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Short-Form Bomanins Mediate Humoral Immunity in *Drosophila*

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Running Title: Bom-Mediated Humoral Immunity

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Abstract

The Bomanins (Boms) are a family of a dozen secreted peptides that mediate the innate immune response governed by the *Drosophila* Toll receptor. We recently showed that deleting a cluster of ten *Bom* genes blocks Toll-mediated defenses against a range of fungi and Gram-positive bacteria. Here we characterize the activity of individual Bom family members. We provide evidence that the Boms overlap in function and that a single *Bom* gene encoding a mature peptide of just 16 amino acids can act largely or entirely independent of other family members to provide phenotypic rescue *in vivo*. We further demonstrate that the *Boms* function in *Drosophila* humoral immunity, mediating killing of the fungal pathogen *Candida glabrata* in an *in vitro* assay of cell-free hemolymph. In addition, we find that the level of antifungal activity both *in vivo* and *in vitro* is linked to the level of *Bom* gene expression. Although Toll dictates expression of the antimicrobial peptides Drosomycin and Metchnikowin, we find no evidence that Boms act by modifying expression of the mature forms of these antifungal AMPs.

Introduction

Innate immune systems provide essential defenses against pathogens in both plants and animals and are the sole basis for defense in all non-vertebrates. Substantial insight into such systems grew out of the discovery of the Toll signaling pathway and its conservation from insects to humans [1,2]. We now have a fairly comprehensive picture of how pathogen invasion of body tissues is recognized and how an ensuing cascade of signaling triggers immune defense activation [3,4]. In many organisms these defenses are demonstrably essential: infection of wild-type hosts by a range of bacteria, fungi, or viruses results in minimal morbidity or mortality, whereas hosts with compromised innate immune function succumb, often rapidly. Nevertheless, a fundamental question remains – what effector activities mediate survival in the absence of a lymphocyte-directed adaptive response?

The study of innate antimicrobial effectors began in the 1920s with the discovery of lysozyme, which Fleming found can lyse bacterial cell walls [5]. In the 1970s, work in insects identified activities in hemolymph that kill bacteria or fungi and that increase in response to activation of innate defenses [6,7], culminating in the identification and isolation of the first three antimicrobial peptides in 1980 [8]. There was a flurry of interest in antimicrobial peptides (AMPs) as potential therapeutic agents and a general assumption that they represent the primary effector mechanism. However, very few mutations have been reported that link inactivation of one or more AMP genes to an immune deficiency. Moreover, in *Drosophila* such a link has been established not for an AMP, but rather for a set of novel effectors – the *Bomanin*, or *Bom*, gene family.

The *Bomanin* genes encode three forms of mature peptide – short, tailed, and bicipital – that are secreted into the hemolymph upon Toll pathway activation [9,10]. Ten of the twelve defined *Bomanin* genes are tandemly arrayed in a cluster at position 55C on the *Drosophila melanogaster* second chromosome. We have demonstrated that excising this cluster disrupts immune defenses to the same extent and with the same specificity as blocking the entire Toll pathway [10]. This finding raises a number of questions regarding Bom peptides as immune effectors. Are they humoral effectors or do they promote cellular immunity? Do they complex with one another or do they act as individual peptides? Is each Bomanin specific for a different pathogen?

In this paper, we explore the effector role of Boms via a two-pronged approach. We begin by using transgenic studies to define the activity of particular *Bom* genes. We then use microbiological and biophysical assays to define the nature of Bom activity *in vitro*. Together, these two approaches reveal that expression of individual Boms in hemolymph can provide both resistance to infection *in vivo* and pathogen killing *in vitro*.

Materials and Methods

Flies and Transgenic Strains

Flies were raised at 25°C on standard cornmeal molasses food. The *w¹¹¹⁸* strain was used as the wild-type. *Bom^{Δ55C}* flies were described previously [10].

All transgenic constructs were based on a genomic fragment – hereafter 55C-Right – that encodes the four 55C *Bom* genes (*Bom3*, *Bom836*, *Bom065*, *Bom068*) remaining in the *Bom*^{Δleft} second chromosome deficiency [10]. Specifically, genomic DNA encompassing region 2R:18,387,209 to 18,391,496 was amplified and cloned into attB-pNot-CaSpeR [11]. Next, individual *Bom* ORFs were deleted using PCR SOEing [12] in such a way as to generate four constructs, each deleted for three of the four *Bom* genes in 55C-Right.

To generate transgenic constructs expressing a single *Bom* gene under control of the *Bom3* promoter (*pBOM3*), the starting point was the 55C-Right construct with *Bom3* intact and the remaining *Bom* ORFs deleted. The *Bom3* ORF was then swapped out for another ORF using PCR SOEing, placing the introduced ORF directly downstream of *pBOM3*.

All constructs were introduced into the *Drosophila* genome by ΦC31-mediated transgenesis [13] at an *attP* landing site located at 86Fb (BDSC stock #24749). Stable lines established for each construct carry homozygous transgenes on the third chromosome in a *Bom*^{Δ55C} background.

CRISPR deletion of 55C region

Cloning and injections were performed using established protocols [14]. A pair of gRNAs designed to delete the region 2R:18,380,931 to 18,391,053 were cloned into pU6-BbsI-chiRNA (Addgene plasmid # 45946). Homology arms (766bp and 840bp left and right, respectively) were cloned into pDsRed-attP (Addgene plasmid # 51019). pBS-Hsp70-Cas9 (Addgene plasmid # 46294) was used as the Cas9 source. Constructs were injected into *w*¹¹¹⁸ flies.

Microbial Culture

Micrococcus luteus was cultured overnight in LB media at 37°C, heat-killed by autoclaving for 20 minutes at 121°C, and then concentrated to OD₆₀₀ = 300 in 20% glycerol. For infection experiments, *Candida glabrata* strain CBS 138 [ATCC 2001] was cultured overnight in YPD media at 37°C and concentrated to OD₆₀₀ = 100 in fresh PBS containing 0.01% Tween. For antimicrobial assay experiments, *C. glabrata* was grown to mid-log phase in YPD and then diluted 1:1000 in YPD.

Drosophila Infection, Survival Assays, and Statistical Analyses

Septic wounding, survival assays, and statistical analyses using GraphPad Prism were conducted essentially as previously described [10].

Bomanin Peptide Preparations

The 16-amino acid mature forms of Bom1 (GNVIINGDCRVCNVHG) and Bom3 (GNVIINGDCRVCNVRA) were synthesized by Lifetein and Biomatik, respectively, using a solid support resin with Fmoc and Boc chemistry. Peptides were delivered at 95% purity, confirmed by HPLC as well as mass spectrometry. Based on previously published mass spectrometry analysis [9], an intramolecular disulfide bond was introduced into both synthetic peptides and Bom1 was amidated at the carboxyl-terminus. Bom1 was dissolved in sterile water, and Bom3 was dissolved in 50% DMSO. Peptide concentrations were determined using the Qubit protein assay (Life Technologies) and were measured to be 43 μM (Bom1), and 56 μM (Bom3). These peptide solutions were introduced neat into the *in vitro* antimicrobial assay.

Hemolymph Preparation and Antimicrobial Assays

Toll pathway activation was achieved by wounding 2-7 day old male flies with heat-killed *M. luteus*. After incubating the flies at 29°C for 24 hours, cell-free hemolymph was collected from groups of seventy flies by a modification of a standard method [15]. Flies were loaded into a Zymo-Spin IC column (Zymo Research) previously washed twice with 200 µl sterile water. The flies were covered with 2 mm glass beads (Walter Stern) and centrifuged at 13,500 rpm atop a collection tube for 20 minutes at 4°C, yielding approximately four µl of cell-free hemolymph.

Antimicrobial assays with synthetic peptide or hemolymph were carried out as prescribed for standard antimicrobial peptide assays [16]. Two µl of peptide, hemolymph, or a combination of the two was mixed with an equal volume of log-phase *C. glabrata* suspended at 1:1000 in YPD. Samples were incubated either at room temperature for 1 hour or on ice for 24 hours. Following incubation, samples were diluted to 100 µl with YPD, spread onto YPD plates, and grown overnight at 37°C. Yeast colonies were counted the next day.

Hemolymph Extraction and MALDI-TOF Analysis

After Toll activation as above, hemolymph samples for MALDI-TOF were extracted from 2-7 day old male flies using glass capillaries, and transferred with a Narishige IM-300 Microinjector into two µl 0.1% trifluoroacetic acid (TFA)/ 50% acetonitrile (ACN). The samples were then mixed 1:1 with α -cyano-4-hydroxycinnamic acid (HCCA) matrix (Agilent: 2037A), and two µl were spotted onto a MALDI plate where sample and matrix cocrystallized *in situ* under a flow of warm air.

MALDI mass spectra were acquired using a Bruker Biflex IV MALDI-TOF mass spectrometer operated in either linear or reflectron mode with positive polarization. Desorption and ionization were achieved using a 337 nm laser. As an external mass calibration for linear mode spectra, equal parts of Peptide Calibration Standard II (Bruker: 8222570) and Protein Calibration Standard I (Bruker: 8206355) were mixed with HCCA matrix and spotted onto the same sample plate. For calibration of reflectron mode spectra, Peptide Calibration Standard II was mixed with HCCA matrix. For each genotype and condition, spectra from at least five individual flies were collected, yielding reproducible results. To identify particular peptides, the m/z values of peaks in our spectra were matched to those of the corresponding peaks in a prior study [9]. Spectra, for which representative examples are shown, were visualized using R 3.3.2, RStudio 1.0.136 and ggplot2 2.2.1.

Results

A Transgenic Assay Reveals the Effector Activity of a Single Bom Peptide

Ten *Bomanin* genes are clustered on the *D. melanogaster* second chromosome (Fig. 1). Our prior studies [10] defined two deletions affecting this cluster, designated 55C for its position on the polytene map. *Bom*^{Δ55C} flies, which lack the entire 55C *Bom* cluster, fail to mount a successful immune response against pathogens normally counteracted by the Toll pathway. In contrast, flies carrying the smaller *Bom*^{Δleft} deletion are susceptible to a number of pathogens, but have wild-type resistance to a relatively weak pathogen, the yeast *Candida glabrata*. Because *Bom*^{Δleft} lacks the six genes on the left (proximal) side of the *Bom* cluster, the four remaining *Bom* genes provided a useful starting point for defining the functional unit of Bom activity.

We generated a transgenic construct, hereafter 55C-Right, spanning the four intact *Bom* genes to the right of the deletion in *Bom*^{Δleft}. By generating flies carrying the 55C-Right transgene on each third chromosome and the *Bom*^{Δ55C} deletion on each second chromosome, we could recapitulate the genome of *Bom*^{Δleft}. Using 55C-Right as a template, we next generated four related constructs, each expressing only a single ORF from the four *Bom* genes originally present. We then introduced two copies of the wild-type or single-gene 55C-Right constructs into the *Bom*^{Δ55C} background and assayed survival after challenge with *C. glabrata* (Fig. 2).

The data in Fig. 2 demonstrate that a single *Bom* gene – either *Bom3* or *Bom065* – can restore resistance to *C. glabrata* in *Bom*^{Δ55C} flies. We note that both *Bom3* and *Bom065* encode short-form Bom peptides, defined as having a mature length of just 16 amino acids [10]. A third short-form *Bom* gene, *Bom068*, provided lesser but still significant rescue. Intriguingly, resistance strength appeared to correlate with *Bom* transcript level, as measured in Toll-induced wild-type adults (Table 1). Thus, resistance was higher for *Bom3* and *Bom065* and lower for *Bom068*.

Robust Expression of Short-Form Boms Conveys Resistance to C. glabrata

By further modifying the 55C-Right construct, we set out to compare the activity of different *Bom* forms when expressed at comparable levels. In particular, we replaced the *Bom3* coding region with that of other *Bom* genes, i.e., fused different *Bom* ORFs to the strongly Toll responsive *Bom3* promoter (*pBOM3*). In this fashion, we generated 55C-Right constructs that should express a single short, tailed, or bicipital Bom at the high levels normally observed for

Bom3 upon Toll pathway activation. We then assayed survival after challenge with *C. glabrata* (Fig. 3).

All three short-form *Bom* genes tested provided full resistance to *C. glabrata* when expressed under control of *pBOM3*. This included *Bom068*, which provided considerably weaker resistance when driven by its own promoter (compare Figs. 2 and 3). These data support the hypothesis that it is the level of expression, rather than the sequence composition, of short-form Boms that determines their contribution to survival. Furthermore, we find no evidence that the short-form Bom peptides vary intrinsically in specificity for *C. glabrata*.

We turned next to representative examples of tailed and bicipital Bomanins. A tailed form, *Bom836*, provided no rescue of *Bom*^{A55C} when controlled by its endogenous promoter. Analysis of endogenous transcript levels following Toll activation revealed far less transcript accumulation for *Bom836* than for either *Bom3* or *Bom065* (see Fig. 2 and Table 1). However, the *Bom836* ORF failed to generate detectable rescue even when fused to *pBOM3* (Fig. 3). Likewise, we did not observe rescue with *pBOM3*-driven expression of a bicipital form, *Bom23* (Fig. 3). Thus, neither a tailed nor a bicipital Bom exhibited activity against *C. glabrata*. For subsequent studies we therefore focused on the short-form Boms, for which our survival assay provides a robust readout *in vivo* for an individual gene product.

Short-Form Boms Mediate Cell-Free Killing of C. glabrata

Having found that expression of a single short-form Bom could rescue adults otherwise immunodeficient against *C. glabrata* infection, we turned our attention to *in vitro* studies. In

initial experiments we assayed synthetic mature short-form Bom peptides under conditions permissive for antimicrobial activity against *C. glabrata*. No such activity was detected with synthetic forms of mature Bom1 or Bom3 (data not shown).

In parallel, we set out to explore the *in vitro* activity of hemolymph, in which many of the Bom peptides are abundantly expressed upon infection. We stimulated the Toll pathway, collected cell-free hemolymph after 24 hours, and mixed it with an equal volume of mid-log *C. glabrata*. After incubation, the mixture was plated and the yeast colonies counted the next day. Two alternative conditions were used for incubation. The first – a one-hour incubation at room temperature – was designed to mimic conditions under which infection would normally occur. The second – a 24-hour incubation at 4°C – was designed to increase assay sensitivity. Wild-type hemolymph was fungicidal under either condition, with no detectable *C. glabrata* colonies formed (Fig. 4).

When we assayed hemolymph from *Bom*^{Δ55C} adults, there was a clear and dramatic difference from the wild type: deleting the 55C *Bom* genes eliminated the fungicidal activity of hemolymph. The *Bom* genes thus mediate a humoral antimicrobial activity against *C. glabrata*. Furthermore, the Bom-dependent activity of hemolymph *in vitro* parallels the Bom-dependent resistance observed *in vivo* against the same pathogen.

Hemolymph Activity Correlates with Bom Gene Dosage

To assay the effect of gene dosage on the level of Bom-dependent hemolymph activity, we took advantage of genetic backgrounds representing different overall levels of Bom expression. We

first assayed hemolymph from *Bom*^{Δ55C}; {55C-Right} flies. Antimicrobial activity was comparable to the wild type under both conditions (Fig. 4). We next assayed hemolymph from *Bom*^{Δ55C}; {*Bom065*} flies, in which expression of a single Bom peptide (Bom065) confers *C. glabrata* resistance in the *Bom*^{Δ55C} background (see Fig. 2). In this case we found no fungicidal activity by *Bom*^{Δ55C}; {*Bom065*} hemolymph at room temperature, but readily detectable and significant killing at 4°C overnight (Fig. 4). Thus, the strength of antimicrobial activity *in vitro*, like resistance *in vivo*, appears to correlate with the overall level of short-form Bom expression.

The Levels and Composition of Antifungal AMPs Appears Bom Independent

Although the Bomanins mediate antimicrobial activity, they could act upstream of known antimicrobial peptides (AMPs) to, for example, bring about accumulation or modification of AMP gene products. In the case of antifungal defenses, there are two AMPs that are robustly induced upon Toll activation and display strong antifungal activity *in vitro*: Drosomycin (Drs) and Metchnikowin (Mtk) [18,19]. (A third AMP, Cecropin, is active against a wide range of pathogens [20], but is inactive against *C. glabrata* [21].) We have previously shown that induction of mRNA expression by Toll does not require Bom function [10]. To determine if Boms act on these AMPs at the protein level, we turned to mass spectrometry.

Hoffmann, Bulet, and colleagues have reported an approach for characterizing the peptide composition of hemolymph from a single *Drosophila* adult [9]. Using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), they identified singly-charged ions for a set of 24 immune induced molecules (IMs). Adopting their approach, we obtained near identical spectrograms (Fig. 5 a, b, compare with Fig. 3 in [9]), enabling us to

identify individual peptides by their m/z ratio. Upon inducing innate immune signaling, the IMs were readily detectable, including Drs (IM19), Mtk (IM17), and three Boms (1, 2 and 3) in the 55C cluster (Fig. 5 b, c). The m/z for an additional 55C Bom peptide, Bom23, falls outside the mass range examined.

Next, we carried out parallel experiments with hemolymph from *Bom*^{Δ55C} flies (Fig. 5 d, e). As expected, no peaks were found with an m/z ratio matching that of Bom1, Bom2, or Bom3. Peaks were present, however, for both Drs and Mtk. In the case of Drs, the signal remained robust and with the same signal maximum. We note, however, that Drs has a relatively high molecular weight, precluding detection of a minor change in m/z in our study.

For Mtk, we did observe a significant difference in peak maxima, with a shift from 3044 m/z in the *w*¹¹¹⁸ control to 3022 m/z in *Bom*^{Δ55C}. This shift, however, is not Bom-dependent. Rather, it reflects a difference in Mtk isoform between the two genetic backgrounds. There are two known isoforms of Mtk, encoding either a His or Arg residue at position 3 in the mature peptide. Both isoforms are active [19]. The m/z values corresponding to the two species exactly match those we detect here.

To verify that the Bom phenotypes do not reflect a difference in Mtk isoform, we first sequenced the *Mtk* gene in the *w*¹¹¹⁸ and *Bom*^{Δ55C} backgrounds. This analysis confirmed the isoform identification by mass spectrometry: Mtk^{R3} in the *w*¹¹¹⁸ background and Mtk^{H3} in the *Bom*^{Δ55C} background. Next, we used CRISPR-Cas9 to generate a *Bom* 55C deletion in the *w*¹¹¹⁸ background, eliminating the polymorphism in Mtk between the control and experimental

samples. Using the newly derived *Bom*^{Δ55C-2} flies, we obtained survival curves after infection identical to those of the previously published TALEN-generated *Bom*^{Δ55C} flies (online suppl. Fig. 1).

Taken together, these studies reveal that deleting the *Bom* 55C cluster has no readily detectable effect on accumulation or post-translational modification of two major Toll-induced antifungal peptides in hemolymph.

Refined Understanding of the Bom Repertoire

Six of the 55C Boms are not detected even in wild-type hemolymph. Why? They are unlikely to represent pseudogenes, since they are well conserved at the amino acid level and lack any obvious nonsense or missense mutations. Instead, we favor two explanations. First, for a Bom with robust expression at the mRNA level, the peptide is likely present but masked. For example, we note that the calculated mass of the amidated form of Bom065 is 1723.8 m/z. This peptide is likely to fall in the shoulder of the robust peak for IM4 (calculated mass 1721.9 m/z) and therefore not be detected. Second, a number of Boms may have been missed simply because their level of immune induced expression is relatively low. Here, Bom068 provides a useful example. Compared to other short-form Boms, *Bom068* mRNA accumulates to only modest levels upon Toll activation (see Table 1). Consistent with this weak expression, we observed modest resistance to *C. glabrata* infection in flies expressing Bom068 from its endogenous promoter in a *Bom*^{Δ55C} background, and were unable to detect the peptide in MALDI-TOF. However, when the *Bom068* ORF was heterologously expressed under control of the strongly Toll responsive *pBOM3* promoter we observed wild-type resistance to *C. glabrata* infection and,

furthermore, ready detection of a peak at 1787 m/z, matching the predicted value in MALDI-TOF for Bom068 that has undergone proteolytic processing but not amidation (Fig. 5 f, g).

Discussion

Individual 55C Bom Genes Mediate Antimicrobial Defense

Transgenic analyses revealed that a single, short-form 55C *Bom* gene provides resistance to *C. glabrata* in the absence of the remaining nine genes from the cluster. Furthermore, every short-form *Bom* gene tested displayed such activity. The highly similar structure of the short-form Boms thus appears to reflect a shared activity found in each. These data make it unlikely that all ten genes of the cluster form a supramolecular complex or are all part of a common activity cascade.

We did not find transgenic activity for tailed or bicipital Boms. Two explanations appear very reasonable. First, these Bom forms might have specificity for particular pathogens other than *C. glabrata*. Bom activity has been observed not just for yeast, but also for representatives of the hyphal fungi and Gram-positive bacteria [10]. Second, the tailed and bicipital Boms might be active only in combination with other Bom family members.

Boms exist in a cluster across the *Drosophila* genus. Why did such a cluster arise if individual genes are active and interchangeable, at least in certain circumstances? Our results indicate that for particularly virulent pathogens, such as *Enterococcus faecalis*, successful immune defense requires very high levels of Bomanins [10]. We hypothesize that a single transcription unit is

insufficient to provide enough Bom peptide in the time frame required. By this model, duplication provided multiple, near-identical genes that could act as a battery for defense. Several lines of evidence support this idea. First, there are multiple, strongly Toll responsive *Bomanin* genes, each immediately downstream of a canonical TATA start site and almost always one or more perfect or near-perfect Toll responsive NF- κ B elements [22]. Second, the 55C-Right construct, inserted at 86Fb and lacking more than half the 55C cluster, expresses each of the four remaining *Bom* genes at the same level as in the full-length cluster, suggesting an absence of control elements shared across the ten genes (online suppl. Table 1).

If duplication was driven by a need for a high overall level of expression, what should we make of the variation we observe in sequence among Bom family members? It could provide sequence specificity for Bom action. It could also provide protection against pathogen countermeasures, varying residues targeted by microbial enzymes or inhibitors. Lastly, variation could also be a simple consequence of duplication and drift.

It is important to note that removing the 55C *Bom* cluster drastically reduces, but does not eliminate, Bom expression. A pair of additional *Bomanins* – *Bom778* (tailed) and *Bom791* (bicipital) – remain intact, as do two additional genes (*IM4*, *IM14*) that are immune induced and encode peptides that bear sequence similarity to the Bomanins. For this reason, caution is required in drawing conclusions with regard to the complete repertoire of the Bomanin family.

Bom Peptides Are Required for the Toll-Directed Humoral Defense

Bomanins are clearly required for the humoral response, as a preparation free from intact cells provides Bomanin-dependent fungicidal activity *in vitro*. Furthermore, this activity acts rapidly, killing *C. glabrata* within an hour under normal growth conditions. An involvement in humoral immunity makes good sense, as the Bomanins are secreted from the fat body into the hemolymph [9]. Indeed, they are loaded into hemolymph at concentrations sufficiently high as to suggest a stoichiometric role in defense rather than a role as cytokines.

To date, synthetic Boms have not been active in our *in vitro* assays. We have no easy explanation. The problem is not one of concentration, since MALDI-TOF revealed that synthetic peptide concentrations were well matched to those of the endogenously expressed forms. Furthermore, the measured mass to charge (m/z) values were also identical. Nor is the problem one of the exogenous peptides requiring a partner protein or other factor, since the synthetic Bomanins are also inactive when mixed with induced *Bom*^{Δ55C} hemolymph (unpublished observations). This failure to observe complementation *in vitro* suggests that the synthetic forms lack a conformation or stable interaction that is required for activity and that is normally provided *in vivo* prior to release into hemolymph.

Loss-of-function mutations in multiple family members provide a powerful tool for dissecting effector function. In this study, for example, we were able to define an essential role for the *Bom* family, assess the significance of gene duplication and divergence, and define effector specificity both *in vivo* and *in vitro*. Whereas small genes of overlapping function are a poor target for traditional mutagenesis, generating mutations in a number of such genes is now both straightforward and feasible. More insights and more surprises lie ahead.

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Disclosure Statement

The authors declare no competing financial interests.

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Figure Legends

Fig. 1. Organization of the *Bom* family cluster at cytogenetic map position 55C. This figure reflects a renaming of the *Bom* family members, with the CG or IM prefixes used previously replaced with the *Bom* prefix while retaining the gene number for both DIMs (one or two digits) and CGs (last three digits) for continuity with prior literature [9,17]. Accordingly, the two remaining *Bom* genes, located outside the 55C region, are now *Bom778* and *Bom791*. For the ten genes shown in this drawing, arrowhead direction denotes gene orientation. Color indicates form of the encoded *Bom* peptide: short (red), tailed (yellow), or bicipital (orange). Diagrams below illustrate the extent of the chromosomal deletions in *Bom*^{Δ55C} and *Bom*^{Δleft}. The bracket at the top of the figure encompasses the *Bom* genes in the 55C-Right genomic construct.

Fig. 2. A single *Bom* gene rescues the *Bom*^{Δ55C} immunodeficiency toward *C. glabrata*. This graph illustrates survival of transgenic adults at indicated intervals post-infection with *C. glabrata*. Each transgene encodes either the four *Bom* peptides of 55C-Right or a single one of those peptides, as indicated. All transgenes were present in two copies in a *Bom*^{Δ55C} background. Each curve represents the pooled results of three independent experiments involving 25 or more flies per genotype. Survival curves were compared using the Gehan-Breslow-Wilcoxon test. Significance is shown relative to the *no transgene* control (*Bom*^{Δ55C}) and adjusted for multiple comparisons (*** = p<0.0001; n.s. = not significant, p>0.05).

Fig. 3. Short-form *Bom* peptides mediate survival to *C. glabrata* infection. As in Fig. 2, this graph plots survival of adults at indicated intervals post-infection with *C. glabrata*. *Bom*

transgenes were expressed under control of either their endogenous promoter or *pBOM3*. All transgenes were present in two copies in a *Bom*^{Δ55C} background. Data were captured and analyzed as in Fig. 2.

Fig. 4. Fungicidal activity of hemolymph is Bom-dependent. Hemolymph activity was assayed for four sets of flies: *w*¹¹¹⁸ (wild-type), *Bom*^{Δ55C}, and *Bom*^{Δ55C} carrying either a 55C-Right (four-gene) or *Bom065* (single-gene) construct. All flies had been pricked one day earlier with heat-killed *M. luteus* to induce Toll-mediated gene expression. Fungicidal activity was assayed by incubating cell-free hemolymph with *C. glabrata* for 1 hour at room temperature (RT) or 24 hours at 4°C before spreading the mixture onto a yeast (YPD) plate. Percent killing was calculated by comparing the colony count for each sample to that for yeast mixed with a *w*¹¹¹⁸ uninduced hemolymph control. Experiments were performed in triplicate; error bars represent standard error of the mean. One-way ANOVA followed by Tukey's test was performed for each incubation condition. Significance is shown relative to the *w*¹¹¹⁸ uninduced control (••• and ♦♦♦ = p<0.0001; n.s. = not significant, p>0.05).

Fig. 5. Analysis of hemolymph from adult *Drosophila* by MALDI-TOF MS. Hemolymph (50 nl) was collected from a single fly from each genotype and analyzed by MALDI-TOF MS using linear (**a, b, d, f**) or reflectron mode (**c, e, g**) (see Materials and Methods). Representative MALDI spectra from five or more such samples are shown for hemolymph from a *w*¹¹¹⁸ fly either uninduced (control) (**a**), or induced 24 h earlier by Toll pathway stimulation with heat-killed *M. luteus* (**b, c**), and induced flies of the following genotypes: *Bom*^{Δ55C} (**d, e**) and *Bom*^{Δ55C}; {*pBOM3-Bom068*} (**f, g**). a.u. = arbitrary units. The y-axis was scaled to the tallest peak within

the window examined. Numbering and naming of induced peptides corresponds to published conventions [9]. As observed previously [9], a prominent peak appears in control spectra that becomes insignificant in the context of Toll induction.

Table 1. Survival to *C. glabrata* infection correlates with total *Bom* transcript level.

Transgene in <i>Bom</i> ^{A55C}	Gene: Present (+) or Absent (-)				Survival at 8 days (see Fig. 2)	Total transcript level from 55C <i>Bom</i> genes [§]
	<i>Bom3</i>	<i>Bom836</i>	<i>Bom065</i>	<i>Bom068</i>		
{55C-Right}	+	+	+	+	96%	59
{ <i>Bom3</i> }	+	-	-	-	95%	18
{ <i>Bom836</i> }	-	+	-	-	1%	3
{ <i>Bom065</i> }	-	-	+	-	93%	24
{ <i>Bom068</i> }	-	-	-	+	25%	9
no transgene	-	-	-	-	6%	none

[§]*Bom* transcript levels, quantified by real-time PCR and shown relative to *rp49*, were measured from 2-7 day old adult males 24 hours after Toll induction with heat-killed *M. luteus* (online suppl. Table 1).

Figure 1.

Author: Lindsay

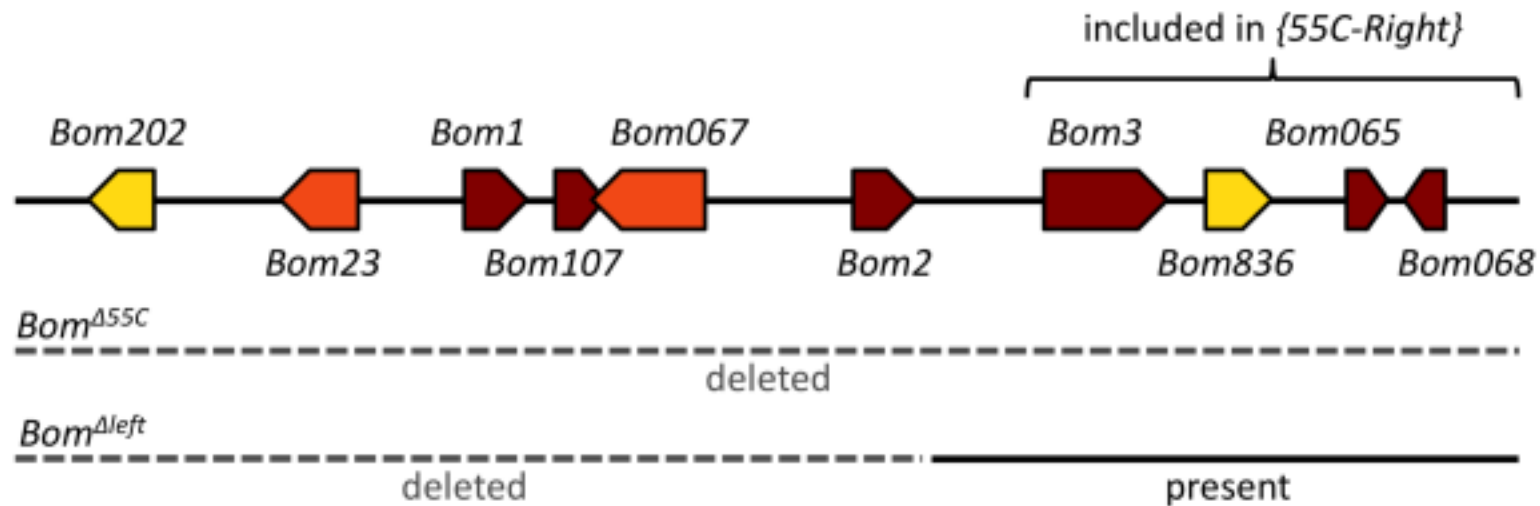


Figure 2.

Author: Lindsay

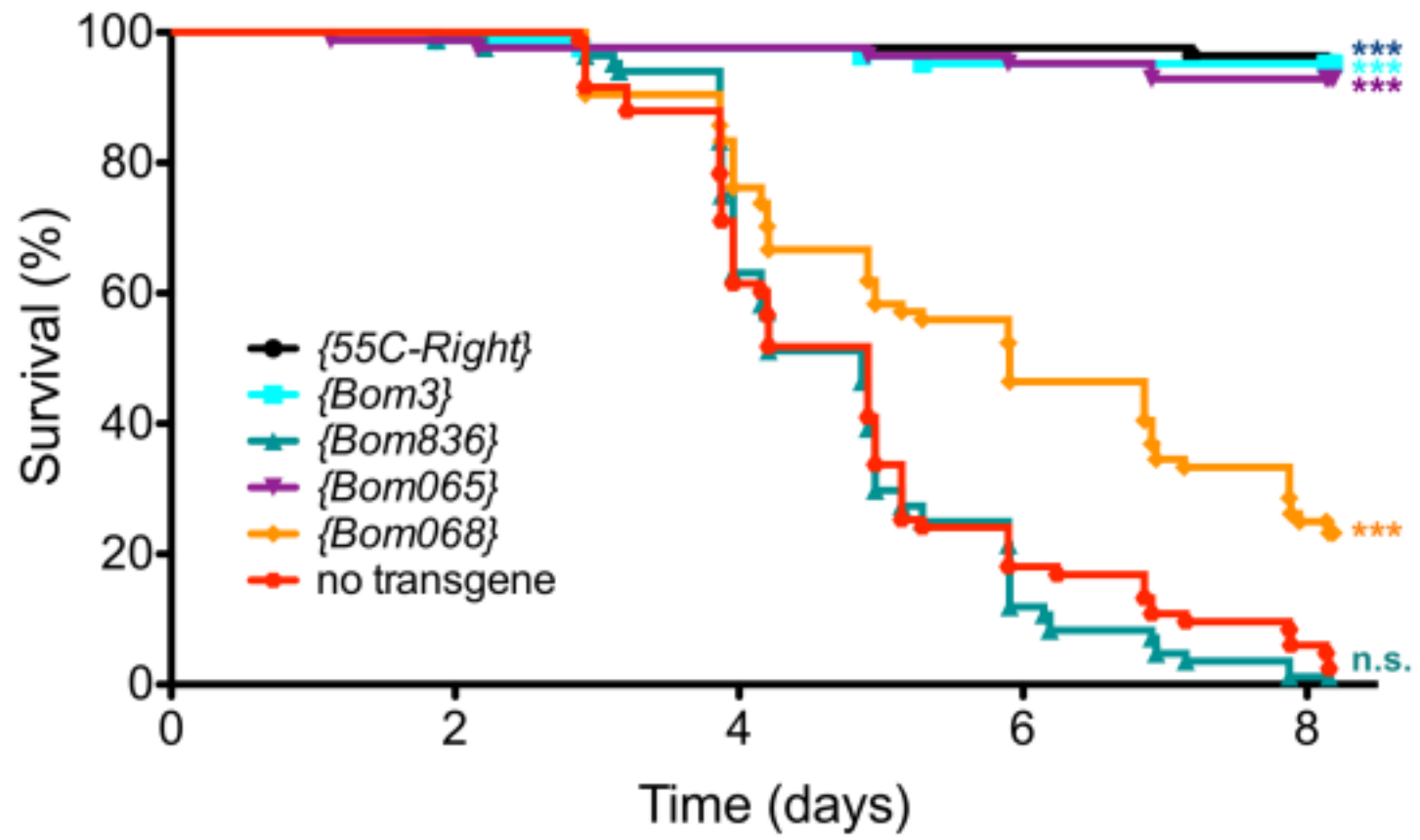


Figure 3.

Author: Lindsay

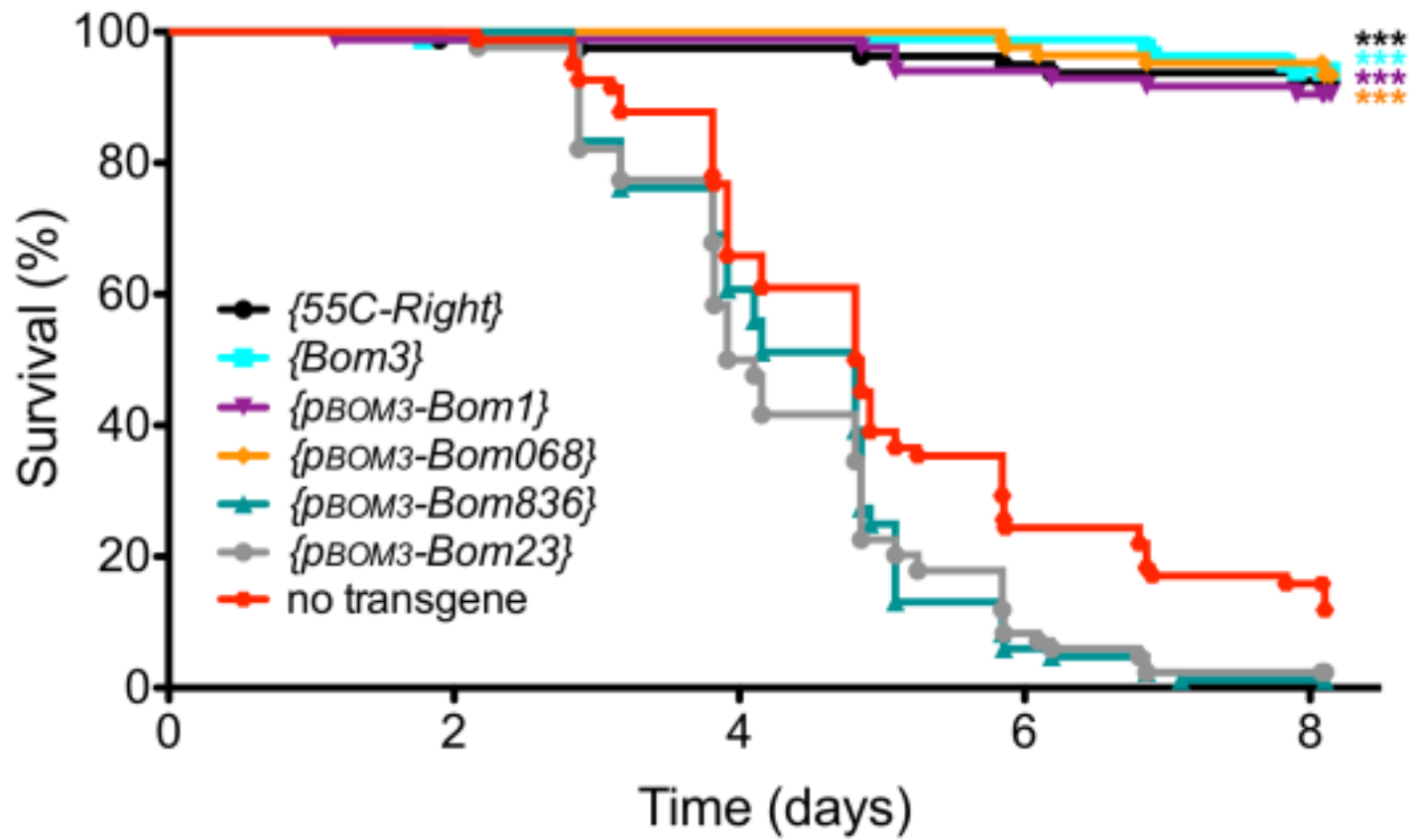


Figure 4.

Author: Lindsay

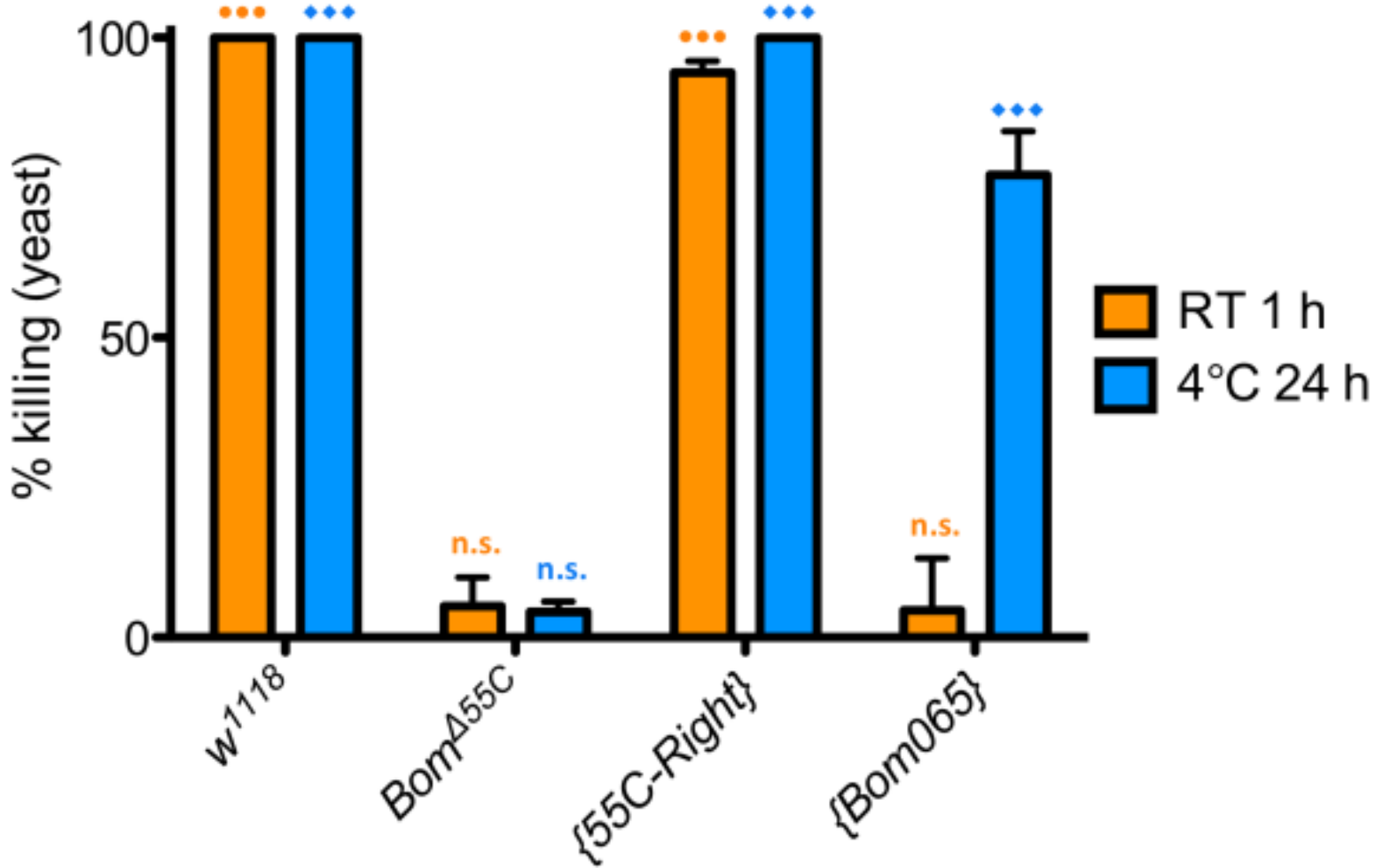
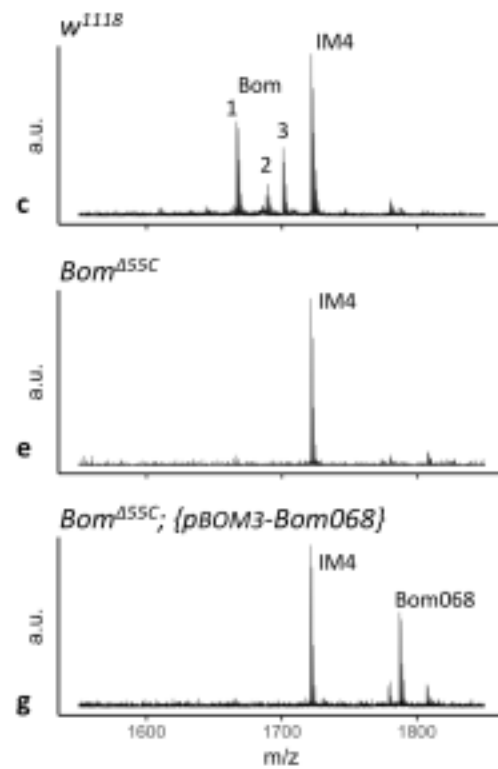
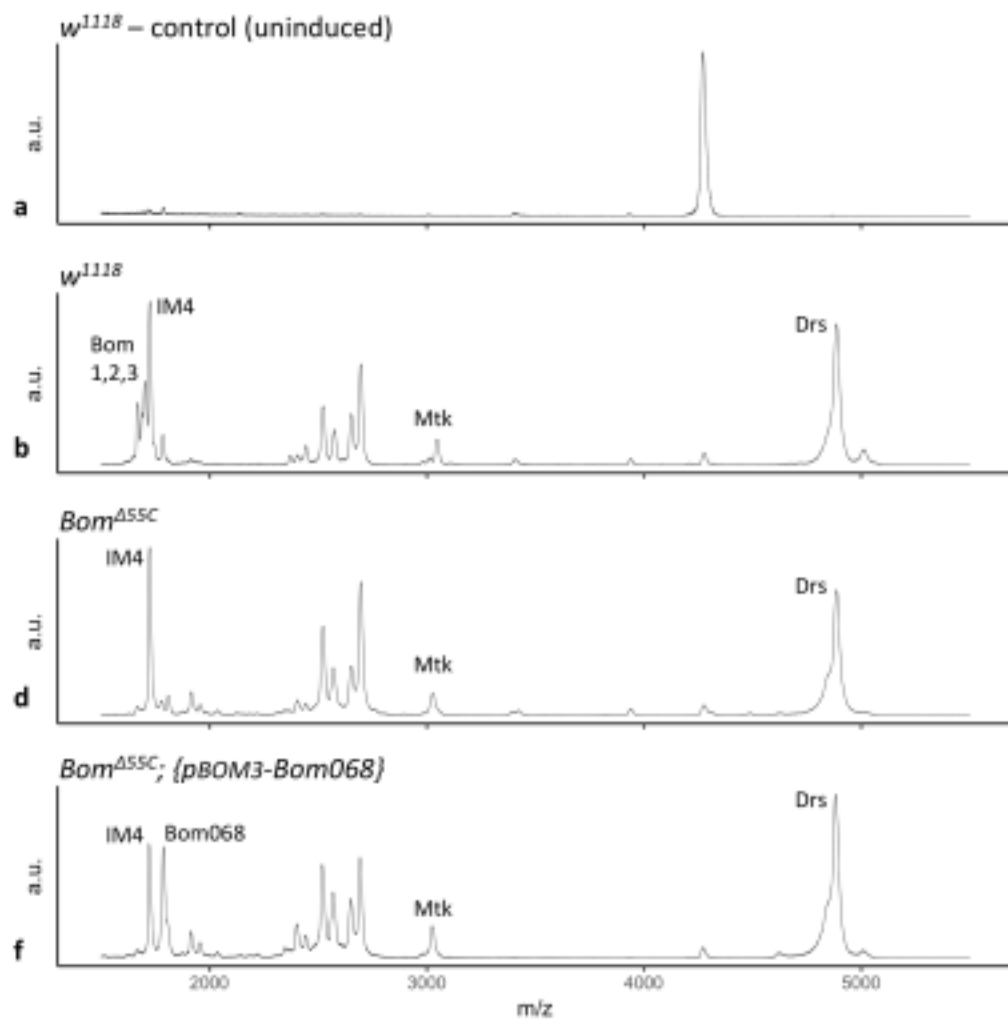


Figure 5.

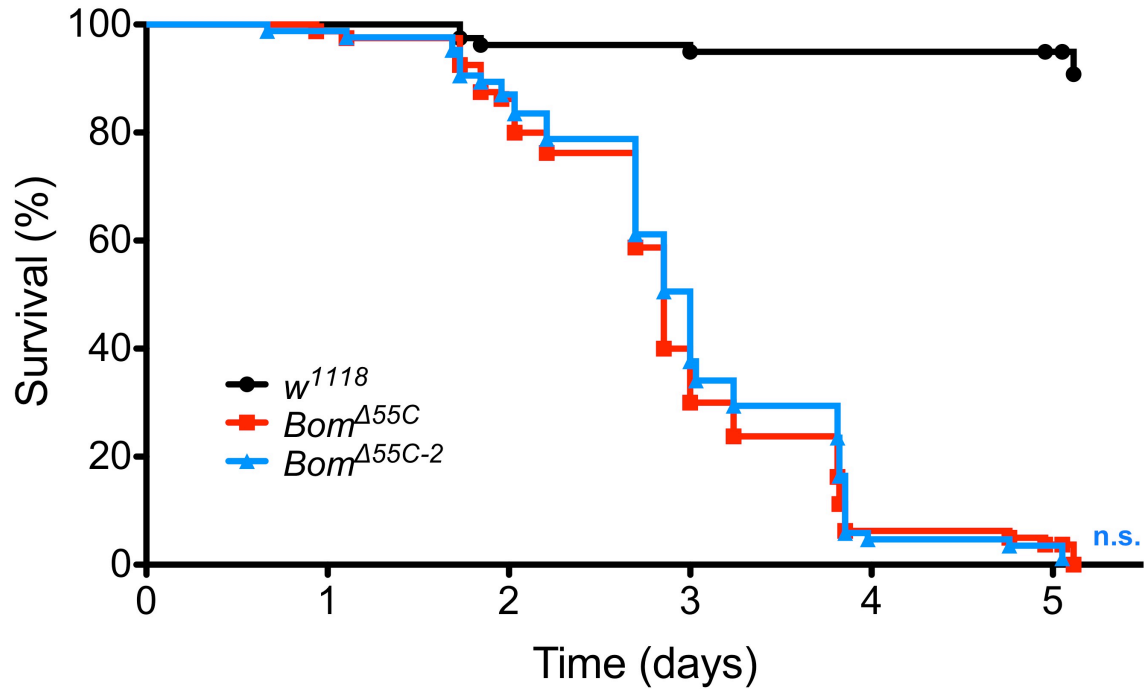
Author: Lindsay



Supplementary Table 1. Induced *Bom* transcript levels measured in wild-type and transgenic flies.

Genotype	Induced <i>Bom</i> transcript level [†]			
	<i>Bom3</i>	<i>Bom836</i>	<i>Bom065</i>	<i>Bom068</i>
<i>w¹¹¹⁸</i>	18 ± 1.1	2.8 ± .26	25 ± 4.0	3.2 ± .04
<i>Bom^{Δ55C}; {55C-Right}</i>	19 ± .78	3.5 ± .36	30 ± .80	6.5 ± .07
<i>Bom^{Δ55C}; {Bom3}</i>	18 ± .84	na	na	na
<i>Bom^{Δ55C}; {Bom836}</i>	na	3.0 ± .34	na	na
<i>Bom^{Δ55C}; {Bom065}</i>	na	na	24 ± 1.2	na
<i>Bom^{Δ55C}; {Bom068}</i>	na	na	na	8.9 ± .48

[†]Total RNA was extracted, using Trizol, from three separate groups of flies (2-7 day old adult males, n =6) for each genotype 24 hours after Toll induction with heat-killed *M. luteus*. RNA was reverse-transcribed (SuperScript II, Invitrogen) and real-time PCR was carried out using iQ SYBR Green Supermix (Bio-Rad) with an iCycler iQ instrument. Transcript levels are shown relative to *rp49*, quantified using the 2^{-ΔΔCt} method, and corrected for primer amplification efficiencies. Standard error of the mean is indicated. “na” = not applicable.



Supplementary Figure 1. *Bom^{Δ55C-2}* phenocopies *Bom^{Δ55C}*. Survival of flies after *C. glabrata* infection was monitored for wild type (*w¹¹¹⁸*), TALEN-generated Δ *Bom55C* (*Bom^{Δ55C}*), and CRISPR-generated Δ *Bom55C* (*Bom^{Δ55C-2}*). Data were captured and analyzed as in Fig. 2. Significance is shown relative to *Bom^{Δ55C}* (n.s. = not significant, $p > 0.05$).