A drosomycin–GFP reporter transgene reveals a local immune response in *Drosophila* that is not dependent on the *Toll* pathway

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A hallmark of the systemic antimicrobial response of *Drosophila* is the synthesis by the fat body of several antimicrobial peptides which are released into the hemolymph in response to a septic injury. One of these peptides, drosomycin, is active primarily against fungi. Using a drosomycin–green fluorescent protein (GFP) reporter gene, we now show that in addition to the fat body, a variety of epithelial tissues that are in direct contact with the external environment, including those of the respiratory, digestive and reproductive tracts, can express the antifungal peptide, suggesting a local response to infections affecting these barrier tissues. As is the case for vertebrate epithelia, insect epithelia appear to be more than passive physical barriers and are likely to constitute an active component of innate immunity. We also show that, in contrast to the systemic antifungal response, this local immune response is independent of the *Toll* pathway.

Keywords: antibiotic peptides/*Drosophila*/GFP/innate immunity/local immune response

Introduction

In past years, several advances have highlighted the primordial role of innate immunity in providing a quick effector response to infections in vertebrates (Janeway, 1989; Fearon, 1997). Furthermore, natural immunity appears not only to trigger the adaptive immune response but also to direct the type of effector response in clonally selected immune cells that is appropriate to fight efficiently against infections (Medzhitov and Janeway, 1997). However, it has become clear that a first line of defense of the organism consists of the local synthesis and release of antimicrobial peptides in tissues, also called barrier epithelia, which are in direct contact with microorganisms. The role of these antimicrobial peptides is illustrated dramatically in the case of cystic fibrosis patients, where

β-defensin expressed in the conducting and respiratory airway is inactivated by the high salt concentrations in the airway surface fluid due to a defect in the cystic fibrosis transmembrane conductance regulator. As a result, the airway may become colonized by microbial pathogens, and chronic inflammation ensues (Smith *et al.*, 1996; Goldman *et al.*, 1997).

In *Drosophila*, a septic wound induces the rapid appearance in the hemolymph of a battery of antibacterial peptides that includes the cecropins (Kylsten *et al.*, 1990; Tryselius *et al.*, 1992), diptericin (Wicker *et al.*, 1990), drosocin (Bulet *et al.*, 1993), insect defensin (Dimarcq *et al.*, 1994), metchnikowin (Levashina *et al.*, 1995), attacin (Asling *et al.*, 1995) and one major antifungal peptide, drosomycin (Fehlbaum *et al.*, 1995). These peptides are synthesized mostly in the fat body, a functional equivalent of the liver, and secreted into the hemolymph. We will refer to this reaction as the systemic antimicrobial response.

Since experimental wounds are restricted to a single point of entry and since all of the disseminated fat body is responding to the attack, it is thought that a signal is transmitted through the hemolymph from the entry site of microorganisms, where non-self recognition presumably occurs, to the fat body. It is generally appreciated today that the insect host defense presents remarkable similarities to vertebrate innate immunity, pointing to a certain degree of common ancestry (reviewed in Hoffmann *et al.*, 1996; Hoffmann and Reichhart, 1997). In this context, we have asked whether antimicrobial peptides are also expressed in barrier epithelia in *Drosophila*. In this study, we address this question for the antifungal peptide drosomycin in larvae and adults of this species.

To monitor the expression of drosomycin in live flies, we designed a transgenic reporter system based on the green fluorescent protein (GFP). This protein fluoresces without specific cofactors when illuminated at the right wavelengths, and can be detected in live animals provided they are reasonably transparent (Chalfie *et al.*, 1994; Wang and Hazelrigg, 1994). Further, the fluorescence can be quantified using fluorimeters or imaging devices. Since there is no enzymatic amplification step, GFP is less sensitive as a reporter gene than the bacterial β-galactosidase gene that has been broadly used so far. However, new generations of mutant GFPs that are brighter may compensate for these shortcomings (Heim *et al.*, 1995; Cormack *et al.*, 1996). Indeed, we show here that S65TGFP is an adequate reporter of inducible gene expression in *Drosophila*.

Using drosomycin–GFP reporter transgenes, we find in some non-experimentally immunized animals an expression of the drosomycin–GFP reporter gene in a variety of epithelial tissues. These observations suggest the existence of a local immune response in *Drosophila* in addition to
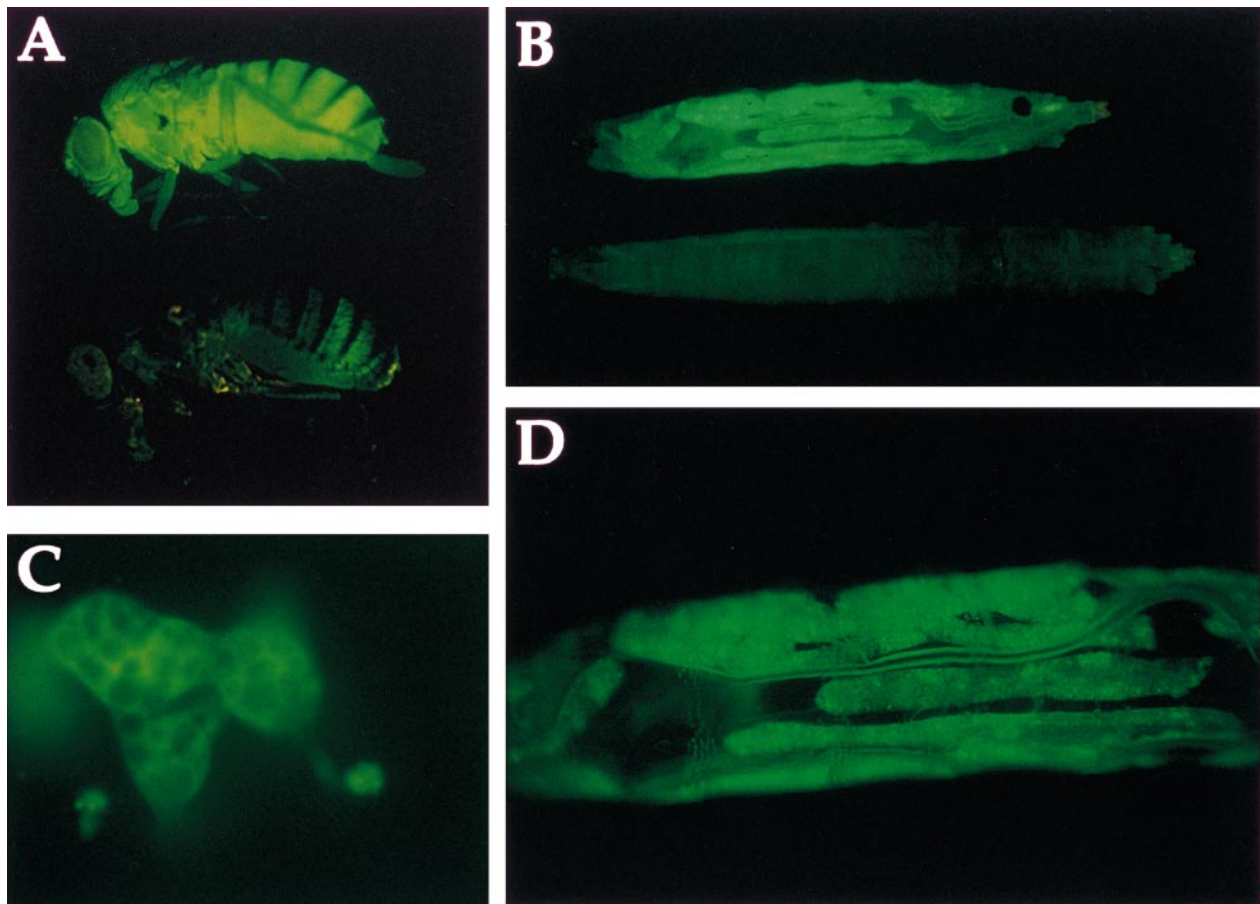
Fig. 1. The drosomycin–GFP reporter gene is expressed in the fat body during the systemic response. (**A**) The transgenic fly shown on top has been immunized 48 h before, whereas the control fly (bottom) from the same line has not. Note the melanized scar on the thorax where the injury was performed. The fluorescence observed in the posterior part of the abdomen of the non-immunized fly corresponds to the constitutive expression of drosomycin in the sperm storage structures of the female (shown in Figure 4A). (**B**) The transgenic larva shown on top has been immunized for 12 h (melanized scar at the posterior end); the bottom larva is a non-immunized control. The reporter gene is expressed in the lobes of the fat body. (**C**) Dissected fat body lobule of an adult undergoing a systemic immune response. The expression of the reporter gene in the disseminated fat body accounts for the fluorescence observed through the cuticle of the insect (A). GFP is found in the cytoplasm and is excluded from lipidic globules. (**D**) Enlargement of the immunized larva shown in (B). The tubular structure that spans most of the larva is one of the two dorsal tracheal trunks. It is illuminated by the neighboring fat body tissue but is not itself fluorescent, as can be seen upon dissection.

the classically described systemic response induced by wounding. Thus, like vertebrate epithelia, insect epithelia may be more than passive physical barriers and are likely to constitute an active component of innate immunity.

Results

The drosomycin–GFP reporter gene is expressed in the fat body and hemocytes during the systemic immune response

We have constructed several drosomycin–GFP reporter genes using either the wild-type or the S65T versions of GFP (Materials and methods). The RNA and protein levels of the reporter constructs induced after immunization are similar to those of the endogenous drosomycin as judged by Northern and Western blot analysis (data not shown). Furthermore, the comparison of the kinetics of protein accumulation as monitored by Western blot analysis with that of its fluorescence monitored quantitatively by fluorometry revealed that S65TGFP becomes fluorescent shortly (1–2 h) after its synthesis in the fat body of the adult (M.-C.Criqui, A.C.Jung and D.Ferrandon, unpublished data). Like the endogenous drosomycin, S65TGFP

accumulates progressively during the systemic response and reaches a maximum 3 days after induction.

To monitor qualitatively the expression of the drosomycin–GFP reporter gene during the systemic immune response, we looked at adult transgenic flies using a dissecting microscope equipped with an epifluorescence illumination module and the relevant set of filters. Through the cuticle, a diffuse green fluorescence was observed all over the immunized animals, whereas control animals displayed only a weak autofluorescence (Figure 1A). Upon dissection of the animal, it appeared that most of this fluorescence was due to the expression of the GFP reporter gene in the disseminated fat body of the adult (Figure 1C). No qualitative differences were noted between different lines carrying independent insertions of the transgenes.

The S65TGFP reporter gene could first be detected 4–6 h after induction; the signal could usually be detected reliably in whole flies after 8 h with a dissecting scope. However, in keeping with the quantitative results described above, the fluorescence was much stronger after 24 h.

In the transparent third instar larva, GFP fluorescence was detected mostly uniformly in all lobes of the fat body of immunized animals starting 4–6 h after injury, whereas

usually no fluorescence could be observed in control animals (Figure 1B and D). These results agree with those obtained by *in situ* hybridization with a drosomycin probe (Fehlbaum *et al.*, 1995).

We also found reporter GFP expression in a subset of larval plasmatocytes and lamellocytes after a septic wound.

Expression of the drosomycin–GFP reporter gene in the larval tracheal system in the absence of experimental immune challenge

While looking at a large number of control non-induced larvae, we noticed that a few larvae displayed an apparently spontaneous expression of the reporter gene. The proportion of drosomycin–GFP-expressing animals was quite variable from one culture vial to the next and was probably dependent on culture conditions such as crowding, age of the culture and/or the microbial environment of the culture medium. Of these larvae, a minor percentage showed an expression in the fat body which reflected a systemic immune response due probably to natural infections. Interestingly, the reporter gene was also detected in different tissues of other larvae, especially in the tracheal epithelia. The most frequent expression pattern was observed in the anterior or posterior spiracles. This expression was often limited to one spiracle of the pair (Figure 2), suggesting a limited response to a local infection. GFP was detected in the epithelium of the tracheal trunk next to the spiracle, but not in the spiracular chamber itself. The extent of this expression was sometimes limited to a ring. In other insects, however, this spiracular expression extended to most of the tracheal dorsal trunk and transverse connectives, indicating that an aerial infection had progressed along this trachea. Rarely, some larvae expressed the reporter gene in the central but not the distal parts of the dorsal trunk. Thus, it was not always possible to correlate an expression in the tracheae with a spiracular expression. Furthermore, some insects displayed a GFP fluorescence only in the transverse connectives, lateral trunk and other smaller tracheae, but not in the dorsal trunk (Figure 2E). The expression of the reporter gene in the tracheal system was almost totally abolished in larvae reared under axenic conditions.

To check that the GFP expression we detect in the tracheal system corresponds to the actual synthesis of the endogenous drosomycin peptide in this tissue, we dissected fluorescent and non-fluorescent parts of tracheal trunks. Strikingly, we could detect a peak corresponding to the drosomycin peptide in the fluorescent tracheae and not in the non-fluorescent ones by MALDI-TOF mass spectrometry on isolated tracheal trunks, thus demonstrating the relevance of the GFP reporter to epithelial tissue expression of drosomycin (Figure 3).

Induction of the expression of the drosomycin–GFP reporter gene following an exposure to microbial agents

To show that the localized expression patterns of the drosomycin–GFP reporter gene in the respiratory system are indeed a local response to a microbial infection, we dipped early third instar larvae in concentrated solutions of various bacteria or fungal spores for 30 min. The larvae were then allowed to recover from the treatment for 5–7 days at 18°C in a normal fly vial. In a typical experiment,

80% of the larvae that had been treated with the Gram-negative bacteria *Erwinia carotovora carotovora* developed a strong expression of the reporter gene throughout the tracheal system. In contrast, only 10% of the control animals that had been dipped in water displayed an expression of the transgene that was limited to the spiracles. In a few experiments, the proportion of control animals reacting to water treatment was somewhat higher than 10%, yet transgene expression was limited to the spiracles and did not propagate to the whole tracheal system. These experiments show that drosomycin can be synthesized in the tracheal system in response to a microbial infection.

Other expression patterns of the drosomycin–GFP reporter gene in larvae and adults in the absence of experimental immune challenge

A few larvae and pre-pupae were found occasionally to express the reporter gene in all cells of the salivary glands (Figure 2F and G). In contrast, all transgenic adults carrying the drosomycin–GFP reporter displayed some fluorescence in the terminal coiled regions of the salivary glands (Figure 4E). The intensity of fluorescence was variable from one fly to the next. We may assume that this expression is weakly constitutive and can be induced strongly. Often, two paired fluorescent patches were seen inside the proboscis; they most likely correspond to expression of the drosomycin–GFP reporter gene in the two small labellar glands whose secretory ducts open at the beginning of the alimentary canal (Figure 4C). Very few individuals displayed expression of the reporter gene in the pseudotracheae of the proboscis (Figure 4D). The salivary and labellar gland expression was sometimes observed already in late pupal stages.

The above results raise the possibility that drosomycin is secreted into the alimentary canal by two sets of secretory organs, the salivary and labellar glands.

Some adults exhibited other expression patterns in the head. For instance, fluorescence was detected fairly often in the maxillary palps. In some cases, it was clear that expression was taking place in the epithelial cells (Figure 4F), whereas in others, the fluorescence appeared more diffuse and could possibly be due to drosomycin–GFP reporter expression in the small maxillary palp trachea. Similar observations were made in the antennal segments.

As in larvae, expression of the drosomycin–GFP reporter gene was sometimes detected in the adult respiratory system, usually of older insects (Figure 4G). Fluorescence was observed in the spiracles, abdominal tracheae, leg tracheae, the head air sacs and tracheae, and probably in the thoracic air sacs. However, the expression in most cases did not extend to the whole respiratory system, but only portions thereof, suggesting a local immune response in the trachea following an aerial infection.

Occasionally, drosomycin–GFP could be detected in the ejaculatory duct, and more rarely in the ejaculatory bulb of some males (Figure 4B). Depending on the fly, the expression in the ejaculatory duct extended more or less anteriorly. These observations suggest that infections can propagate along the genital ducts and induce a local synthesis of drosomycin to fight off this infection.

In a few adults, several rows of three cells beneath the dorsal abdominal cuticula were found to express the
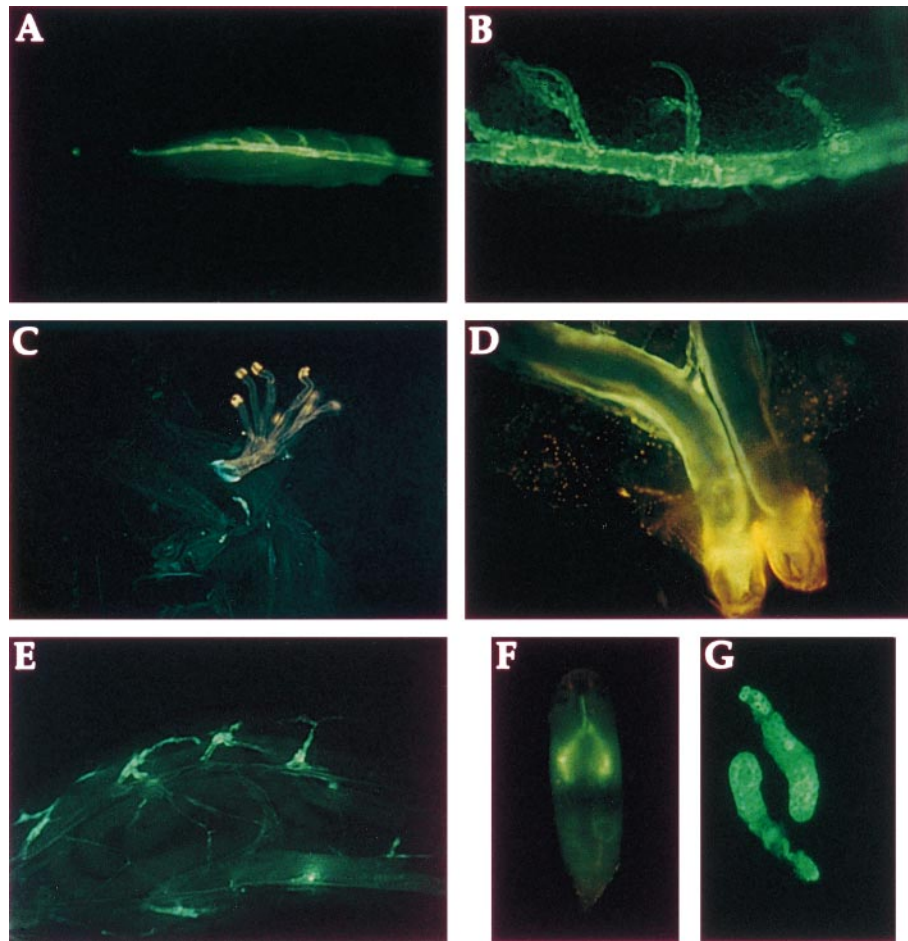
Fig. 2. Localized expression of the drosomycin–GFP reporter gene in larvae. (**A**) This transgenic larva was found in a culture vial and expresses the reporter gene in the right dorsal tracheal trunk in the absence of any experimentally applied immune challenge. Note that only one of the two tracheal trunks is affected. The fluorescent dot on the left corresponds to the expression of the reporter gene in the right anterior spiracle of the larva. The fluorescence is rarely found in the whole tracheal trunk and usually only spans a portion of the trunk. Since these expression patterns are not found in all larvae of a line and since all lines display expression of the reporter genes in a variable proportion of the larvae, these expressions are not the result of an enhancer-trap effect of the transposon insertions. (**B**) Enlargement of (A) showing that the reporter gene is expressed in the tracheal epithelium that secretes and surrounds the tracheal cuticle. The fluorescence is also found in the transverse connectives that emanate from the dorsal trunk to irrigate the tissues of the larva. (**C**) Anterior spiracle of a non-immunized larva observed at high magnification. The fluorescence forms a ring at the base of the spiracle. This ring, when present, sometimes extends further posteriorly along the dorsal tracheal trunk, depending on the larva. (**D**) Posterior spiracles of a non-immunized larva observed at high magnification. Only the distal part of the left dorsal trunk expresses the reporter gene; the right tracheal trunk is partially illuminated by the left dorsal trunk but does not itself express the drosomycin–GFP gene. A description of spiracular structures can be found in Manning and Krasnow (1993). (**E**) Expression of the reporter gene in the transverse connectives and the lateral branches of the tracheal system. There is no expression in the dorsal trunks through which presumably all infections should propagate. One possibility is that the cuticular sheath is thicker in the dorsal trunk and that microorganisms would break through the thinner cuticle found in the transverse connectives more easily, thus triggering a local response there. Another non-exclusive explanation we can bring forward relies on phenomena occurring during larval molts. During embryogenesis, the tracheal system originates from metameric invaginations in the epidermis (reviewed in Manning and Krasnow, 1993). These invaginations become non-functional larval spiracles that are connected to the tracheal system through the spiracular branches. During molts, the old cuticle is degraded in each metamere and is dragged out through the corresponding spiracular branches and spiracles that re-open at that time. Thus, it is possible that infections could draw profit from these events to enter the respiratory tract through the usually collapsed spiracular branch. (F and G) Expression of the reporter gene in the salivary glands of a pupa that had been dipped when at the third larval instar into a concentrated solution of *Aspergillus fumigatus*. This expression pattern is also observed, albeit rarely, in non-immunized larva. The salivary glands and the salivary duct can be seen through the pupa (**F**). All cells of the dissected salivary glands express the reporter gene at an equal level (**G**).

drosomycin–GFP reporter gene (Figure 4H). This expression pattern also included two thin continuous rows of epidermal cells that partially surround the dorsal part of the eyes, as well as two paired groups of abdominal cells that lie on the ventral side at the frontier with the thorax. To our knowledge, these groups of cells have not been identified so far.

All the expression patterns recapitulated in Table I were never induced by wounding experiments that trigger the systemic response.

The drosomycin–GFP transgene reveals a constitutive expression of drosomycin in the female sperm storage organs

All adult females carrying the drosomycin–GFP reporter gene in the different transgenic lines displayed a bright source of fluorescence that could be seen through the cuticle of the posterior part of the abdomen. Upon dissection, the fluorescence was found in the female sperm storage structures: the two spermathecae and the seminal receptacle (Figure 4A). In keeping with this expression,
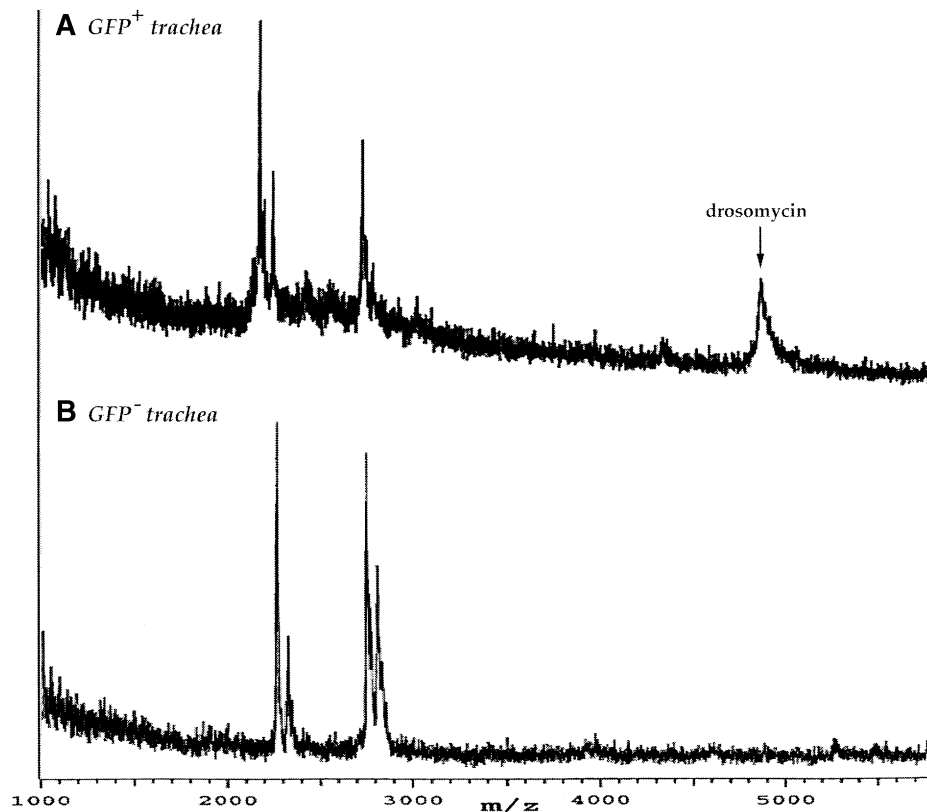
Fig. 3. Detection of endogenous drosomycin in fluorescent tracheae by MALDI-TOF mass spectrometry. The tracheal trunks of larvae were dissected under the fluorescence dissecting microscope. Fluorescent or non-fluorescent tracheal portions were deposited individually on different probes for mass spectrometry analysis. (**A**) Results obtained with a drosomycin–GFP-expressing trachea. (**B**) Data obtained with a non-fluorescent trachea. The peak at m/z 4910 (mass/charge) corresponds to the singly charged ions of drosomycin as controlled with recombinant drosomycin in the same conditions of analysis (Materials and methods). In some cases, the fluorescent and non-fluorescent portions of the same trachea were analyzed, and similar results obtained.

endogenous drosomycin RNA was also detected by *in situ* hybridization in these organs (L.Michaut and R.Lanot, unpublished observations). This signal is not dependent on copulation since it was also found in virgin females. Further, it was present in females grown under axenic conditions. We conclude that this expression is constitutive.

Genetic analysis: a pathway other than Toll?

The systemic antifungal response in the adult has been shown to be under the control of the *spätzle/Toll/tube/pelle/cactus* pathway (Lemaitre *et al.*, 1996). The inducible expression of drosomycin is abolished in *spätzle* (*spz*), recessive lack-of-function *Toll* (*Tl*), *tube* (*tub*) and *pelle* (*pll*) mutants. Drosomycin is constitutively expressed in *cactus* (*cact*) and *Tl* dominant gain-of-function mutants (Lemaitre *et al.*, 1996). Most of this gene cassette, which also establishes the primary dorso-ventral pattern (reviewed in Morisato and Anderson, 1995), has been conserved during evolution and appears to control the activation of the NF-κB transcription factor in the vertebrate inflammatory responses (Ingham, 1994; Kopp and Ghosh, 1995; Verma *et al.*, 1995; Baeuerle and Baltimore, 1996). When this pathway is altered, flies succumb to a fungal, but not to a bacterial infection (Lemaitre *et al.*, 1996). We therefore investigated whether the local expression of drosomycin that is detected in several tissues of ectodermal origin is also controlled by this pathway.

We first looked at insects heterozygous for the drosomycin–GFP reporter transgene and a dominant mutation of *Tl*, *Tl^{10B}*, that results in a constitutively activated TL receptor (Figure 5A–D). Consequently, the drosomycin gene was transcribed strongly in the absence of an immune challenge. In *Tl^{10B}* larvae, we could detect, as expected, a strong GFP expression in all lobes of the fat body; furthermore, some but not all plasmatocytes and lamellocytes were also positive. The intensity of the fluorescence was considerably stronger than in wild-type larvae 12 h after immune challenge, which is likely to result from the continuous accumulation of the stable reporter protein in these tissues since embryogenesis. Strikingly, no constitutive expression could be detected in the tracheal system nor in the salivary glands. However, as in the case of the wild-type, expression of the reporter gene in the tracheae or spiracles could be observed in some larvae, probably in response to a local infection.

In young *Tl^{10B}* adults, a strong fluorescence was at first limited to the abdomen. However, this signal disappeared within 2 days, whereas a weaker fluorescence appeared everywhere in the animal. Upon dissection, it turned out that the cells responsible for the intense fluorescence in the abdomen were larval fat body cells that had not yet lysed. The adult fat body replaces the larval one during the first 2 days of adult life. As in larvae, no constitutive expression of the drosomycin–GFP reporter was detected in tissues of *Tl^{10B}* adults where a local expression is
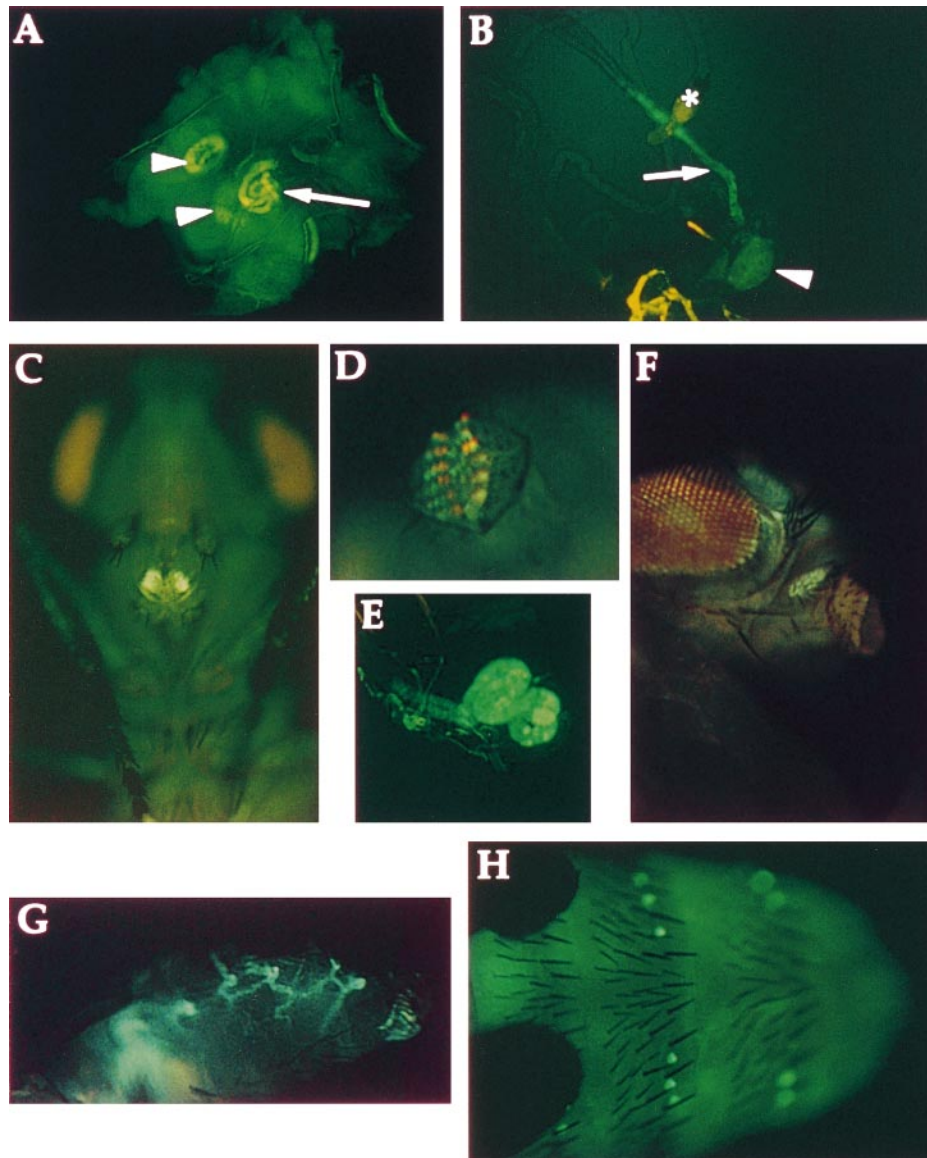
Fig. 4. Localized expression of the drosomycin–GFP reporter gene in adults. (**A**) Constitutive expression of the reporter gene in the seminal receptacle (arrow) and spermathecae (arrowheads) of an immunized adult. The fluorescence in the sperm storage structures is much brighter than that of the surrounding fat body and is probably due to the continuous accumulation of the reporter protein in these structures. The second spermatheca is not in the focal plane of the picture. (**B**) Expression of the reporter gene in the male reproductive tract. In this non-immunized male, the fluorescence is found in the anterior ejaculatory duct (arrow), the ejaculatory bulb (arrowhead) and the posterior ejaculatory duct (not shown). The extent of the expression in the ejaculatory bulb is variable and, when present, may be limited to the posterior part of the ejaculatory duct. The yellow fluorescence observed in the tip of the Malpighi tubules marked by an * is due to the autofluorescence of these structures. (**C**) Expression of the drosomycin–GFP reporter in the labellar glands. The fluorescence is found inside the labellum in paired structures. This expression pattern is frequent. (**D**) High magnification of the bottom of a labellum. Drosomycin–GFP expression is found in the pseudotracheae that conduct liquid food to the tip of the labrum. (**E**) Strong expression of the reporter gene in the adult salivary gland. The fluorescence is limited mostly to the terminal coiled portion of the salivary gland. (**F**) Expression of the drosomycin–GFP in epidermal cells of the maxillary palp. The expressing cells might be sensilla basiconica, i.e. chemoreceptors for taste and smell. In other adults, the fluorescence in the maxillary palp appears more diffuse and may be due to expression in the maxillary palp trachea. (**G**) Expression of the reporter gene in the abdominal tracheal system observed through the cuticle. The fluorescence starts at the abdominal spiracle and extends to the tracheal system. (**H**) Expression of the reporter gene in abdominal sets of cells. Two groups of three cells express drosomycin–GFP at the posterior region of each tergite. Other groups of non-identified cells on the head and ventral abdomen are not shown (see text).

sometimes seen in wild-type adults. These results indicate that in both the larva and the adult, the activation of the *Tl* pathway is not sufficient to trigger expression of drosomycin in these ectodermal tissues.

Next, we determined whether the *Tl* pathway is necessary for the local reporter gene expression or the constitutive expression in the female reproductive tract. To this end, we looked at the expression pattern of the reporter gene in *Tl* and *spz* lack-of-function mutants where the systemic antifungal response is impaired (Figure 5E–I). The spermathecae and seminal receptacles of *Tl* and *spz* females that also carry one or two copies of the GFP reporter transgene constitutively displayed a bright fluorescence. Local expressions in the trachea, salivary and labellar gland, ejaculatory duct, labial palps and pseudotracheae of the proboscis were detected in some *Tl*

Table I. Local expression of the drosomycin–GFP reporter transgene in the absence of experimentally applied immune challenge

	Tissue	Inducible expression[a]	Constitutive expression[b]
Larval respiratory system	Tracheal epithelium	**	
	Spiracles	***	
Larval digestive system	Salivary glands	*	
Adult respiratory system	Tracheal epithelium	**	
	Spiracles	**	
Adult digestive system	Salivary glands	***	#
	Labellar glands	**	
	Pseudotrachea	*	
	Crop	*	
	Rectal ampulla	*	
Adult cuticle	Maxillary palps	**	
	Antenna	*	
	Rows of three abdominal cells[c]	*	
Reproductive system	♀ spermathecae and seminal receptacle	?	###
	♂ ejaculatory duct	*	
Systemic expression	Fat body	*	

[a]The frequency of each expression is scored semi-quantitatively from * to ***. However, these should be considered only as indications, since the actual frequencies vary considerably and are dependent on the vial culture conditions, rendering a statistical analysis meaningless.
[b]The strength of the constitutive expression is indicated: # weak expression; ### strong expression, the intensity of which is stronger than that of the systemic expression in the fat body.
[c]These non-identified group of cells are shown in Figure 4H and described in the Results.

or *spz* adults, as in wild-type. Likewise, expression of the transgene in a *spz* background was also observed in the tracheae of homozygous larvae and in the salivary glands of early pupae. Thus, the local expression of drosomycin–GFP was not eliminated in these mutants, whereas the systemic immune response in the fat body was abolished.

Discussion

Here we report the expression pattern in live *Drosophila* of the major antifungal peptide drosomycin using several GFP reporter transgenes. The GFP reporter gene expressed under the control of the drosomycin promoter appears to reproduce faithfully the transcription pattern of the endogenous drosomycin gene, both quantitatively and qualitatively. Further, since the endogenous and the reporter protein accumulate at roughly the same rate, and since both proteins are resistant to most proteases, it appears likely that the GFP fluorescence accurately mirrors the actual synthesis of the endogenous drosomycin peptide (see Figure 3 for instance).

A local immune response in the respiratory system of *Drosophila*

In *Drosophila*, a septic injury induces the rapid synthesis in the fat body of a cocktail of antimicrobial peptides that are secreted in the hemolymph where they neutralize invading microorganisms (Hoffmann and Reichhart, 1997). This reaction constitutes the systemic response to infection. Our data suggest strongly that *Drosophila* is also able to fight off infections at a local level in epithelia that are in contact with the external environment without mobilizing the systemic response. The expression of the drosomycin–GFP reporter gene in the tracheal system clearly illustrates this phenomenon. First, in most cases, the expression pattern is asymmetrical, both in adults and in larvae, being for instance limited to one of the tracheal trunks of the larva. Furthermore, the GFP signal is detected in the transverse connectives that are connected to the

GFP-expressing parts of the dorsal trunk, and not in those connected to non-expressing parts (Figure 2). Second, the expression is often confined to the entry points of the tracheal system, i.e. the anterior or posterior spiracle, suggesting that drosomycin is synthesized there as a response to a local infection of the aerial pathway. In this view, expression patterns that extend to the whole tracheal trunk may be the results of infections that have not been stopped by the spiracular synthesis of drosomycin. Third, local expression of the reporter gene in the tracheal system of larvae grown under axenic conditions is almost totally abolished. Finally, it is possible to induce the expression of the drosomycin reporter gene systematically throughout the tracheal system by dipping larvae in concentrated microbial solutions. Thus, drosomycin is expressed in the tracheal system as a local immune response to an infection of the respiratory system. However, we cannot exclude that other stimuli, e.g. wounding, can also trigger a local expression in epithelia.

A local immune response in 'barrier epithelia'

Strikingly, we detect reporter gene expression in most tissues that are in contact with the external environment, suggesting that drosomycin is expressed there as a local response to infections, as is the case for the respiratory system.

The digestive tract may be a route of entry for infectious microorganisms as they are likely to be abundant in food. We did not detect any expression of drosomycin–GFP in the gut, in contrast to what has been found in the mosquito *Anopheles gambiae* and in the blood-sucking fly *Stomoxys calcitrans*, which express defensins in the anterior midgut (Dimopoulos *et al.*, 1997; Lehane *et al.*, 1997). However, the reporter gene is frequently strongly expressed in the distal part of the salivary gland of the adult, and less frequently in the labellar glands. These observations suggest that drosomycin may be secreted into the food before reabsorption by the fly to prevent infections of the digestive tract.
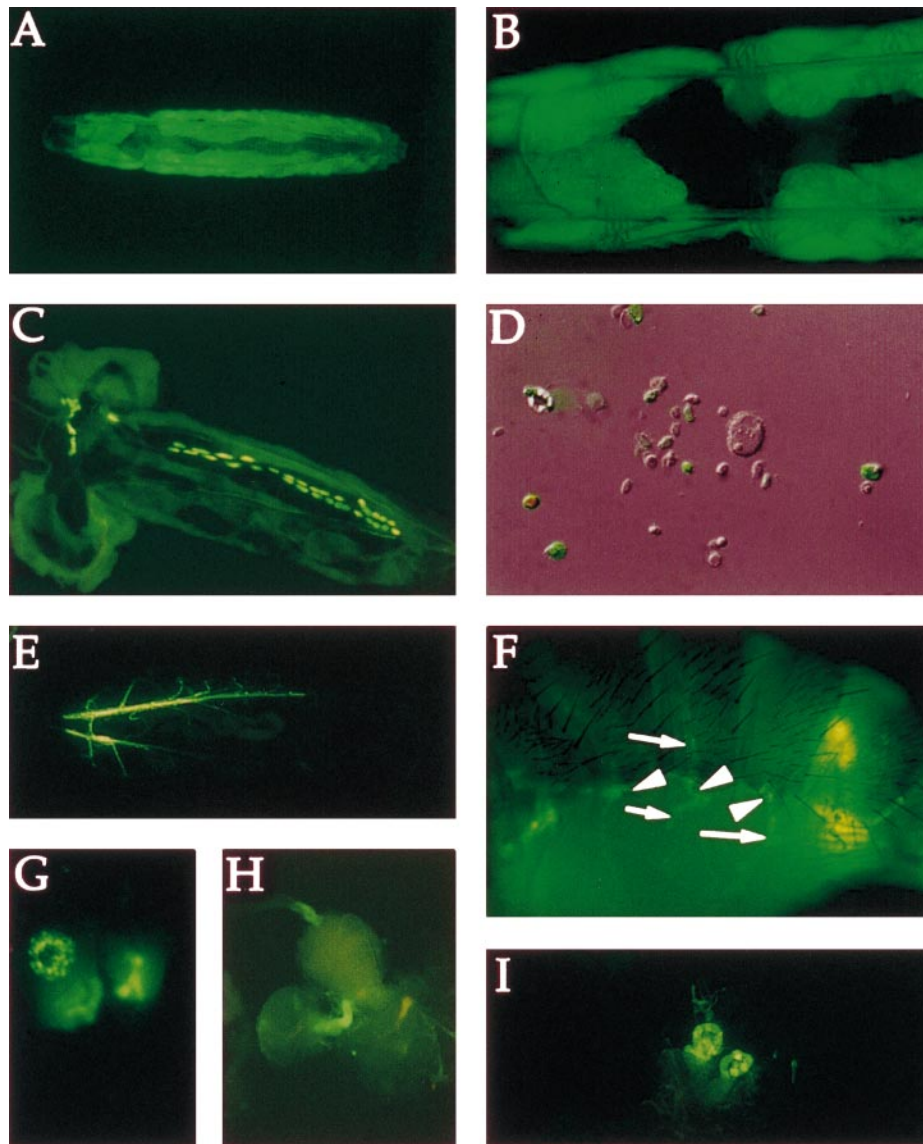
Fig. 5. The localized expression of the drosomycin–GFP reporter transgene is not dependent on the *Tl* pathway. (A–D) *Tl^{10B}* larvae in which the TL receptor is constitutively activated. The non-secreted GFP reporter gene is constitutively expressed in the fat body (**A** and **B**), but not in the tracheae (**B**). In contrast, the secreted drosomycin–GFP fusion (**C**) accumulates in the two rows of pericardial cells, and in garland cells (left of the dissected larva). It is not known whether the endogenous drosomycin peptide is also taken up by these nephrocytes. A variable fraction of plasmatocytes expresses the reporter gene (**D**), as was found in the case of the injury-induced systemic response. (E–I) Variable expression patterns found in non-immunized *spz^{rm7}/spz^{rm7}* larvae and adults. Similar expression patterns were observed in *Tl^{632}/Tl^{9QRE}* adults. (**E**) First instar larva displaying an expression in the tracheal system. (**F**) Expression of the reporter gene in the adult tracheal system as observed through the cuticle. Arrows show the tracheae, and arrowheads the spiracles. (**G**) Dissected spermathecae and seminal receptacle showing constitutive expression of the reporter gene. (**H**) Expression of the reporter gene in the posterior (middle) and anterior (top) ejaculatory duct. The ejaculatory bulb does not express the reporter gene. (**I**) Strong expression in the coiled portions of the salivary glands.

The great reproductive abilities of insects are central to their evolutionary success. The strong constitutive expression of drosomycin in the female sperm storage structures may reflect an evolutionary constraint to prevent infection of these structures by sperm infected during copulation. Furthermore, other antibiotic peptides have been found in the *Drosophila* reproductive tract. Drosocin has been observed in the calix and oviducts of fertilized females (Charlet *et al.*, 1996). Andropin is constitutively synthesized by the male ejaculatory duct and may be transferred to the female genital tract (Samakovlis *et al.*, 1991). Indeed, an antibacterial activity has been found in the ejaculatory duct that is transferred to females in the sperm (J.Postlethwait, personal communication). In the medfly *Ceratitis capitata*,

ceratotoxins are constitutively expressed in the accessory gland under the control of juvenile hormone, and their expression is enhanced by mating (Rosetto *et al.*, 1996; Manetti *et al.*, 1997). It is not clear whether the increased expression is due to mating *per se* or to microbial contamination during copulation. In the *Drosophila* male, drosomycin expression in the ejaculatory duct is more likely to constitute a local immune response to protect the male genital tract from an ongoing infection, since this expression is not constitutive, is not induced by mating and its extent is variable from one individual to another, most displaying no response however.

We have detected a local expression of drosomycin–GFP in epithelial tissues of the trachea and of the salivary

glands, but not in the cuticular epithelium, except for the maxillary palps. Due to the size of the larvae, it appears difficult to reproduce abrasion experiments that have been performed on the silkworm *Bombyx mori* which have demonstrated a localized synthesis of cecropins in the cuticular epithelium (Brey *et al.*, 1993). Except for one single case, we never detected a local epidermal response at the wounding site. In naturally occurring and post-challenge systemic responses, we cannot exclude that fluorescence in the fat body would have masked such a response. The results obtained with the silkworm abrasion experiments indicate that a local immune response is not limited to drosomycin or to *Drosophila*. Preliminary results obtained with the GFP reporter gene system suggest that other antimicrobial peptides also display a local immune response in *Drosophila* (S.Ohresser, personal communication).

Characteristics of the local immune response

One striking feature of the localized drosomycin response is that it does not involve the systemic response. This implies that epithelial cells carry receptors that are able to recognize non-self determinants, presumably conserved structural patterns found on the surface of microorganisms (Janeway, 1989). These activated receptors subsequently induce a transduction cascade that results in the local synthesis and secretion of drosomycin by these very same cells. It is interesting in this respect that this pathway does not involve the *spz–Tl* signaling pathway that is used in the systemic response, a process that may be mediated by putative cytokines in the hemolymph. Alternatively, proteases secreted by microorganisms could trigger the localized response by altering a receptor on the cell surface of these tracheal cells. The case of the up-regulated adult salivary gland drosomycin–GFP expression may be an exception in that it is unlikely that the distal salivary gland cells directly detect the infection. One may wonder whether the infection might be detected at the level of the maxillary palps cells that would in turn signal to the salivary glands.

The *Drosophila* systemic antifungal response in adults has been shown to be controlled by the *spätzle/Toll/cactus* gene cassette (Lemaitre *et al.*, 1996). The constitutive activation of the *Tl* pathway is not sufficient to trigger expression in the tracheal system or in the salivary glands; the absence of the putative ligand SPZ, or of its receptor TL, does not prevent the localized expression in both the adult and the larva. Therefore, the local host response is regulated by another, as yet undetermined, genetic pathway. Furthermore, the dissection of the drosomycin promoter has revealed that the systemic and local immune responses are controlled by distinct regulatory elements (L.Michaut and E.Levashina, unpublished data). In this respect, it is worth noting that the systemic response takes place in tissues of mesodermal origin (fat body and hemocytes), whereas the local expression which we detect is found in epithelia of ectodermal origin.

The local host response: a common strategy in the animal kingdom?

Similarities between the insect systemic immune response and the mammalian acute phase response of inflammation have been underscored. Here, we report a localized immune response in barrier tissues that displays striking parallels with vertebrate mucosal immunity. Indeed, several antimicrobial peptides, α- and β-defensins, have been found in different mammalian epithelia, including the lingual epithelium (LAP), the aerial tract mucosa (TAP, β-defensin 1), the submaxillary salivary glands, kidney, prostate, testis, female genitourinary tract (β-defensin 1) and Paneth cells of the intestine (α-defensins) (Diamond *et al.*, 1993, 1996; Schonwetter *et al.*, 1995; Ouelette and Selsted, 1996; Russell *et al.*, 1996; Zhao *et al.*, 1996). A recent study reported widespread expression of bovine β-defensins in a variety of epithelial tissues. Interestingly, cows suffering from a chronic gut infection with *Mycobacterium paratuberculosis* displayed an intense expression in the ileal mucosal region. Similarly, β-defensins were markedly up-regulated in the lung after an experimental respiratory infection with *Pasteurella haemolytica* (Stolzenberg *et al.*, 1997). Thus, vertebrate defensins are thought to be important components of the innate immune response in mucosal barriers; the role of β-defensins in the immune defense of the respiratory airway is illustrated dramatically in cystic fibrosis patients (Smith *et al.*, 1996; Goldman *et al.*, 1997). Strikingly, drosomycin is expressed in analogous tissues, among them the tracheal epithelia, salivary glands and genital tract. For lack of adequate mutants, we cannot yet demonstrate that the local drosomycin expression in these tissues is necessary for a local host defense. However, this is very likely since mutants in which the systemic antifungal response is abolished are more susceptible to fungal infections (Lemaitre *et al.*, 1996).

Epithelia that are in contact with microorganisms constitute both a physical and an immune barrier. The secretion of antimicrobial peptides locally helps to prevent the spreading of infections and thus provides a first line of defense, the local immune response, which does not require the triggering of a systemic immune response. This strategy is energetically inexpensive for dealing with frequent infections propagated by contact or by minor wounds. Indeed, during the systemic response, drosomycin is found in concentrations as high as 100 µM that are likely to exert an important metabolic toll. Similarly, during the mammalian hepatic acute phase response, the major acute phase reactants such as C-reactive protein or serum amyloid A protein can be induced up to 1000-fold over normal levels to reach a 1 mg/ml concentration (reviewed in Steel and Whitehead, 1994). Indeed, it has been shown recently that the artificial selection in *Drosophila melanogaster* of improved resistance against the parasitoid *Asobora tabida* through the cellular arm of immunity is correlated with a reduced larval competitive ability under a high competition environment (Kraaijeveld and Godfray, 1997). These observations indicate that an improved immune resistance comes with a selective cost. Selection is likely to have favored the constitutive expression of antimicrobial peptides in tissues that are continually exposed to infections or that are especially important, e.g. those of the reproductive tract. Future studies will reveal whether local immune responses have originated early in evolution and have been thereafter conserved, or whether insect and mammals have developed a similar strategy independently.

Materials and methods

Plasmids

We constructed two sets of reporter genes carrying GFP: one expressing only GFP, and the other a GFP fusion to the drosomycin pre-propeptide so that it would be secreted. Using PCR-directed mutagenesis, we created a *Bam*HI site after the GFP stop codon contained in plasmid pTU#58 (a kind gift of M.Chalfie) (Chalfie *et al.*, 1994). Next, we added at this site 800 bp of drosomycin downstream sequences (drosomycin terminator) contained in a *Bam*HI–*Xho*I fragment. Using site-directed mutagenesis, we created an *Nhe*I site after the starting ATG (non-secreted form) or after position 59 of the drosomycin coding sequences (secreted fusion). *Sal*I–*Nhe*I fragments containing 2450 bp of the drosomycin promoter (up to the starting ATG or amino acid 59) were fused to the drosomycin–GFP 3′ end sequences. Finally, both cassettes were inserted as *Sal*I–*Xho*I fragments at the *Xho*I site of P-element-mediated transformation vector pCasPer NNSXS, a modified version of pCasPer (Ferrandon, 1994), yielding plasmids pJM608 [drosomycin promoter–GFP–drosomycin downstream sequences (non-secreted)] and pJM612 [drosomycin promoter–59 amino acids of pre-prodrosomycin–GFP–drosomycin downstream sequences (the secreted fusion protein contains 33 amino acids of the drosomycin peptide)]. It turned out that the product of the wild-type GFP transgene could be detected by its fluorescence only 2 days after induction. This is most likely due to a long delay in the formation of the fluorophore in the fat body since the protein itself is synthesized much earlier (M.-C.Criqui, A.C.Jung and D.Ferrandon, unpublished observations). We therefore tested the S65T mutant of GFP.

To reconstitute the GFP S65T version, we mutated the GFP gene contained in plasmid pJM703 using PCR-directed mutagenesis (Higuchi *et al.*, 1988), with the following primers: IMU 161 TGTCACTACTTTC **A**CT TATGGTGTTCA and IMU 162 TGAACACCATAAG **T**GA AA GTAGTGACA. This mutated PCR fragment was cut with *Nco*I and *Nde*I and inserted in an *Nco*I–*Nde*I-cut pJM704 plasmid, yielding plasmid pJM705 in which the GFPS65T gene is included in a pBluescript KS+ vector. A partial GFP S65T *Nhe*I–*Hpa*I fragment was excised from pJM705 and cloned either into an *Nhe*I–*Hpa*I-cut pJM608 or an *Nhe*I–*Hpa*I-cut pJM612 transformation vector, yielding respectively pJM802 (non-secreted version) and pJM804 (N-terminal end drosomycin–GFP fusion).

Fly strains

All fly stocks are described in Lindsley and Zimm (1992). w⁻ flies were used as wild-type flies and were also used as recipients for transformation. Transgenic lines were established as described (Driever *et al.*, 1990). We obtained at least nine independent lines for construct pJM802, and 16 for pJM804, with transposons located on all three major chromosomes. Northern blot analysis showed that both the endogenous and the reporter genes are expressed at similar levels with the same kinetics of accumulation (data not shown). To obtain *Tl* mutant flies, we crossed *Tl^632* (thermosensitive mutation) and *Tl^9QRE*, both of them in a homozygous transgenic reporter (802 or 804) background at 20°C, and we analyzed the phenotypes of the transheterozygous flies at 29°C. *spz* alleles were *spz^rm7* and *spz^097*. The systemic antifungal response is abolished in these mutant backgrounds (Lemaitre *et al.*, 1996).

Microscopic observations

Live flies and larvae were anesthetized (adults with ether; larvae on ice) and viewed under epifluorescent illumination (excitation filter 480/40 nm; dichroic filter 505 nm LP; emission filter 510 nmLP) with a Leica MZ12 dissecting scope. Dissections were performed in phosphate-buffered saline (PBS) under the dissecting scope and were viewed using either the dissecting microscope or a Leitz Diaplan epifluorescence microscope using a fluorescein isothiocyanate (FITC) filter set. Photographs were taken on a 400 ASA Fujicolor film. They were numerized, enhanced if necessary and assembled using Photoshop 3.0 (Adobe). We are confident that the observed expression patterns reflect the actual synthesis patterns of the endogenous drosomycin since similar expression patterns have been observed by *in situ* hybridization with a drosomycin probe (systemic response, larval salivary glands, sperm storage structures). See also Figure 3.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)

Instrumentation. MALDI mass spectra were acquired on a Bruker (Bremen, Germany) BIFLEX™ matrix-assisted laser desorption time-of-flight mass spectrometer equipped with SCOUT™ High Resolution Optics, an X–Y multisample probe, a gridless reflector and the HIMAS™ linear detector. Ionization was accomplished with the 337 nm beam from a nitrogen laser. All spectra were obtained in the linear positive ion mode and externally calibrated with a mixture of three standard peptides (synthetic drosocin without sugars, synthetic metchnikowin and recombinant drosomycin with MH+ at m/z 2199.5, 3046.4 and 4890.5, respectively). Once the conditions to detect drosomycin in the trachea were found (higher laser energy than for external calibration), a recombinant drosomycin sample was analyzed again under these conditions and found to display an m/z between 4900 and 4910.

Sample preparation. From a 1/1 mixture of α-cyano-4-hydroxycinnamic acid (4HCCA, 40 mg/ml in acetone, Sigma) and nitrocellulose (NC, 40 mg/ml in acetone, Millipore) half diluted with isopropanol, 0.5 µl was deposited on the probe tip and allowed to air dry. The drosomycin–GFP⁻ or –GFP⁺ tracheae were dissected under the dissecting microscope with epifluorescence illumination, rinsed in PBS and then directly loaded onto this NC/4HCCA bed and covered by 0.5 µl of a second matrix solution, which is 4HCCA at 7 mg/ml in H_2O, 0.1% trifluoroacetic acid (TFA)/acetonitrile (1/1, v/v). After air drying, this preparation was washed with 1 µl of 0.1% TFA.

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