid sequences allow for the generation of PCR primers to confirm the orientation of the inserted genes or gene fragments. The plasmid sequences will further aid in the confirmation of bacterial and plant transformation (Klink et al. 2009; Youssef et al. 2013a). The sequence information would be useful for site-directed mutagenesis of the plasmid to engineer in restriction sites or edit the gene sequence (Hallak et al. 2017; Bahramnejad et al. 2019; Castel et al. 2019). Editing the sequence to incorporate different promoters that drive different levels of expression may be useful for comparative studies especially if high levels of expression are toxic to the plant tissue (Austin et al. 2019). The features of the pRAP15 and pRAP17 plasmids have advantages over the original pKSF plasmid from which the FMV-Sgt promoter was derived, namely the eGFP and TetR reporters and being Gateway® compatible (Bolivar et al. 1977; Bhattacharyya et al. 2002; Karimi et al. 2002).

## The pRAP15 and pRAP17 plasmids accomplish efficient plant transformation

The new analyses show that the pRAP plasmids provide high percentages of transformation using the root-inducing K599,



**Figure 7.** Defense MAPK gene expression analysis. Gene expression has been analyzed using the equation  $2^{-\Delta\Delta CT}$  (\* statistically significant,  $p < 0.05$ , Student's *t*-test) (Livak and Schmittgen 2001).



**Table 5.** BAK1 homolog count in various agriculturally important plants.

Organism	BAK1 homologs
<i>G. max</i>	19
<i>B. rapa</i>	14
<i>S. tuberosum</i>	9
<i>S. lycopersicum</i>	7
<i>G. hirsutum</i>	11
<i>B. vulgaris</i>	8
<i>Z. mays</i>	9
<i>O. sativa</i>	6
<i>T. aestivum</i>	10
<i>H. vulgare</i>	7
<i>S. bicolor</i>	6
<i>S. officinalis</i>	8
<i>M. esculenta</i>	10
<i>E. guineensis</i>	5

examining data obtained from 12 different studies where the transformation efficiency has not been reported. The overexpression studies performed in *G. max*<sub>[Williams 82/PI 518671]</sub> that used pRAP15 as a control have examined the transformation data from 5,660 plants. Concomitantly, analyses of data from the pRAP15 transformation experiments where it had been engineered to harbor target genes for overexpression, also in *G. max*<sub>[Williams 82/PI 518671]</sub>, examined 9,033 plants. The analyses show that approximately a 70% transformation efficiency occurring in plants expressing the eGFP reporter. Plants expressing the eGFP reporter also exhibit the expression of the targeted transgene as demonstrated by RT-qPCR and visual reporting (RFP, *uidA*) (Sheen et al. 1995; Klink et al. 2009; Matsye et al. 2012). Similar analyses have examined the pRAP17 control in transformation experiments of *G. max*<sub>[Peking/PI 548402]</sub>, examining 4,494 control plants. Concomitantly, 9,096 plants that have been engineered to harbor the pRAP17 RNAi plasmid containing different target genes or sub-fragments. The analyses show that approximately a 70% transformation efficiency as revealed by eGFP expression and RT-qPCR can be expected under these conditions as well. Smaller studies using pRAP17 to genetically transform the *G. max*<sub>[Williams 82/PI 518671]</sub> genotype also show approximately a 70% transformation efficiency like what was observed for the pRAP15 overexpression studies. However, another small study that used pRAP17 to genetically transform the *G. max*<sub>[DT97-4290/PI 642055]</sub> genotype resulted in a 50-53% transformation efficiency. This result may indicate differences exist in the ability of K599 to transform different *G. max* genotypes since the plasmids have been able to effectively transform *A. thaliana*, *A. cepa* and *G. hirsutum*. However, the efficiency is still high in terms of the user's expectation of retrieving transformed plants. The results have shown it is possible to express *G. max*, *A. thaliana* and *H. glycines* genes with equal effectiveness.

### The pRAP15 and pRAP17 plasmids affect the expression of their targets

The results analyzed here have shown that the engineering in of target DNA into the pRAP15 and pRAP17 plasmids leads to the ability of the transgenic roots to effectively alter the infection of pathogens in ways that would be expected. Furthermore, what has not been discussed in these analyses are, as expected, many genes that have been expressed in pRAP15 that had no effect on pathogen success (Matthews et al. 2013, 2014; McNeece et al. 2019; Lawaju et al. 2020). These results, therefore, argue against the induced expression of some non-

specific basal defense pathway occurring because of transgenic expression of any gene or gene fragment within the pRAP15 or pRAP17 platforms. The expression of *H. glycines* fructose-1,6-diphosphate aldolase (HgALD) as an inverted hairpin repeat in pRAP17 in *G. max* led to an 83% reduction in its cognate RNA in the target pathogen *H. glycines* while the *H. glycines*  $\beta$ -actin transcript abundance was unaffected (Youssef et al. 2013a). However, in some cases, the expression of a gene does influence the expression of other genes in a manner that appears to have the hallmarks of co-regulation (Pant et al. 2014; Sharma et al. 2016; McNeece et al. 2019). Accompanying the presented experiments has been quality control measures that examine the level of expression of the targeted gene. For the experiments employing pRAP15, RT-qPCR leads to the measurement of induced expression of the targeted gene as compared to controls. Importantly, the expression of the transgene is maintained during pathogen infection and defense process (Matsye et al. 2012; Sharma et al. 2016). Furthermore, experiments employing pRAP17 lead to suppressed gene expression of both plant and pathogen targets as compared to controls as shown through RT-qPCR. The suppressed expression of the transgene is also maintained throughout the course of the engineered susceptible reaction (Sharma et al. 2016). Consequently, the function of the plasmids is reliable throughout the course of the study. These experiments were imperative in understanding the function of the biological role of one of these targeted genes, XTH43 (Pant et al. 2014; Niraula et al. 2021). Related experiments employing RNAi have also demonstrated the effectiveness of the pRAP17 plasmid. Experiments performed in *A. thaliana* have obtained the characteristic 'bleached' plants in RNAi experiments targeting its PDS3 (Youssef et al. 2013b). PDS is necessary for carotenoid production, required for chloroplast membrane stabilization as well as the quenching of reactive oxygen species (ROS) in the chloroplast (Lütke-Brinkhaus et al. 1982; Dekker and Duke 1995). However, this result was not directly tested to determine chlorophyll content or an impact on chloroplast structure.

What has not been demonstrated in any of these experiments is confirmation of whether the plant cellular biological product relating to the transgenically-targeted gene is altered in content or structure. A cell wall biochemical analysis demonstrated that XTH43 overexpression in the *H. glycines*-susceptible genotype *G. max*<sub>[Williams 82/PI 518671]</sub> shortened the xyloglucan chains while creating more of them and increased the amount of xyloglucan (Niraula et al. 2021). The outcome of the experiments explained the failure of the *H. glycines*-parasitized root cell (syncytium) to expand, accompanying its defense response. In contrast, the RNAi roots showed the opposite effect (Niraula et al. 2021). XTH43-RNAi experiments performed in the *H. glycines*-resistant *G. max*<sub>[Peking/PI 548402]</sub> had roots with xyloglucan characteristics similar to what is found in the *H. glycines*-susceptible genotype *G. max*<sub>[Williams 82/PI 518671]</sub> (Niraula et al. 2021). The analysis was the first biochemical study to show a specific cell wall modification caused by a *G. max* gene (XTH43) engineered for its overexpression to detrimentally affect *H. glycines* parasitism (Niraula et al. 2021). The aforementioned results demonstrated at least one aspect of the defense process (cell wall modification) while reinforcing the observation of the involvement of the vesicle transport system in the process (Matsye et al. 2011,

2012; Pant et al. 2014; Sharma et al. 2016; Niraula et al. 2021). Through these experiments on XTH43, the involvement of the vesicle transport system in defense to *H. glycines* parasitism is further strengthened. The involvement is strengthened because one of the genes composing the major *H. glycines* resistance locus, *rhg1*, is  $\alpha$ -SNAP which functions with a multisubunit proteinaceous structure known the soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptor (SNARE) in plant defense processes (Collins et al. 2003; Matsye et al. 2012; Pant et al. 2014; Sharma et al. 2016).

### The pRAP plasmids function to inhibit fungal pathogenesis

Overexpression was examined in experiments seeking to identify *G. max* genes that function in its defense response to the fungal pathogen *M. phaseolina*. *M. phaseolina* is a devastating pathogen of *G. max* and over 500 other plants including many important agricultural crops (Su et al. 2001; Ramezani et al. 2007; Wrather and Koenning 2009). *G. max* lacks extensive resistance capability to *M. phaseolina* so the approach of the experiments was to take genes that are induced during the defense response it has toward *H. glycines* and overexpress them to see if they would also function to suppress *M. phaseolina* infection (Lawaju et al. 2018). The approach was taken because *H. glycines* infection makes the pathogenicity of *M. phaseolina* in *G. max* worse so it was believed that any defense response toward these two pathogens would involve similar gene sets (Todd et al. 1987; Winkler et al. 1994; Lawaju et al. 2018). Lawaju et al. (2018) expressed *NDR1-1*, *ENHANCED DISEASE SUSCEPTIBILITY 1-2 (EDS1-2)* (Glyma.06G187200), *NONEXPRESSOR OF PRI-2 (NPR1-2)* (Glyma.09G020800) and *TGA2-1* (Glyma.10G296200) leading to a range of 30-35% reduction in infection in the *M. phaseolina*-susceptible *G. max*<sub>[Williams 82/PI 518671]</sub>. They were the first genes shown to have a role in the defense process that *G. max* has toward *M. phaseolina* (Lawaju et al. 2018). RNAi of these same genes have been performed in the partially *M. phaseolina*-resistant *G. max*<sub>[DT97-4290/PI 642055]</sub> reported by Smith and Carvil (1997). RNAi of *NDR1-1*, *EDS2-2*, *NPR1-2* and *TGA2-1* led to a 41% increase in pathogenicity of *M. phaseolina* (Lawaju et al. 2018). The combination of increased resistance in the overexpression lines performed in the *M. phaseolina*-susceptible *G. max*<sub>[Williams 82/PI 518671]</sub> and increased susceptibility in the RNAi lines in the partially *M. phaseolina*-resistant *G. max*<sub>[DT97-4290/PI 642055]</sub> provided evidence the genes function in the genetic pathway(s) leading to defense (Pant et al. 2014; Lawaju et al. 2018). The broad applicability of the pRAP15 plasmid has been further examined in ectopic expression experiments (Youssef et al. 2013a; Matthews et al. 2014). In these experiments, pRAP15 was used to ectopically express components of salicylic acid defense signaling and a variety of other *A. thaliana* defense genes (Youssef et al. 2013a; Matthews et al. 2014; Maldonado et al. 2014b). The achieved results were a high level of resistance, up to 77%, to different pathogens including *H. glycines* and *M. incognita* (Youssef et al. 2013a; Matthews et al. 2014; Maldonado et al. 2014b). A similar approach used to overexpress *G. max* genes in *G. hirsutum* has led to a suppression of infection of up to 97% for *M. incognita* (Pant et al. 2015b; McNeece et al. 2017; Niraula et al. 2020a). Plant

transformation efficiencies as estimated by eGFP expression have been reported between 40% for pRAP17 engineered with the *H. glycines* gene HgALD to 60% for the empty pRAP17 plasmid (Youssef et al. 2013b). However, an extensive analysis over many genes had not been performed so it was unclear how well these results would compare over an analysis of many genes. The reported transformation efficiency analyzed here for both the empty pRAP15 and pRAP17 plasmids, as well as those harboring transgenes, is about 70%, beyond the earlier reported efficiencies (Youssef et al. 2013b).

### The *G. max* PRR co-receptor BAK1 (BAK1-1) PTI gene functions in *G. max* defense to *H. glycines*

Prior studies have demonstrated a defense role for the *G. max* PTI cytoplasmic kinase *BIK1* (Pant et al. 2014; McNeece et al. 2019). The *A. thaliana* BIK1 associates with several different membrane-spanning PRR co-receptors that function in PTI, including BAK1. However, under normal growth conditions, BAK1 associates with BRASSINOSTEROID-INSENSITIVE 1 (BRI1) to regulate growth (Clouse et al. 1996; Li et al. 2002). Analyses in the *G. max*-*H. glycines* pathosystem so far have not examined PTI receptors. Analyses targeting *BAK1-1* became logical because of the demonstrated effectiveness of the *G. max* *BIK1-6* against *H. glycines* parasitism (Pant et al. 2014). BlastP searches of the *G. max* proteome under its default settings with the *A. thaliana* BAK1 protein sequence led to the identification of 19 paralogs annotated as BAK1, somatic embryogenesis receptor kinase (SERK), NSP1-INTERACTING KINASE (NIK) 1, 2 or 3, or a receptor protein tyrosine kinase. SERK functions in different processes including defense (Hecht et al. 2001). NIK1 functions in antiviral defense (Carvalho et al. 2008). The annotation of these groups is somewhat interchangeable as the *A. thaliana* SERK3 is BAK1 (Nam and Li 2002). Of the 19 *G. max* BAK1 paralogs, 11 had probe sets on the Affymetrix microarray used to determine gene expression occurring within the syncytium root cells undergoing the defense response to *H. glycines*. Under our criteria only *BAK1-1* and *BAK1-2* and *BAK1-14* exhibit expression at any of the 3 time points studied during the defense response ( $p < 0.05$ ). *BAK1-1* and *BAK1-2* which were the top 2 BlastP matches to the *A. thaliana* BAK1 are expressed at the time point prior to soil infestation with *H. glycines* as well as 3 and 6 dpi. That *BAK1-1* and *BAK1-2* are expressed prior to infection made each of them reasonable candidates to examine their role in defense. *BAK1-14* is expressed later in the defense response by 6 dpi and therefore may have a specialized defense function. A functional transgenic analysis of the *G. max* *BAK1-1* would clarify these observations.

*BAK1-1* was studied in functional transgenic analyses because it was the top match to the *A. thaliana* BAK1 protein sequence and had expression during the defense response. The transgenic *BAK1-1*-OE replicates, on average exhibited a 72% transformation efficiency with similar efficiencies observed for *BAK1-1*-RNAi and the pRAP15 and pRAP17 controls. These values are similar to what has been observed from the analyses of previously reported transformation efficiencies reported here. Furthermore, the results of the RT-qPCR analyses revealed that *BAK1-1* could be increased in its relative expression by 6.33-fold ( $p < 0.05$ , Student's *t*-

test) in the overexpressing roots as compared to its pRAP15 control. This observation is lower than the overall average of 11.24-fold ( $p < 0.05$ , Student's *t*-test) found for 84 different overexpressed genes spanning the prior analyses that used pRAP15. In contrast, *BAK1-1* RNAi roots exhibited decreased transcript abundance of  $-5.78$ -fold ( $p < 0.05$ , Student's *t*-test) in comparison to its pRAP17 control. This level of decreased transcript abundance is greater than the  $-3.84$ -fold ( $p < 0.05$ , Student's *t*-test) observed for 68 different genes whose RT-qPCR data has been analyzed. The results further demonstrate that there is variability in the level of effect that can be expected and this effect is likely caused by how these genes are regulated within the plant genome (Albrecht et al. 2008).

With the *BAK1-1*-overexpressing and RNAi roots in hand, it was possible to examine their effect on *H. glycines* parasitism. FI studies of the *BAK1-1* overexpressing roots resulted in a statistically significant 67% reduction in *H. glycines* parasitism in the wr analysis and a 68% reduction in the pg analysis ( $p < 0.05$ , MWW). The similarity in outcomes for the wr and pg analyses demonstrated that transgene expression had no effect on root growth under our conditions. Furthermore, the *BAK1-1*-RNAi roots experienced a statistically significant 4.8-fold increase in *H. glycines* parasitism in the wr analysis and a 4.9-fold increase in the *BAK1-1*-RNAi roots in the pg analyses ( $p < 0.05$ , MWW). Again, the similarity in the wr and pg results demonstrate there are no effects on root growth under our conditions. These results indicate that *BAK1-1* performs an important defense role in the *G. max*-*H. glycines* pathosystem. Taken together with the observation that *G. max* *BIK1-6* overexpression decreases *H. glycines* parasitism by 87%, the result indicates that *BAK1-1* may function along with various PRR co-receptors during the defense response. The *A. thaliana* *BAK1* is known to function along with different types of PRRs including FLAGELLIN-SENSITIVE 2 (FLS2), EF-Tu RECEPTOR (EFR) as well as the DAMP PEPTIDE 1 RECEPTOR (At-PEPR1) during plant defense processes (Li and Chory 1997; Veronese et al. 2006; Zipfel et al. 2006; Chinchilla et al. 2007; Zhang et al. 2010; Liu et al. 2013). Alternatively, other *G. max* *BAK1* genes contribute to the defense process. The *G. max* *BAK1-2* and *BAK1-14* both are shown to be expressed within syncytia undergoing the defense response. Other *G. max* *BAK1* genes lacking expression data could also function in the defense process.

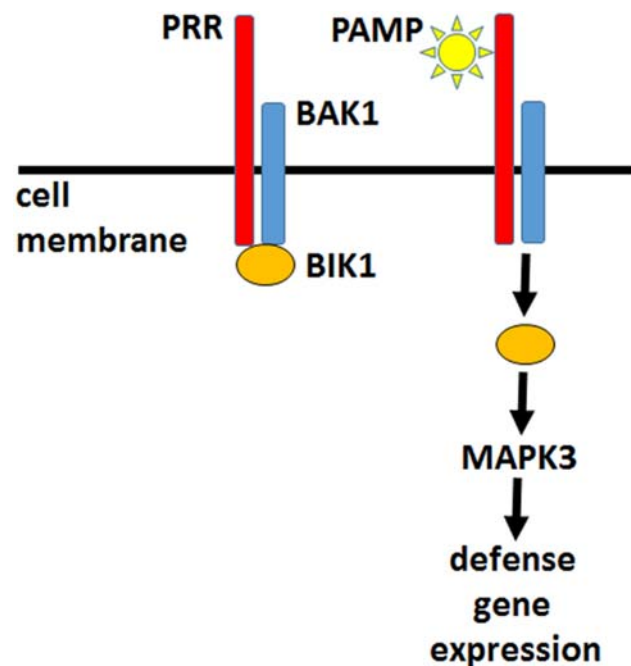
### The *G. max* *BAK1* functions as part of a signaling hub

The effectiveness that *BIK1-6* has in the defense response that *G. max* has toward *H. glycines* demonstrates it also likely functions as a signaling hub. Consistent with this observation, *G. max* *BIK1-6* overexpression leads to the induced expression of *MAPK3-1* and *MAPK3-2* while its RNAi decreases their relative transcript abundance (McNeece et al. 2019). *MAPK3* functions as a major signal transduction hub in plant defense processes, ultimately transducing signals to the nucleus to alter transcription of defense genes (Bi and Zhou 2017). The *A. thaliana* *BIK1*-binding protein *BAK1* functions through the MAPK pathway during the defense response and pathogen effector inhibition of *BAK1* impairs MAPK signaling (Zhou et al. 2014). The results presented here show that *G. max* has a *BAK1* membrane receptor homolog (*BAK1-1*) that functions in PTI that has a role in

its defense to *H. glycines* parasitism. Furthermore, the presence of *BAK1* homologs in many different plants, including those of agricultural significance, points to their likely important role in their basic biology and defense processes (Bajwa et al. 2013; Gao et al. 2013; Khew et al. 2015; Gravino et al. 2017; Wang et al. 2018b).

### Model

The results obtained here have led to the development of a model depicting the *G. max* *BAK1-1* as it functions in the defense response (Figure 8). In this model, the *G. max* *BAK1-1* is shown in close apposition to a PRR co-receptor. The PRR co-receptor could be a homolog of FLS2, EFR, PEPR1 or other yet to be identified co-receptor, each binding to *BIK1-6*. The co-receptor would detect the PAMP, leading to activation of *BAK1-1* and subsequently *BIK1-6*. Alternatively, some other molecule could be recognized by a *BAK1* co-receptor such as a damage associated molecular pattern (DAMP) (Gravino et al. 2017). Pathogen activation releases *BIK1-6* that then is responsible for the activation of *MAPKs* and ultimately defense gene expression. The work presented here is complimentary to previous studies that have shown the *G. max* ETI membrane receptor *NDR1-1* functions in defense to *H. glycines* with both *BIK1-6* and *NDR1-1* capable of inducing *MAPK3* expression (Aljaafri et al. 2017; McNeece et al. 2017, 2019). Furthermore, the result contributes to the understanding of both PTI and ETI functioning in defense to *H. glycines* through an increase in expression of *MAPKs* that increase the expression of proven defense genes (McNeece et al. 2019). This result is consistent with the important role that the *A. thaliana* *BAK1* has in plant defense by transmitting signals from *BIK1* and other kinases to *MAPKs* or the NADPH oxidase *RbohD* (Liang and Zhou 2018; Xue et al.



**Figure 8.** Model. (A), A yet to be identified PRR interacting with *G. max* *BAK1-1* which interacts with *BIK1-6* is poised to become activated. (B), A PAMP is recognized by the PRR, leading to *BAK1-1* and *BIK1-6* activation, releasing *BIK1-6*. *BIK1-6* induces the expression of defense genes. It is possible that another *BIK1* paralog functions in place of *BIK1-6*. Based off Pant et al. (2014), McNeece et al. (2019).

2020). The qPCR analysis of the *G. max* MAPK3-1 and MAPK3-2 showing their relative transcript abundance in the *BAK1-1*-OE and RNAi roots have revealed that *BAK1-1* influences their expression. The same observation has been made in RT-qPCR analyses of *BIK1-6*-OE and RNAi roots for MAPK3-1 and MAPK3-2 relative transcript abundance (McNeece et al. 2019). The *BAK1-1* results appear to indicate that important signals in the defense response that *G. max* has toward *H. glycines* are transduced through *BAK1-1*. Experiments identifying nematode associated molecular patterns (NAMPs) that are present in different plant parasitic nematode species, including *H. glycines*, have shown the NAMP ascr#18 activates MAPK3, and MAPK6 (Manosalva et al. 2015). Furthermore, ascr#18 treatment increased the relative transcript abundance for the MAPK-related *Flg22-INDUCED RECEPTOR KINASE1 (FRK1)* and the calcium-dependent protein kinase-related *PHOSPHATE-INDUCED1 (PHI1)* PAMP-related microbe-associated molecular pattern (MAMP)-triggered immunity (MTI) marker genes, consistent with a role for *BAK1* in the process (Boudsocq et al. 2010). Recent experimental evidence in *A. thaliana* has shown the RING-H2 FINGER A3A (RHA3A) and RHA3B E3 ubiquitin ligases mediate the monoubiquitination of BIK1 (Ma et al. 2020). This process has been shown to be an essential prerequisite for BIK1 release from the FLS2-BAK1 complex leading to immune signaling activation (Ma et al. 2020). The conserved nature of PTI and ETI indicate that homologs of these genes exist in the other important agricultural plant species described earlier including cassava, maize, rice, wheat, sorghum, rape seed, potato, tomato, oil palm, sugarcane and sugar beet and in some cases have been described (Gao et al. 2013; Bajwa et al. 2013; Zhang et al. 2015; Khew et al. 2015; Wang et al. 2018b; Yu et al. 2020).

### Future applications of the pRAP system of plant transformation vectors

The results presented here, generated over 10 years of time demonstrate the utility of the pRAP plasmids in transgenic research. The plasmids have been used primarily to understand the interactions that *G. max* and *G. hirsutum* have with parasitic nematodes and fungal pathogens. Newly emerging pathogens are a constant threat in agriculture providing an opportunity to identify and generate resistance before they become a major problem (Fisher et al. 2020). At one point, pathogens like *H. glycines* fit the definition of an invasive species since it arrived to the U.S. from elsewhere. However, there are newly emerging pathogens of great threat. For example, *Fusarium oxysporum* f.sp. *vasinfectum* race 4 (FOV4) has become a major threat to cotton production. Recent studies have been undertaken to understand its epidemiology and identify resistance (Ulloa et al. 2013; Wang et al. 2018a; Abdelraheem et al. 2020; Srivastava et al. 2021). The transgenic analysis of charcoal rot presented by Lawaju et al. (2018), through the use of the pRAP plasmids, provides a model for understanding and developing resistance for FOV4. Furthermore, the approach of candidate gene testing in the *G. hirsutum* for FOV7 has demonstrated that important genes (i.e. *G. hirsutum* *GLUTAMATE RECEPTOR LIKE 4.8 (GLR4.8)*) that underlie resistance can be identified (Liu et al. 2021). Consequently, model approaches used in one pathosystem have applicability to

solve newly emerging ones or ones where the candidate genes are already identified (Smigocki et al. 2013; Li and Smigocki 2016, 2018).

### Disclosure statement

No potential conflict of interest was reported by the author(s).

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