

The Toll and Imd pathways are the major regulators of the immune response in *Drosophila*

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Microarray studies have shown recently that microbial infection leads to extensive changes in the *Drosophila* gene expression programme. However, little is known about the control of most of the fly immune-responsive genes, except for the antimicrobial peptide (AMP)-encoding genes, which are regulated by the Toll and Imd pathways. Here, we used oligonucleotide microarrays to monitor the effect of mutations affecting the Toll and Imd pathways on the expression programme induced by septic injury in *Drosophila* adults. We found that the Toll and Imd cascades control the majority of the genes regulated by microbial infection in addition to AMP genes and are involved in nearly all known *Drosophila* innate immune reactions. However, we identified some genes controlled by septic injury that are not affected in double mutant flies where both Toll and Imd pathways are defective, suggesting that other unidentified signalling cascades are activated by infection. Interestingly, we observed that some *Drosophila* immune-responsive genes are located in gene clusters, which often are transcriptionally co-regulated.

Keywords: Imd/innate immunity/oligonucleotide microarrays/Toll

Introduction

Innate immunity plays a very important role in combating microbial infection in all animals. The innate immune response is activated by receptors that recognize surface determinants conserved among microbes but absent in the host, such as lipopolysaccharides, peptidoglycans and mannans (Medzhitov and Janeway, 1997). Upon recognition, these receptors activate multiple and complex signalling cascades that ultimately regulate the transcription of target genes encoding effector molecules. Importantly, different pathogens elicit specific transcription programmes that can now be investigated by using microarray technology (De Gregorio *et al.*, 2001; Huang *et al.*, 2001; Irving *et al.*, 2001).

Drosophila is devoid of an adaptive immune system and relies only on innate immune reactions for its defence. Genetic and molecular approaches have shown that

Drosophila is a powerful model system to study innate immunity, which seems to be remarkably conserved from flies to mammals (Hoffmann and Reichhart, 2002; Tzou *et al.*, 2002a). To combat microbial infection, *Drosophila* activates multiple cellular and humoral responses including, for example, proteolytic cascades that lead to blood coagulation and melanization, the production of several effector molecules such as antimicrobial peptides (AMPs) and the uptake of microorganisms by blood cells (Tzou *et al.*, 2002a). AMPs are made in the fat body, a functional equivalent of mammalian liver, and secreted in the haemolymph, where they directly kill invading microorganisms (Hoffmann and Reichhart, 2002). Genetic analyses have shown that AMP genes are regulated by the Toll and Imd pathways (Hoffmann and Reichhart, 2002; Tzou *et al.*, 2002a). The Toll pathway is activated mainly by Gram-positive bacteria and fungi and controls in large part the expression of AMPs active against fungi, while the Imd pathway responds mainly to Gram-negative bacteria infection and controls antibacterial peptide gene expression (Hoffmann and Reichhart, 2002; Tzou *et al.*, 2002a). However, most of the AMP genes can be regulated by either pathway, depending on the type of infection, and the selective activation of Toll or Imd by different classes of pathogens leads to specific AMP gene expression programmes adapted to the aggressors. Thus, the control of AMP genes by the Toll and Imd pathways provides a good model to study how recognition of distinct microbes generates adequate responses to infection.

The Imd and Toll pathways do not appear to share any intermediate components and mediate differential expression of AMP-encoding genes via distinct NF- κ B-like transcription factors (Hoffmann and Reichhart, 2002; Tzou *et al.*, 2002a). Upon infection, the Toll pathway is activated in the haemolymph by an uncharacterized serine protease cascade that involves the serpin Necrotic and leads to the processing of Spaetzle, the putative Toll ligand. Binding of Spaetzle to Toll activates an intracellular signalling cascade, involving the adaptor proteins dMyD88 and Tube, and the kinase Pelle, that leads to degradation of the I κ B-like protein Cactus and the nuclear translocation of the NF- κ B-like transcription factors Dif and Dorsal (Hoffmann and Reichhart, 2002; Tzou *et al.*, 2002a). An extracellular recognition factor, peptidoglycan recognition protein (PGRP)-SA, belonging to a large family of proteins that bind to peptidoglycan has been implicated in the activation of the Toll pathway in response to Gram-positive bacteria but not fungi (Michel *et al.*, 2001). These data support the idea that the Toll pathway is activated by soluble recognition molecules that trigger distinct proteolytic cascades converging to Spaetzle.

Recently, several studies have led to the genetic and molecular identification of seven components of the Imd

pathway (Hoffmann and Reichhart, 2002; Tzou *et al.*, 2002a). The ultimate target of the Imd pathway is Relish, a rel/NF- κ B transactivator related to mammalian P105. Current models suggest that this protein needs to be processed in order to translocate to the nucleus. Its cleavage is dependent on both the caspase Dredd and the fly I κ B-kinase (IKK) complex. Epistatic experiments suggest that dTAK1, a MAPKKK, functions upstream of the IKK complex and downstream of Imd, a protein with a death domain similar to that of mammalian receptor-interacting protein (Hoffmann and Reichhart, 2002; Tzou *et al.*, 2002a). Recently, three independent studies have shown that a putative transmembrane protein, PGRP-LC acts upstream of Imd and probably functions in sensing microbial infection (Choe *et al.*, 2002; Gottar *et al.*, 2002; Rämét *et al.*, 2002b). The *Drosophila* Toll and Imd pathways share many features with the mammalian TLR/IL-1 and TNF-R signalling pathways that regulate NF- κ B, pointing to an evolutionary link between the regulation of AMP gene expression in flies and the mammalian innate immune response (Hoffmann and Reichhart, 2002; Tzou *et al.*, 2002a).

We identified 400 *Drosophila* immune-regulated genes (DIRGs) through a microarray analysis of the transcription programmes induced by septic injury and by natural fungal infection (De Gregorio *et al.*, 2001). Many of these genes were assigned to functions related to the immune response including, in addition to the AMP response, microbial recognition, phagocytosis, melanization, coagulation, reactive oxygen species (ROS) production, wound healing and iron sequestration. Although the regulation of AMP genes by Imd and Toll pathways has been studied extensively, little is known about the role of these two pathways in the control of other genes regulated by infection in *Drosophila*. In this study, we have characterized further the role of the Toll and Imd pathways in the *Drosophila* host defence. To study the contribution of each pathway in the resistance to infection, we first compared the susceptibility of flies carrying mutations affecting the Toll, Imd or both signalling cascades with several types of bacterial and fungal infection by a survival test. Secondly, we analysed, using northern blots, the expression of AMP genes after different types of infection in the same mutants. Finally, we monitored by microarray analysis the effect of mutations affecting the Toll and Imd cascades on the transcriptional reprogramming induced by septic injury. Our study demonstrates that the Toll and Imd pathways are the major regulators of the immune response in *Drosophila* adults.

Results

Contribution of the Toll and Imd pathways to resist microbial infection

It has been shown that mutants of the Imd pathway are more susceptible than wild-type flies to Gram-negative bacterial infection, while mutants of the Toll pathway are more susceptible to fungal and Gram-positive bacterial infection (Lemaitre *et al.*, 1996; Rutschmann *et al.*, 2002). Three *Drosophila* lines carrying mutations affecting both the Toll and Imd pathways (*imd;spz*, *imd;Tl* and *dif;kenny*), have been reported to be sensitive to both bacterial and fungal infections (Lemaitre *et al.*, 1996;

Leulier *et al.*, 2000; Rutschmann *et al.*, 2000, 2002). However, these double mutant lines probably retain limited Toll or Imd activity because the *imd* allele is a hypomorph and *dif* mutants retain Dorsal activity (Georgel *et al.*, 2001). We have generated double mutant *Drosophila* lines by recombining two strong alleles of the Toll pathway (*spz: spz^{tm7}* and *Tl: Tl^{1-RXA/Tl⁶³²}*) with a null allele of *relish* (*rel: rel^{E20}*). The comparison of the susceptibility to microbial infection of flies deficient for either the Toll (*spz* or *Tl*) or Imd (*rel*) pathway and flies mutated for both *rel,spz* or *rel,Tl* allows us to analyse in detail the contribution of each pathway to host defence.

Wild-type Oregon^R (wt), single (*rel*, *Tl* and *spz*) and double mutant (*rel,spz* and *rel,Tl*) adult flies were injected with Gram-negative bacteria (*Escherichia coli*), Gram-positive bacteria (*Micrococcus luteus* and *Enterococcus faecalis*) and fungi (*Aspergillus fumigatus*) or were naturally infected with the spores of the entomopathogenic fungus *Beauveria bassiana* (Figure 1). As previously observed, *Tl* and *spz* mutants are resistant to *E.coli* injection while *rel* flies are highly susceptible, dying within 3 days (Lemaitre *et al.*, 1996; Hedengren *et al.*, 1999; Leulier *et al.*, 2000). Surprisingly, both double mutants (*rel,spz* and *rel,Tl*) are more susceptible than *rel* to *E.coli* infection, suggesting that the Toll pathway triggers a significant response against Gram-negative bacteria (Figure 1A). This is in agreement with a previous study showing that *dif;kenny* double mutants die earlier than *kenny* flies after infection by *E.coli* (Rutschmann *et al.*, 2002). To study the contribution of Toll and Imd pathways to resist Gram-positive bacteria infection, we injected two bacterial strains: *M.luteus*, which does not kill flies deficient in the Toll or Imd pathway (Leulier *et al.*, 2000); and *E.faecalis*, which kills *spz* flies very rapidly (Rutschmann *et al.*, 2002). Interestingly, we noticed that double mutant lines are very sensitive to infection by *M.luteus* (Figure 1B) and that *rel,spz* double mutants are slightly more susceptible than *spz* flies to *E.faecalis* infection (Figure 1C). These data confirm that the Toll pathway is the most important pathway in fighting Gram-positive bacterial infection but indicate that the Imd pathway can also play a significant role. Finally, we observed that *rel,spz* and *rel,Tl* are almost equally as susceptible as single mutants in the Toll pathway (*Tl* or *spz*) to injection of *A.fumigatus* and to natural infection by *B.bassiana*, suggesting that the Imd pathway is not essential for the antifungal response (Figure 1D and E).

Next, we analysed the effect of mutations affecting Imd and Toll pathways on the expression of AMP genes after injection of *E.coli*, *M.luteus* or *A.fumigatus*. Figure 2 shows a northern blot analysis of two antibacterial peptide genes (*attacin* and *diptericin*) and two antifungal peptide genes (*drosomycin* and *metchnikowin*). The double mutants *rel,spz* and *rel,Tl* failed to show induction of AMP genes. In fact, the only AMP transcript detectable in these flies is the antifungal *drosomycin*, which is present at a level similar to that in unchallenged flies. *Diptericin* is regulated by the Imd pathway, while *metchnikowin*, *attacin* and *drosomycin* are regulated by both pathways. Interestingly, the contribution of each pathway to the expression of each AMP gene depends on the type of infection. For example, in agreement with previous studies (Leulier *et al.*, 2000), *drosomycin* expression is affected

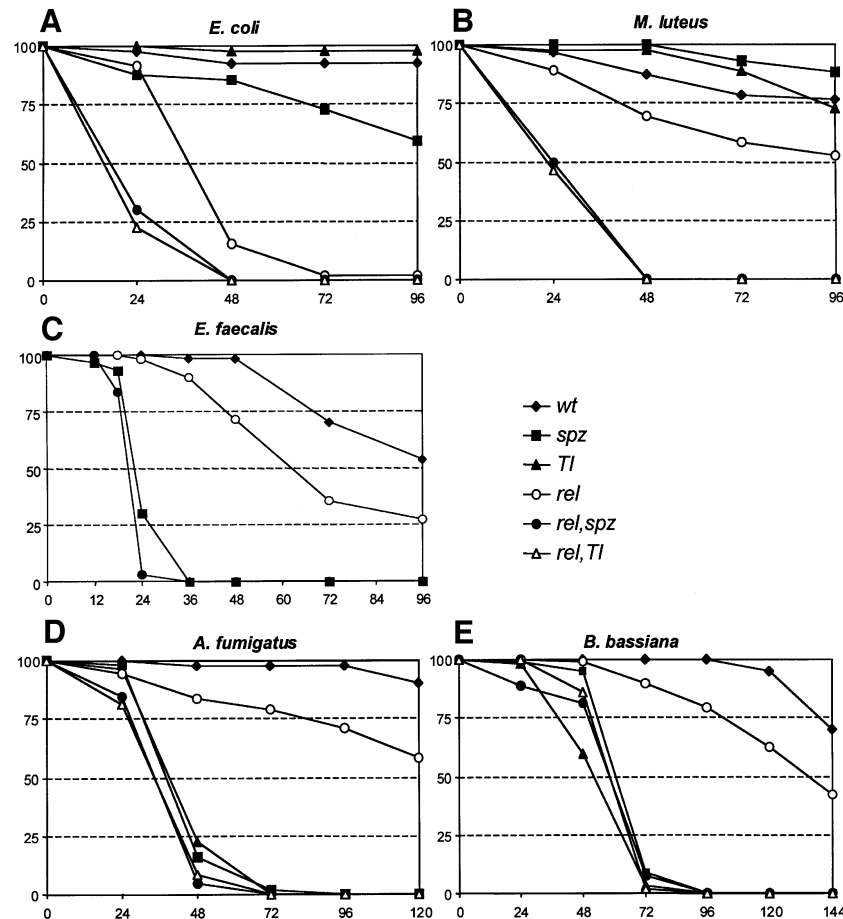


Fig. 1. Contribution of Toll and Imd pathways to the resistance to bacterial and fungal infections. Wild-type (*wt*), single (*spz*, *Tl* and *rel*) or double mutant (*rel,spz* and *rel,Tl*) adult flies were subjected to septic injury using *E.coli* (A), *M.luteus* (B), *E.faecalis* (C) and *A.fumigatus* (D) or to natural infection using *B.bassiana* (E). The graphs show the survival rate (%) at specific times after infection (h). All infection experiments were performed at 29°C, except for *E.faecalis* infection that was conducted at 25°C. *Tl*-deficient flies were not subjected to *E.faecalis* infection because they do not display a strong phenotype at 25°C (see Materials and methods). The presence of the *ebony* mutation in the *rel* mutant line may explain the slight susceptibility of *rel* flies after fungal infection (Leulier *et al.*, 2000).

similarly by the *rel* and Toll pathway mutants after *E.coli* infection, but is regulated predominantly by the Toll pathway during *M.luteus* or *A.fumigatus* infections (Figure 2).

The results obtained by northern blot analysis correlate with the data from survival experiments. The contribution of the two pathways to the control of the antibacterial peptides (Figure 2) is consistent with the augmented sensitivity to bacterial infection of double mutant flies versus single mutants (Figure 1A–C). The level of the antifungal peptide Drosomycin transcript after fungal infection is very similar in the double mutant flies (*rel,spz* and *rel,Tl*) compared with *Tl* and *spz* single mutants (Figure 2C), consistent with a similar resistance to *A.fumigatus* and *B.bassiana* displayed by these four lines (Figure 1C and D). Importantly, the *Tl* and *spz* alleles alone, or in combination with *rel*, display the same behaviour in all survival experiments performed (Figure 1) and have a similar pattern of AMP gene expression (Figure 2), suggesting that Spaetzle is the sole extracellular activator of the Toll pathway in response to microbial infection. However, we noticed that *attacin* and *dipericin* expression after *A.fumigatus* infection is

reduced in *Tl* but not in *spz* flies (Figure 2C). We extended the analysis of *A.fumigatus* infection to *pelle*, *tube* and *dif* mutants (data not shown), which display the same AMP expression profile as *spz*, suggesting that the effect observed in *Tl* flies is due to the genetic background of the strain used. The complete survival and northern analysis presented here was extended to a strong allele of *pelle* alone or in combination with *rel*, which gave similar results to *spz* and *Tl* alleles (data not shown).

The Toll and Imd pathways control the majority of *Drosophila* immune-regulated genes

To identify which of the 400 previously identified DIRGs are controlled by the Imd and/or Toll pathways, mRNA samples from *spz*, *rel* and *rel,spz* adult males, collected after septic injury with a mixture of *E.coli* and *M.luteus*, were hybridized to Affymetrix DrosGenome1 GeneChips capable of measuring mRNA levels for nearly every gene in the *Drosophila* genome. The gene expression profiles obtained for the mutant flies were compared with our previous analysis of wild-type flies. Since double mutants start to die within 1 day after bacterial infection (Figure 1A and B), we limited our analysis to the first 6 h of the

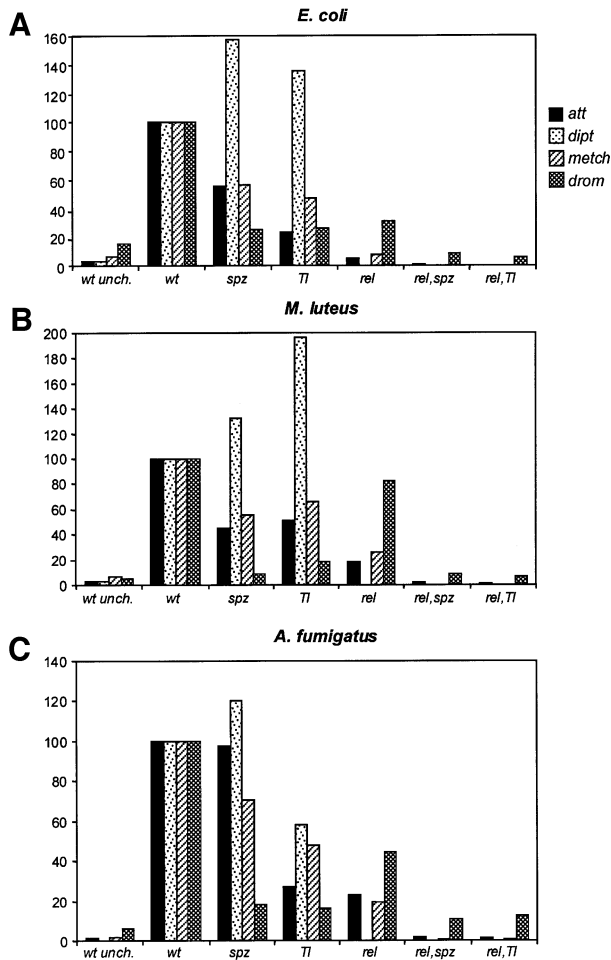


Fig. 2. Contribution of the Toll and Imd pathways to the induction of AMP genes. Total RNA was extracted from unchallenged wild-type flies (wt unch.) or 6 h after infection of wild-type (wt) single (*spz*, *Tl* and *rel*) or double mutant (*rel,spz* and *rel,Tl*) adult flies with *E.coli* (A), *M.luteus* (B) and *A.fumigatus* (C). Expression levels of *attacin* (*att*), *dipterizin* (*dipt*), *metchnikowin* (*metch*) and *drosomycin* (*drom*) were measured by northern blotting. The signal of each AMP-encoding gene was quantified by PhosphorImager and normalized with the corresponding value of the *rp49* gene. The graphs show the amount of each AMP transcript relative to the level measured in wild-type challenged flies that was set to 100. Northern blot analysis was carried out as in Leulier *et al.* (2000).

immune response (time points: 0, 1.5, 3 and 6 h), ensuring that the changes in expression profiles are not an indirect consequence of the sickness of the flies. In addition to loss-of-function mutants, we also observed the genome-wide changes in gene expression of uninfected *Tl^{10b}/+* flies carrying a gain-of-function allele that constitutively activates the Toll pathway. Each time series was observed in duplicate, while the *Tl^{10b}* allele was assayed three times. Complete results can be found at <http://www.fruitfly.org/expression/immunity/>.

General statistics and hierarchical cluster analysis

Out of the 400 DIRGs previously identified, the majority (283) display a significant change in the expression pattern in the first 6 h (for each gene $P < 0.0025$) (Figure 3A).

Using an automated approach, we determined for each gene whether the gene expression profile is significantly different in a mutant background compared with wild-type (see Materials and methods). We observed that half of the 162 up-regulated genes examined (86) are not induced in *rel,spz* double mutants, 32 are partially affected and only 44 are still fully induced in this background (Figure 3A, upper table). Similar data were obtained from the analysis of 121 down-regulated genes, the majority of which are dependent (46) or partially dependent (27) on Relish and Spaetzle for their regulation, while 48 genes show no significant difference in *rel,spz* compared with wild-type flies (Figure 3A, lower table). Within the group of DIRGs affected in *rel,spz* flies, we could distinguish four categories based on their differential response to *spz* and *rel* mutations (see Venn diagrams in Figure 3A). Genes affected in *rel* but not in *spz* flies are probably controlled by the Imd pathway. In contrast, genes affected in *spz* flies but not in *rel* are probably controlled by the Toll pathway. We also found genes that are affected in both single mutants, which are probably regulated by both Imd and Toll pathways, and genes that are affected only in double mutant flies, suggesting that the two pathways play redundant roles in their regulation (Figure 3A). The tables in Figure 3A also show that 34 induced and 12 repressed DIRGs are regulated in *Tl^{10b}* flies in the absence of infection. Interestingly, 34 of them are significantly affected in the *rel,spz* background and 23 in *spz* flies. It does appear that the Imd pathway may be less important in repressing DIRGs, as very few repressed DIRGs are only dependent on Relish (Figure 3A).

To analyse the gene expression profile in more detail, we hierarchically clustered all 400 DIRGs including previous data obtained after *B.bassiana* infection (time points: 0, 12, 24 and 24 h) and the complete kinetics of wild-type flies after septic injury (time points: 0, 1.5, 3, 6, 12, 24 and 48 h) (Figure 3B). We observe that most of the genes induced after fungal infection display a late or sustained response after septic injury and are not induced in *spz* and *rel,spz* flies, while they are fully induced in *rel* mutants and are up-regulated in unchallenged *Tl^{10b}* flies (Figure 3B, cluster UP6). These data strongly suggest that this group of genes is controlled by the Toll pathway. In addition to the UP6 cluster, Spaetzle can regulate acute phase genes (UP5). In contrast to Spaetzle, Relish controls predominantly early and sustained phase genes, which are not induced by fungal infection or in *Tl^{10b}* flies (UP4). Up-regulated genes independent from Relish and Spaetzle (clusters UP1 and UP3) are generally weakly induced by fungal infection and not affected by *Tl^{10b}*.

The analysis of repressed DIRGs shows that Spaetzle can regulate both early (D5 and D9) and late/sustained (D1 and D3) phase genes, while Relish partially controls a small number of early phase genes (D8). A large group of late phase genes repressed after fungal infection are regulated by both Relish and Spaetzle (D10). Interestingly, a second group of genes strongly repressed after fungal infection (D2) are not affected by *rel* and *spz* mutations but affected in *Tl^{10b}* flies.

Our analysis shows that the Toll and Imd pathways regulate the majority of the immune-responsive genes. However, the presence of genes not affected, or only partially affected, in the *rel,spz* background suggests that

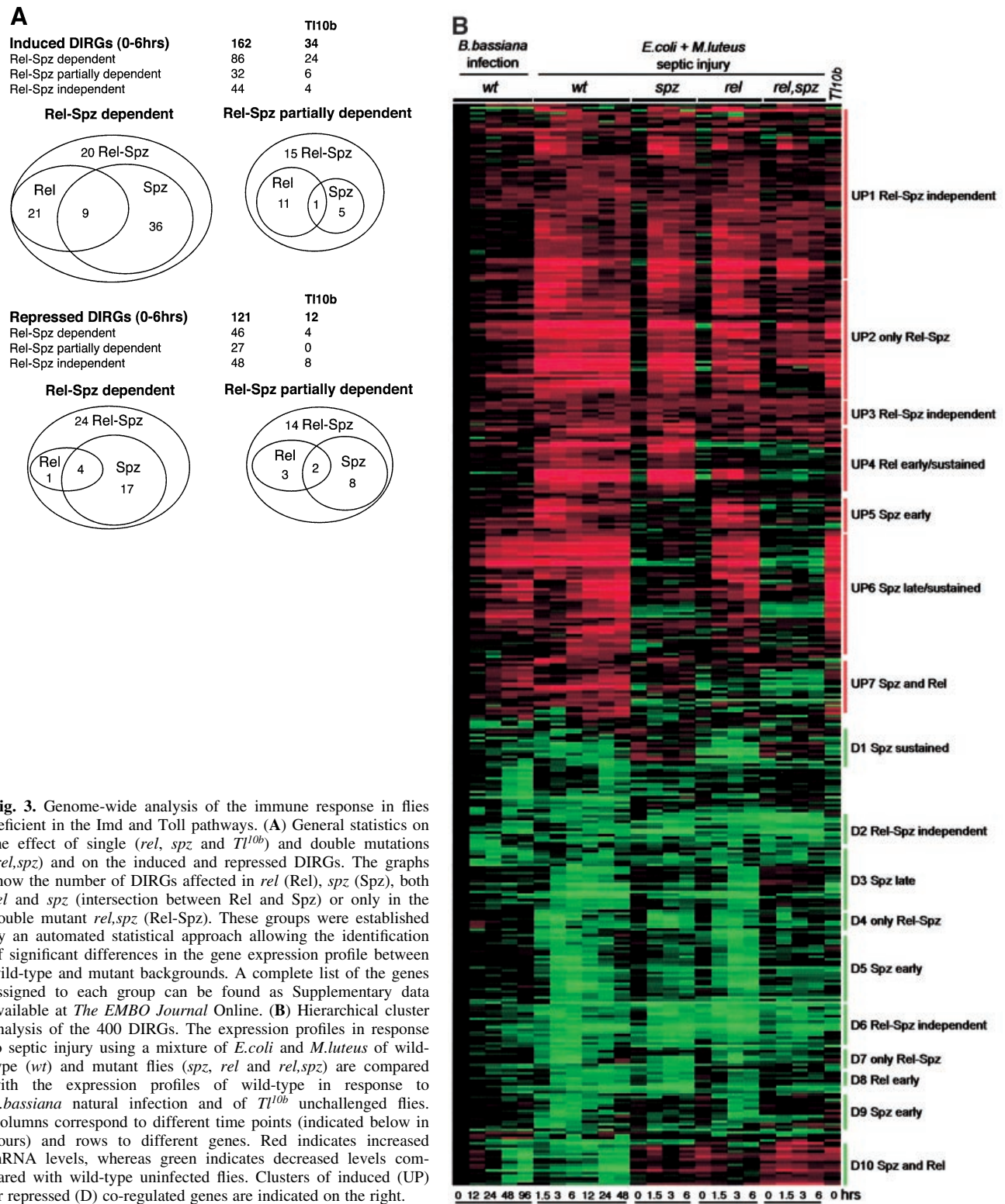


Fig. 3. Genome-wide analysis of the immune response in flies deficient in the Imd and Toll pathways. **(A)** General statistics on the effect of single (*rel*, *spz* and *Tl^{10b}*) and double mutations (*rel,spz*) and on the induced and repressed DIRGs. The graphs show the number of DIRGs affected in *rel* (Rel), *spz* (Spz), both *rel* and *spz* (intersection between Rel and Spz) or only in the double mutant *rel,spz* (Rel-Spz). These groups were established by an automated statistical approach allowing the identification of significant differences in the gene expression profile between wild-type and mutant backgrounds. A complete list of the genes assigned to each group can be found as Supplementary data available at *The EMBO Journal* Online. **(B)** Hierarchical cluster analysis of the 400 DIRGs. The expression profiles in response to septic injury using a mixture of *E. coli* and *M. luteus* of wild-type (*wt*) and mutant flies (*spz*, *rel* and *rel,spz*) are compared with the expression profiles of wild-type in response to *B. bassiana* natural infection and of *Tl^{10b}* unchallenged flies. Columns correspond to different time points (indicated below in hours) and rows to different genes. Red indicates increased mRNA levels, whereas green indicates decreased levels compared with wild-type uninfected flies. Clusters of induced (UP) or repressed (D) co-regulated genes are indicated on the right.

other pathways regulate the *Drosophila* immune response. Consistent with the survival experiments (Figure 1), we found that most of the genes induced by fungal infection are regulated by the Toll pathway after septic injury, without contribution of the Imd pathway, and that the two pathways contribute to the control of many genes induced only by bacterial infection.

Target genes of Toll and Imd pathways

To address which immune reactions are controlled by Relish, Spaetzle, both Relish and Spaetzle or by a still unknown mechanism, we examined the effect of the *rel* and *spz* mutations on the expression of selected DIRGs (Table I). Unlike our previous automated analysis, we used a less stringent approach. In Table I, we considered each

gene affected by a mutation when we detected at least a 2-fold change in one time point in the mutant line compared with the corresponding time point in wild-type flies. As previously shown by northern blot analysis (Lemaitre *et al.*, 1996; Hedengren *et al.*, 2000), we found that most AMP genes are regulated by both Relish and Spaetzle, with the exceptions of *attacin D* and *diptericin A*, which are controlled only by Relish. Most of our results correlate with previous analyses. However, in contrast to northern blot analysis, we failed to detect an effect of the single *spz* mutation on the induction of *drosomycin*, and of the *rel* mutation on *attacin A* activation, suggesting that the cRNA probes from these genes can saturate the oligonucleotide microarray.

Among the genes regulated only by the Imd pathway (affected in *rel* flies, but not in *spz*), we found several PGRPs encoding genes (PGRP-LB, SB1, SD and one PRGP-like), which play a role in the detection of bacteria (Werner *et al.*, 2000). We also identified three genes encoding enzymes involved in the melanization process (Pale, Punch and Dhpr) and one encoding a putative prophenoloxidase-activating enzyme (proPO-AE). Finally, we identified genes coding for an uncharacterized serpin (Sp4), one induced and three repressed serine proteases (see also Table I, part 2), one factor involved in iron metabolism (Zip3), one stress response peptide (TotM) and Imd.

In the group of genes controlled only by the Toll pathway (affected in *spz* mutants and not in *relish*), we found most of the up-regulated genes of the Toll pathway itself (*necrotic*, *spaetzle*, *Toll*, *pelle*, *cactus* and *Dif*). These findings extend previous data showing that Cactus regulates its own expression (Nicolas *et al.*, 1998). In this group, we also found genes encoding two short proteins with significant similarities to the N-terminal domain of Gram-negative-binding proteins (GNBPs), that have been isolated as proteins that bind to β -1-3 glucan, a component found on the cell wall of fungi (Kim *et al.*, 2000). Therefore, these two new GNBPs are potential candidates for the recognition protein that activates the Toll pathway in response to fungal infection. The Toll pathway also controls genes encoding three uncharacterized serine proteases, two serpins, one kunitz-type serine protease inhibitor, three putative proPO-AEs that may play a role in the melanization reaction and one peroxidase gene that could mediate the production of ROS. Finally, we identified several genes coding for unknown small peptides, including the IM2 family, that are induced by *B.bassiana* infection and could function as new antifungal effector molecules controlled by the Toll cascade. Spaetzle also regulates several repressed DIRGs (Table I, part 2) including genes encoding serine proteases, serine protease inhibitors and one lysozyme. It has been reported that infection inhibits the expression of cytochrome P450 detoxification genes in vertebrates (Renton, 2000). Interestingly, we found that in *Drosophila*, the Toll pathway mediates the repression of seven cytochrome P450 genes and of other detoxification enzymes (*sodh-1*, *CG3699* and *CG7322*) in response to bacterial infection. The *rel* mutation used in this study (*rel^{E20}*) abolishes the transcription of *relish* mRNA (Hedengren *et al.*, 1999); therefore, we limited the analysis of *relish* expression to the *wt*, *spz* and *TI^{10b}* lines. Interestingly, we found a partial

effect of the *spz* mutant on *relish* expression at the 6 h time point.

The genes regulated by the Imd and Toll pathways can be divided into three groups as shown in Table I: group A genes are affected only in *rel,spz* flies; group B genes are affected in both *rel* and *spz* flies; and group C is composed of AMP genes weakly affected in *rel* but strongly affected in *rel,spz* flies. Imd and Toll pathways are redundant in the regulation of genes in group A. This group includes genes probably involved in melanization (putative proPO-AE, *yellow f*; *Cp19*), one component of the Toll pathway (*dorsal*), two components of the JNK pathway (*d-Jun* and *puc*) involved in wound healing (Rämet *et al.*, 2002a) and one putative chitin-binding lectin (*idgf3*) that could recognize endogenous chitin at the injury site. Both Imd and Toll pathways affect the genes in groups B and C. This groups include three PGRP genes (*PGRP-SA*, *SC2* and *LC*); two complement-like genes (*Tep2* and *Tep4*) and one complement-binding receptor gene that could be involved in phagocytosis; one fibrinogen-like gene potentially involved in coagulation; one gene involved in melanization (*Ddc*); and one transferrin gene mediating iron sequestration. In addition, we found in this category genes encoding five up-regulated and two down-regulated serine proteases, two serine protease inhibitors and several unknown small peptides highly induced by infection (Table I, parts 1 and 2).

Among the DIRGs independent of Imd and Toll pathways (not affected in the double mutant *rel,spz*), we identified genes encoding a putative binding lectin (*Idgf1*), a putative coagulation factor (annexin IX), one enzyme potentially involved in melanization (laccase-like), two homologous small peptides and several serine proteases (one up-regulated and seven repressed).

Genes responding to microbial infection can be located in co-regulated genomic clusters

The identification of a large number of DIRGs, coupled with the analysis of the mutations affecting the Imd and Toll pathways, allowed us to examine on a large scale the chromosomal localization of co-regulated genes. An automated statistical analysis helped us to identify 36 DIRGs significantly clustered in the genome. A few examples of genomic clusters identified through this method are given in Figure 4A. In addition, we found other associations of DIRGs not identified by the automated analysis, which are shown in Figure 4B. Finally, Figure 4C shows an example of DIRGs that, although not associated, are encoded in the same cytological region (*spaetzle*, *Toll*, *pelle* and *CG5909* in 97A4-F4). Some of the clusters include copies of homologous genes (three *IM-2*-like genes; *attacins A* and *B1*; three *cecropin* genes; and *Dif* and *dorsal*), whose association can be explained by duplication events. However, we identified several gene clusters whose members do not share sequence similarities.

Interestingly, most of the genes inside each cluster (Figure 4A and B) or in the same genomic region (Figure 4C) share a similar type of regulation. For example, the genes *Ady43A* and *CG11086* are both induced upon infection by the Toll pathway, suggesting that they are targets of the same transcription factors (either *Dif* or *Dorsal*) (Figure 4C). In support of this

Table I. Effect of mutations affecting the Toll and Imd pathways on the expression of selected DIRGs

CG number	Name	Function	<i>wt</i>	<i>rel</i>	<i>spz</i>	<i>rel,spz</i>	<i>T1^{10b}</i>	<i>B.b.</i>
1. Induced DIRGs								
Genes regulated by Relish								
CG14704	PGRP-LB	Peptidoglycan recognition	++S	0	++	0	0	0
CG9681	PGRP-SB1	Peptidoglycan recognition	++++S	++	++++	++	++	0
CG7496	PGRP-SD	Peptidoglycan recognition	++++S	++	++++	+	+	0
CG4437	PRGP-like	Peptidoglycan recognition	+S	0	+	0	0	0
CG2056		Ser-protease	+L	0	+	0	0	0
CG9733	proPO-AE	Ser-protease/melanization	++A	0	++	0	-	0
CG9453	Sp4	Serpin	+S	0	+	0	0	0
CG10118	Pale	Melanization	+S	0	+	0	0	+
CG9441	Punch	Melanization	++A	0	++	0	0	0
CG4665	Dhpr	Melanization	+A	0	+	0	0	0
CG7629	Attacin D	Antimicrobial peptide	+++++S	0	+++++	0	0	0
CG12763	DiptericinA	Antimicrobial peptide	++++S	--	++++	-	++	0
CG15829		Unknown peptide (82 amino acids)	++A	0	++	0	0	0
CG14027	TotM	Stress response	++++S	++	++++	++	0	+
CG6898	Zip3	Iron metabolism	+S	0	+	0	0	0
CG5576	imd	IMD pathway	+A	0	+	0	0	0
Genes regulated by Relish and Spaetzle								
(A)								
CG4559	Idgf3	Chitin binding/wound healing	+S	+	+	0	0	0
CG6639		Ser-protease	+L	+	+	0	+++++	++++
CG3505	proPO-AE	Ser-protease/melanization	++S	++	++	+	++	+
CG6687		Serpin	++L	+++	++	+	+++	++
CG18525	Sp5	Serpin	+S	+	+	0	0	0
CG18550	yellow f	Melanization	+L	+	+	0	+++	+
CG6524	Cp19	Melanization	+S	+	+	0	0	+
CG10810	Drosomycin	Antimicrobial peptide	++S	++	++	+	++	++
CG8175	Metchnikowin	Antimicrobial peptide	++S	++	++	+	++	++
CG10146	Attacin A	Antimicrobial peptide	++++S	++++	++++	+	+++	++
CG18372	Attacin B1	Antimicrobial peptide	+++S	+++	+++	+	+++	+
CG12494		Unknown peptide (61 amino acids)	+L	+	+	0	0	0
CG8846	Thor	Translation initiation	+A	+	+	0	0	0
CG6667	Dorsal	Toll pathway component	+L	+	+	0	0	0
CG2163	Pabp2	Poly(A) binding	+S	+	+	0	0	+
CG2275	d-Jun	JNK pathway/wound healing	+A	+	+	0	0	0
CG7850	Puc	JNK pathway/wound healing	+A	+	+	0	0	0
(B)								
CG11709	PGRP-SA	Peptidoglycan recognition	+++S	+	++	+	+	0
CG14745	PGRP-SC2	Peptidoglycan recognition	+S	-	-	--	-	0
CG4432	PGRP-LC	Peptidoglycan recognition	+S	0	0	0	0	0
CG7052	Tep2	Complement-like	+++S	+	0	0	+	+
CG10363	Tep4	Complement-like	+S	0	0	0	0	+
CG4823		Complement binding	+S	0	0	0	0	+
CG11842		Ser-protease	+L	0	0	0	++	+
CG9645		Ser-protease	+S	0	0	-	0	++
CG5909		Ser-protease	++A	0	+	0	0	++
CG15046		Ser-protease	++A	+	+	0	0	0
CG6361		Ser-protease	+S	0	0	0	0	0
CG11331		Serpin	++A	++	+	0	0	+
CG3604		Kunitz family	++A	0	+	0	0	0
CG10697	Ddc	Melanization	+++A	+	++	+	0	0
CG5550	Fibrinogen-like	Coagulation	+++A	++	++	+	0	0
CG12965		Unknown peptide (45 amino acids)	++++S	++	0	-	+++	++
CG9080		Unknown peptide (121 amino acids)	++A	0	++	--	0	+
CG10812	Drosomycin B	Antimicrobial peptide	+L	0	0	-	0	+
CG1385	Defensin	Antimicrobial peptide	++++S	-	+++	0	+	+
CG1878	Cecropin B	Antimicrobial peptide	+++++A	++	+++	0	0	0
CG1373	Cecropin C	Antimicrobial peptide	+++++A	++	++++	-	0	0
CG6429		Unknown peptide (124 amino acids)	+++A	++	++	+	0	0
CG4269		Unknown peptide (102 amino acids)	++S	0	0	-	0	0
CG17278		Unknown peptide (49 amino acids)	+A	0	0	0	0	0
CG8157		Unknown peptide (113 amino acids)	+S	0	0	0	0	0
CG3666	Transferrin	Iron metabolism	+++A	++	++	+	0	0

Table I Continued

CG number	Name	Function	<i>wt</i>	<i>rel</i>	<i>spz</i>	<i>rel,spz</i>	<i>T1^{10b}</i>	<i>B.b.</i>
CG3132	β-galactosidase	Lysosomal enzyme	++S	0	+	0	0	0
CG7279	Lip1	Lipase	+S	0	0	0	0	0
CG4267		Lipase	++S	+	+	0	+	0
(C)								
CG10816	Drosocin	Antimicrobial peptide	++++A	++	++++	+	++	+
CG10794	Diptericin B	Antimicrobial peptide	+++S	++	+++	+	++	0
CG1365	CecropinA1	Antimicrobial peptide	+++S	++	+++	-	0	0
CG1367	CecropinA2	Antimicrobial peptide	++++S	++	++++	0	0	0
CG4740	Attacin C	Antimicrobial peptide	+++++S	+++	+++++	++	+++	+
Genes regulated by Spaetzle								
CG13422	GNBP-like	Recognition	+++S	+++	+	0	+++	++
CG12780	GNBP-like	Recognition	+S	+	0	0	0	-
CG8215		Ser-protease	+L	+	0	0	++++	++
CG9631		Ser-protease	+L	+	0	0	+	0
CG18563		Ser-protease	+L	+	0	0	++++	
CG1102	proPO-AE	Ser-protease/melanization	+S	+	0	0	++	0
CG3066	proPO-AE	Ser-protease/melanization	++S	++	+	+	+	0
CG16705	proPO-AE	Ser-protease/melanization	+S	+	0	0	++	+
CG7219		Serpin	++++A	++++	0	0	+	0
CG16713		Kunitz family	++S	++	+	+	++	+
CG18106	IM2	Unknown	++S	++	0	0	+++	++
CG15065	IM2-like	Unknown	+A	+	0	0	0	+
CG18108	IM2-like	Unknown	+++S	+++	0	0	+++	++
CG15066		Unknown peptide (134 amino acids)	+++S	+++	-	--	++	++
CG16978		Unknown peptide (96 amino acids)	+++A	++++	++	++	0	+
CG5791		Unknown peptide (98 amino acids)	++L	++	--	--	++	++
CG4250		Unknown peptide (121 amino acids)	++L	++	0	0	+++	+
CG8913		Peroxidase	++S	++	+	0	++	+
CG4757		Lipase, carboxylesterase	++L	++++	0	0	+++++	++++
CG6675		Lipase	++A	++	0	0	+	++
CG9434	Frost	Cold response	+++A	+++	++	++	0	+
CG1857	Necrotic	Toll pathway component	++S	++	0	0	++	+
CG6134	Spaetzle	Toll pathway component	++A	++	0	0	0	0
CG5490	Toll	Toll pathway component	+S	++	0	0	+	0
CG5974	Pelle	Toll pathway component	+S	++	0	0	0	0
CG6794	Dif	Toll pathway component	+A	+	0	0	0	+
CG5848	Cactus	Toll pathway component	++A	++	0	0	+	+
CG11992	Relish	Imd Pathway	++++A		+++		+	+
Genes not regulated by Relish and Spaetzle								
CG4472	Idgf1	Chitin binding/wound healing	++A	++	++	++	0	0
CG6467		Ser-protease	+S	+	+	+	0	+
CG5730	Annexin IX	Coagulation	+S	+	+	+	0	0
CG3759	Laccase-like	Melanization	+S	+	+	+	0	0
CG13324		Unknown peptide (112 amino acids)	++A	++	+++	++	0	0
CG13323		Unknown peptide (112 amino acids)	+A	+	++	+	0	0
2. Repressed DIRGs								
Genes regulated by Relish								
CG18180		Ser-protease	-L	0	-	0	0	-
CG18179		Ser-protease	-L	0	-	+	+	-
CG9672		Ser-protease	-L	0	-	0	0	0
Genes regulated by Spaetzle								
CG16756		Lysozyme-like	-A	-	0	0	0	0
CG12351		Ser-protease	-L	---	0	0	--	-
CG18030		Ser-protease	--S	----	--	--	0	--
CG7532		Ser-protease	-L	-	0	0	0	0
CG6953	Fat-spondin	kunitz family	-A	-	0	0	0	0
CG6067		Ser-protease	--A	--	0	0	0	0
CG3775		Neprilysin-like	--A	--	0	0	0	0
CG8540	Cyp316a1	Cytochrome P450	-S	-	0	0	0	-
CG14032	Cyp4ac1	Cytochrome P450	-S	-	0	0	0	0
CG8453	Cyp6g1	Cytochrome P450	-A	-	0	0	0	0
CG6816	Cyp18a1	Cytochrome P450	-A	-	0	0	0	0

Table I Continued

CG number	Name	Function	<i>wt</i>	<i>rel</i>	<i>spz</i>	<i>rel,spz</i>	<i>Tl^{10b}</i>	<i>B.b.</i>
CG10833	Cyp28d1	Cytochrome P450	-S	-	0	0	0	0
CG8345	Cyp6w1	Cytochrome P450	-A	--	0	0	0	0
CG3540	Cyp4d14	Cytochrome P450	-A	-	0	0	0	0
CG1982	Sodh-1	Detoxification	-- -A	---	-	0	0	0
CG3699		Detoxification	-L	-	0	0	--	0
CG7322		Detoxification	-A	-	0	0	0	0
Genes regulated by Relish and Spaetzle								
CG8871		Ser-protease	-L	-	--	0	0	0
CG4178	Lsp1	Larval serum protein 1	--S	--	--	0	--	0
CG6806	Lsp2	Larval serum protein 2	--L	--	--	0	--	-
CG15231		Unknown peptide (42 amino acids)	--S	-	0	0	--	--
CG16749		Ser-protease	-S	-	-	0	0	0
CG2060	Cyp4e2	Cytochrome P450	-A	0	0	0	0	0
Genes not regulated by Relish and Spaetzle								
CG8579		Ser-protease	-S	--	--	-	-	-
CG4812		Ser-protease	--A	--	--	--	-	0
CG12385		Ser-protease	-S	-	-	--	0	-
CG8869		Ser-protease	--S	--	--	--	-	-
CG10475		Ser-protease	--S	---	--	--	-	--
CG17951	Ser99Dc	Ser-protease	-S	--	--	--	-	0
CG8867	Ser4	Ser-protease	-L	--	--	--	0	0
CG8562		Zinc carboxy peptidase	-A	--	-	-	-	0
CG17814		Peritrophic membrane	-A	-	-	-	-	0
CG11853	Takeout	Ligand binding	--S	--	---	---	---	-
CG8577	PGRP-SC1b	Peptidoglycan recognition	-A	--	--	---	-	-

Differential response to septic injury of selected DIRGs in wild-type (*wt*), *relish* (*rel*), *spaetzle* (*spz*) and *relish,spaetzle* double mutants (*rel,spz*) is compared with the effect of the *Tl^{10b}* gain-of-function mutation in uninfected flies and with the response to *B.bassiana* natural infection (*B.b.*). 0, gene expression unchanged; +, gene expression up-regulated; -, gene expression down-regulated. Each + and each - corresponds to one log₂ unit change in expression level relative to uninfected wild-type flies. A, acute response gene; S, sustained response gene; L, late response gene. Genes regulated by both Relish and Spaetzle are divided into three groups (A) genes affected only in *rel,spz* flies; (B) genes affected in both *rel* and *spz* flies; (C) genes affected in *rel* and *rel,spz* flies. It should be noted that we used a less stringent threshold to subcategorize the genes in this table (2-fold change in one time point) compared with the automated approach used in Figure 3A.

finding, a cluster of three Dorsal optimal binding sites has been identified recently between *Ady43A* and *CG11086* (Markstein *et al.*, 2002).

Discussion

To identify the target genes of the Toll and Imd pathways in response to microbial infection, we have compared the gene expression programmes induced by septic injury in wild-type and mutant adult male flies using oligonucleotide microarrays. In parallel, we have monitored the survival rate and the expression level of various AMP genes after infection by various microorganisms. For the Toll pathway, we selected a strong homozygous viable allele of *spz* (*rm7*). We observed that the *spz*, *Tl* and *pII* mutations alone or in combination with *rel* (Figures 1 and 2; data not shown) have similar effects on both the survival rate and pattern of AMP gene expression after microbial infection. These findings suggest that the effects of *spz* mutation on the transcription programme induced by infection reflect the role of the entire Toll pathway in the immune response. For the Imd pathway, we selected a null viable allele of *relish* (*E20*). Similarly to the Toll pathway, previous comparative studies did not reveal any striking difference between mutations in *relish* and null mutations in the genes encoding the other members of the Imd pathway such as *kenny*, *ird5* and *dredd*, with the sole

exception of mutations in *dTAK1*, which have a slightly weaker phenotype (Leulier *et al.*, 2000; Rutschmann *et al.*, 2000; Lu *et al.*, 2001; Vidal *et al.*, 2001). Again, these data suggest that the effects of *rel* mutation on the immune response reflect the role of the whole Imd pathway. However, we cannot exclude other pathways, including Toll, from having a minor role in Relish activation.

Based on known data on AMP gene expression, we predicted redundant functions for the Imd and Toll pathways in the control of some of their target genes; thus, we generated a double mutant *rel,spz* strain devoid of all Toll and Imd activity. Finally, we extended the microarray analysis to a gain-of-function allele of Toll (*Tl^{10b}*) that is constitutively active even in the absence of infection. The *Drosophila* lines used in this study are not isogenic, thus some of the changes in the gene expression programmes might arise from the genetic background. In addition, developmental or physiological defects induced by the mutations could also affect the adult expression profile. *Tl^{10b}* flies, for example, show a melanotic tumour phenotype (Lemaitre *et al.*, 1995). However, *spz* and *rel* adults do not show any detectable defect (Hedengren *et al.*, 1999); therefore, we believe that most of the changes in the expression profiles in these mutants reflect the direct or indirect effects of the Toll and Imd pathways on transcriptional reprogramming during the immune response.

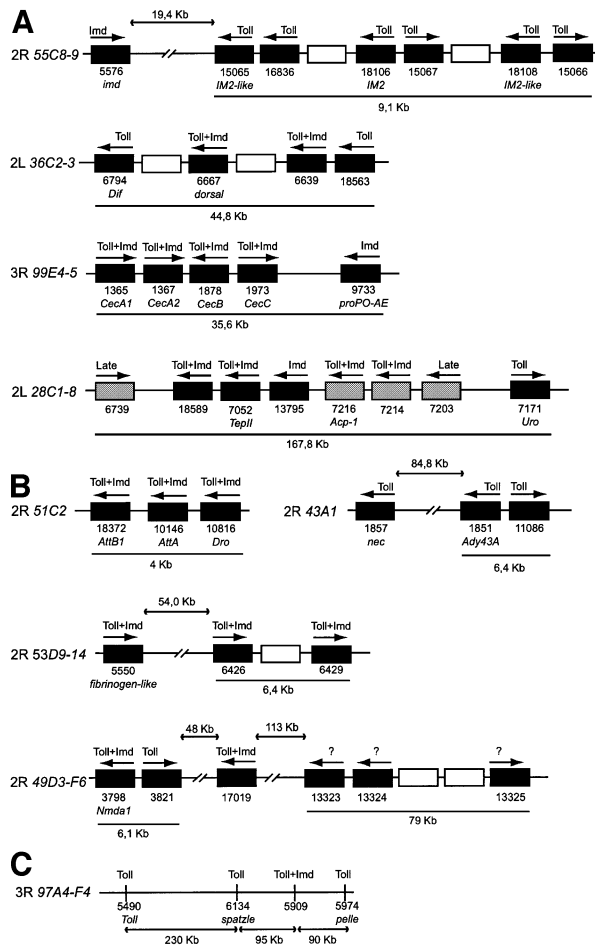


Fig. 4. Genomic organization of co-regulated genes. **(A)** Examples of local genomic clusters derived from the automated analysis described in Materials and methods. **(B)** Examples of other associated DIRGs. Each black rectangle corresponds to one infection-induced gene, grey rectangles to infection-repressed genes and white rectangles represent genes not regulated by infection. The CG number and the name of the gene (when known) are given below. The arrow on the top of each rectangle indicates the direction of transcription. On each arrow is indicated the type of regulation. The distances between non-clustered genes and the sizes of local gene clusters are given in kb. The cytological location of each group of genes is indicated on the left. **(C)** Schematic representation of DIRGs grouped in the same cytological region.

The septic injury experiments were performed using a mixture of Gram-positive and Gram-negative bacteria. This type of infection activates a wide immune response and allows the simultaneous analysis of several categories of immune-responsive genes (De Gregorio *et al.*, 2001). However, it has been shown that Toll and Imd pathways are activated selectively by different classes of microorganisms; thus, the use of a bacterial mixture might increase the redundancy of the two pathways in the control of common target genes.

In our previous microarray analysis, we observed a very high correlation with published data: 34 out of 35 genes induced by infection identified by northern blot were also detected as up-regulated with the microarray approach (see www.cnrs-gif.fr/cgm/immunity/). Here, we found that the effects of mutations in Toll and Imd pathways on most of the AMP-expressing genes and on several genes

expressing regulatory factors (*necrotic*, *cactus* and *relish*) corroborate previous studies using northern blots (Nicolas *et al.*, 1998; Levashina *et al.*, 1999; unpublished data). However, we failed to detect the partial effects of single mutations *spz* or *rel* on the induction of a subset of AMP genes, suggesting that some genes expressed at high levels (like AMP genes) have saturated binding to the arrays, preventing accurate measurements.

Toll and Imd control the majority of the *Drosophila* immune response

The microarray analysis demonstrates that the functions of Toll and Imd pathways in *Drosophila* immunity can be extended beyond the regulation of AMP genes. The majority of the DIRGs are affected by the mutations in the Toll or Imd pathways (Figure 3). Many of these genes are unknown (see www.fruitfly.org/expression/immunity/ for a complete list); others can be assigned to several immune functions (Table I). The susceptibility of the Imd and Toll pathway mutants to different types of microbial infection suggested a control of the antifungal response by the Toll pathway: a major role for the Toll pathway for the response to Gram-positive bacteria with a minor contribution of Imd, and a predominant role of Imd with a minor contribution of Toll to the resistance against Gram-negative bacteria (Figure 1). In agreement, microarray analysis shows that the Toll pathway controls most of the late genes induced by fungal infection and cooperates with the Imd pathway for the control of genes implicated in several immune reactions such as coagulation, AMP production, opsonization, iron sequestration and wound healing. Interestingly, *defensin*, which encodes the most effective antimicrobial peptide directed against Gram-positive bacteria (Tzou *et al.*, 2002c), is co-regulated by both the Imd and Toll pathways. Our hierarchical cluster analysis of the expression profiles combining the effect of the mutations after septic injury with the response to fungal infection provides a wealth of information that may help to elucidate the function of some of the uncharacterized DIRGs. Until now, the increased susceptibility to infection of Imd- or Toll-deficient flies has been attributed to the lack of expression of AMP genes, and it has been shown recently that the constitutive expression of single AMP genes in *imd;spz* double mutant flies can increase the survival rate of some types of bacterial infection (Tzou *et al.*, 2002c). Our finding that the Toll and Imd pathways are the major regulators of the *Drosophila* immune response now suggests that other immune defence mechanisms might contribute to the increased susceptibility to infection displayed by mutant flies.

Interactions between the Imd and Toll pathways

The interactions between the Toll and Imd pathways are more complex than merely regulating the same target genes. In agreement with northern blot analysis (unpublished data), we show that the transcriptional control of *relish* in response to infection receives a modest input from the Toll pathway, revealing an additional level of interaction between the two cascades. The activation of Toll may increase the level of Relish to allow a more efficient response to bacterial infection. This finding is in agreement with previous observations showing that in mutants where the Toll pathway is constitutively active

(*Tl^{10b}*), all the antibacterial peptides genes, including *dipericin*, are induced with more rapid kinetics than in wild-type flies (Lemaitre *et al.*, 1996). Furthermore, the higher susceptibility to *E.coli* infection of the *rel,spz* double mutant compared with the *rel* single mutants flies indicates that Toll also has a direct, Relish-independent effect on the resistance to infection by Gram-negative bacteria (Figure 1A). Northern blot analysis shows that *relish* induction in response to infection is significantly reduced in *dTAK1* and *dredd* mutants, indicating that the Imd pathway undergoes autoregulation (unpublished results). Interestingly, the Imd pathway can influence the Toll pathway through the control of *PGRP-SA*, which encodes a recognition protein essential for the activation of the Toll pathway by Gram-positive bacteria (Michel *et al.*, 2001). Again, it is interesting to notice that this interaction between the Toll and Imd pathways correlates with the contribution of both pathways to fight infection with Gram-positive bacteria (Figure 1B and C). Interestingly, all the genes encoding components of the Toll pathway required for both antibacterial and antifungal responses (*necrotic*, *spaetzle*, *Toll*, *pelle*, *cactus* and *Dif*) are not controlled by the Imd pathway and are subjected to autoregulation.

Other pathways controlling the *Drosophila* immune response

The Rel/NF- κ B proteins Dif, Dorsal and Relish, which are the transactivators induced by the Toll and Imd pathways, bind to the κ B sites present in the promoters of target genes, such as AMP genes, regulating their expression. Therefore, the analysis of the promoters of the DIRGs controlled by Toll or Imd pathways could help to identify all the direct NF- κ B targets during infection. However, some of the effects of mutations affecting the Toll or Imd pathways that we monitored by microarray analysis might be mediated by the regulation of other transcription factors or signalling cascades. It has been shown recently in larvae that the *Tep1* gene is regulated by the JAK–STAT pathway and can be activated by the Toll pathway, suggesting that Toll can control, at least partially, the JAK–STAT cascade (Lagueux *et al.*, 2000). Here we report that two genes encoding components of the JNK pathway (*puc* and *d-Jun*) are partially regulated by Toll and Imd in response to septic injury.

The presence of DIRGs independent of or only partially dependent on both the Imd and Toll pathways suggests the presence of other signalling cascades activated after septic injury. Potential candidates are MAPK and JAK–STAT pathways. Beside their developmental functions, the MAPK pathways have been implicated in wound healing (JNK) and the stress response (MEKK) (Sluss *et al.*, 1996; Inoue *et al.*, 2001; R  met *et al.*, 2002a). The JAK–STAT pathway, as we mentioned above, controls the *Drosophila* complement-like gene *Tep1* (Lagueux *et al.*, 2000). The stimuli that trigger these cascade are not known and it is not clear if these cascades are activated by exogenous or host factors. Interestingly, in vertebrates, the JAK–STAT pathway is activated by cytokines during the immune response. The microarray analysis of mutants in these pathways might help to reveal their exact contribution to the *Drosophila* immune response. Our observation that Toll and Imd pathways control most of the DIRGs raises

the question of whether these two pathways are the sole signalling cascades directly activated by microbial elicitors, while the other signalling pathways are triggered by other stimuli associated with infection such as wound, stress, cytokine-like factors and Toll and Imd activities.

Co-regulated genomic clusters

In vertebrates, many genes involved in the immune response are grouped in large chromosomal complexes. The recent completion of the *Drosophila* genome did not reveal any striking chromosomal organization beside clustering of genes belonging to the same family, probably reflecting recent duplication events (Khush and Lemaitre, 2000). In this study, we observed that some of the genes responding to microbial infection are located in the same cytological region or are associated in transcriptionally co-regulated genomic clusters. Interestingly, microarray analysis of circadian gene expression in *Drosophila* has led to the identification of similar clusters of genes (McDonald and Rosbash, 2001). Other microarray analyses might reveal the importance of the genome organization in the definition of adequate transcription programmes in response to a variety of stimuli.

Materials and methods

Drosophila stocks

Oregon^R flies were used as a wild-type standard. Exact genotypes of the flies analysed in this study are: *spaetzle^{rm7}/spaetzle^{rm7}* (*spz*); *Tl^{1-RXA}, e/Tl^{r632}* (*Tl*); *relish^{E20}, e/relish^{E20}, e* (*rel*); *pll⁷/pll⁷⁸* (*pll*); *spaetzle^{rm7}, relish^{E20}/spaetzle^{rm}, relish^{E20}* (*rel,spz*); *Tl^{1-RXA}, relish^{E20} e/Tl^{r632}, relish^{E20}* (*rel,Tl*); and *Tl^{10b}, e/+* (*Tl^{10b}*). *spaetzle^{rm7}, Tl^{1-RXA}* and *relish^{E20}* are strong or null alleles of *spz*, *Tl*, and *rel*; *Tl^{r632}* is a thermosensitive allele of *Tl* with a strong phenotype at 29°C (Lemaitre *et al.*, 1996; Hedengren *et al.*, 1999). *rel,spz* flies were obtained by recombining *spaetzle^{rm7}* and *relish^{E20}* on the third chromosome. The alleles *Tl^{1-RXA}* and *Tl^{r632}* were recombined with *relish^{E20}*, and the resulting double mutant lines were crossed to generate the line *rel,Tl*. The *Tl^{10b}* allele is a gain-of-function allele of Toll. *Tl^{10b}/TM3* males were crossed to wild-type female flies, and *Tl^{10b}/+* males were subjected to microarray analysis.

Infection experiments

For septic injury and natural infection experiments, we used *Drosophila* adults, aged 3–4 days, at 25°C. Septic injury was produced by pricking the thorax of the flies with a needle previously dipped into a concentrated culture of *E.coli*, *M.luteus* and *E.faecalis* or in a suspension of *A.fumigatus* spores (Tzou *et al.*, 2002b). Natural infection was initiated by shaking anaesthetized flies in a Petri dish containing a sporulating culture of the entomopathogenic fungus *B.bassiana*. For survival experiments, 60 flies were infected in the morning and incubated at 29°C (except for *E.faecalis* infection that was performed at 25°C). For northern blotting and microarray analysis, flies were incubated at 25°C and collected at specific times after infection.

Analysis of mRNA expression using oligonucleotide arrays

Microarray analysis was performed with Affymetrix *Drosophila* GeneChips using poly(A) RNA from adult males as previously described (De Gregorio *et al.*, 2001). To identify genes that show changes between conditions, *t*-tests were performed. Due to the limited number of arrays used, we agglomerated all infected time points and treated them equivalently. We did restrict our analysis to equally represented time points, those from 90 min to 6 h after septic infection. It should be noted that this method of analysis prevents us from observing real differences between genotypes for genes that are particularly dynamic. Five sets of tests were performed for the 400 DIRGs with the following comparisons: wild-type uninfected samples ($n = 5$) with wild-type bacterially infected ($n = 12$); wild-type infected ($n = 12$) with *spz* infected ($n = 6$); wild-type infected ($n = 12$) with *rel* infected ($n = 6$); wild-type infected ($n = 12$) with *rel,spz* infected ($n = 6$); and wild-type uninfected ($n = 5$) with *Tl^{10b}* uninfected ($n = 3$). To mitigate false positives, the *P*-value for each *t*-test

was considered significant if it was <0.0025 so that we expect approximately one false positive for each of the five sets of tests.

DIRG gene clusters were identified in the genome by first finding all DIRG genes that were adjacent in the genome. Forty-three such pairs of DIRGs exist. Using the binomial distribution, it was calculated that a total of four genes (including the pair) within a 16 gene window (seven genes on either side of each pair) was significant at $P < 0.05$. A total of six gene clusters comprising a total of 36 genes met these criteria.

Supplementary data

Supplementary data for this paper are available at *The EMBO Journal* Online.

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