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(54) Title: METHODS

(57) Abstract: The present invention relates to methods of screening for potential insecticidal agents using genes encoding insect P450 enzymes. In particular the invention relates to methods of using insect *cyp* genes and/or their promoters to identify and/or optimise potential insecticidal agents. Such methods employ transgenic cells and/or whole organisms in which one or more *cyp* gene is deleted or under-expressed.



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METHODS

The present invention relates to methods of screening for potential insecticidal agents using genes encoding insect P450 enzymes. In particular the invention relates to methods of using insect *cyp* genes and/or their promoters, as well as the products of said *cyp* genes, to identify and/or optimise potential insecticidal agents. The methods employ transgenic cells and/or whole organisms in which one or more *cyp* gene is deleted or under-expressed as well as transgenic cells, cell-lines and/or organisms that over-express one or more *cyp* genes or express one or more *cyp* genes in a heterologous manner. The invention further extends to the transgenic cells, cell-lines and/or organisms used in the methods of the invention as well as to DNA constructs comprising one or more *cyp* gene and DNA constructs comprising the promoter of a *cyp* gene.

Cytochrome P450 monooxygenases (CYPs) comprise a superfamily of enzymes that are found in varying numbers in almost all living organisms from bacteria to mammals. These enzymes are known to catalyse a variety of different types of chemical reaction, including for example aromatic and aliphatic oxidation, hydroxylation, dealkylation, epoxidation, oxidative deamination, desulphuration and dehalogenation reactions. In addition to monooxygenase activity, the P450s also demonstrate reductase, peroxidase and oxidase activities. It is thus unsurprising that, given their wide range of catalytic activities, these enzymes have been shown to be involved in the metabolism of a wide range of xenobiotics.

Different classes of xenobiotic compounds have been, and are being, used as insecticides. However, resistance to a large number of these compounds has emerged in most insect species. The completion of the sequencing of the *Drosophila* genome has revealed a total of 90 P450 or *cyp* genes (Adams *et al.* 2000 Science 287(5461):2185-2195).

Studies have shown that several insect *cyp* genes play a role in resistance to insecticides (Scott 1999, Insect Biochem. & Mol. Biol. 29:757-777) and molecular studies in *Drosophila* have shown a positive correlation between insecticide resistance and over-expression of one or more *cyp* genes (Maitra *et al.* 2000 Gene 248:147-156). Accordingly there exists a need to develop novel insecticides that are resistant to degradation by such P450 enzymes.

One conventional method of identifying novel insecticidal compounds relies on screening compounds against wild-type insects and assessing whether the compound has

any deleterious effect on the insect. When potential insecticidal compounds are screened for activity against wild-type insects, only very few are shown to be highly insecticidally active and it is routinely only these compounds that are taken forward into further development as a potential insecticide. The vast majority of compounds, which either
5 exhibit only a low level of insecticidal activity or which have no insecticidal activity at all, are often discarded.

The apparent lack of insecticidal activity of a compound screened against wild-type insects may be due to the fact that the compound is truly incapable of acting as an insecticide. Alternatively, the apparent lack of insecticidal activity may be due to the
10 compound being metabolised by the insect. It is thought that some insect *cyp* genes (in particular *cyp6* genes of *Drosophila* and *Musca* species; Maitra *et al. supra*) are constitutively expressed at a low level in the wild-type insect. Thus the low level of expression of particular P450 genes and concomitant low level of residual activity of the P450 enzymes coded therefore could be contributing to the apparent lack of insecticidal
15 activity of a compound. It is thus likely that some of the compounds that are routinely discarded, due to their lack of insecticidal activity against wild-type insects, have the potential to be insecticides if they were modified so that they were no longer susceptible to P450-mediated metabolism. This disadvantage of the conventional method of screening (i.e. the fact that weak chemical leads are often discarded) is addressed by the
20 current invention which uses transgenic insects and insect cells wherein the level of expression, or the activity of a P450 gene has been reduced relative to the level of expression or activity of the P450 gene in a wild-type insect or insect cell, in order to screen for and identify such weak chemical leads.

Thus in a first aspect of the invention there is provided a transgenic insect or insect
25 cell wherein the level of expression of a P450 gene has been reduced relative to the level of expression of said P450 gene in a non-transgenic insect or insect cell.

For the avoidance of doubt the term "transgenic" as used herein in reference to an insect or insect cell, describes an insect or an insect cell wherein the genetic material of said insect or insect cell has been altered using recombinant DNA technology. Thus, a
30 transgenic insect or insect cell of the invention does not include a naturally occurring or chemically produced mutant insect or insect cell wherein the level of expression of a P450 gene has been reduced relative to the level of expression of said P450 gene in a non-mutant or wild-type insect or insect cell.

Preferably the P450 gene whose level of expression is reduced in a transgenic insect or insect cell of the invention is one of the 90 *cyp* genes found in *D. melanogaster* (i.e. *cyp4g1*, *cyp4d1*, *cyp4d14*, *cyp4d2*, *cyp4ae1*, *cyp4g15*, *cyp28c1*, *cyp311a1*, *cyp318a1*, *cyp4s3*, *cyp306a1*, *cyp18a1*, *cyp308a1*, *cyp6v1*, *cyp6t1*, *cyp307a2p*, *cyp309a1*, *cyp309a2*,
 5 *cyp28d2*, *cyp28d1*, *cyp4ac1*, *cyp4ac2*, *cyp4ac3*, *cyp6a16*, *cyp4d21*, *cyp4e3*, *cyp28a5*, *cyp303a1*, *cyp310a1*, *cyp6t2p*, *cyp6w1*, *cyp6a2*, *cyp6u1*, *cyp9b1*, *cyp9b2*, *cyp4ad1*, *cyp4e2*, *cyp4e1*, *cyp6a14*, *cyp6a15p*, *cyp6a13*, *cyp4p2*, *cyp4p1*, *cyp4p3*, *cyp49a1*, *cyp12d1*, *cyp6g1*, *cyp6g2*, *cyp6t3*, *cyp301a1*, *cyp9h1*, *cyp6a22*, *cyp6a17*, *cyp6a23*, *cyp6a19*, *cyp6a9*, *cyp6a20*, *cyp6a21*, *cyp6a8*, *cyp317a1*, *cyp4aa1*, *cyp12b2*, *cyp6d2*,
 10 *cyp9c1*, *cyp4d20*, *cyp302a1*, *cyp307a1*, *cyp316a1*, *cyp4d8*, *cyp314a1*, *cyp312a1*, *cyp12c1*, *cyp305a1*, *cyp313b1*, *cyp12e1*, *cyp315a1*, *cyp313a5*, *cyp313a2*, *cyp313a3*, *cyp9f2*, *cyp9f3p*, *cyp313a4*, *cyp304a1*, *cyp6d5*, *cyp313a1*, *cyp12a4*, *cyp12a5*, *cyp6d4*, *cyp6a18*, or *cyp4c3*, or a homologue thereof. The nucleotide sequences encoding the above *cyp* genes may be obtained from the FlyBase database, which is a database of genetic and molecular
 15 data for *Drosophila*. The database is maintained by a consortium of researchers funded by the National Institutes of Health, U.S.A., and the Medical Research Council, London and the database may be accessed at: <http://flybase.bio.indiana.edu/>.

In an embodiment of the present invention the P450 gene is selected from the group consisting of the *cyp6* gene family from *D. melanogaster* and homologues thereof.
 20 The *cyp6* genes from *D. melanogaster* include: *cyp6v1*, *cyp6t1*, *cyp6a16*, *cyp6w1*, *cyp6a2*, *cyp6u1*, *cyp6a14*, *cyp6a15*, *cyp6a13*, *cyp6g1*, *cyp6g2*, *cyp6t3*, *cyp6a22*, *cyp6a17*, *cyp6a19*, *cyp6a9*, *cyp6a20*, *cyp6a21*, *cyp6a8*, *cyp6d2*, *cyp6d5*, *cyp6d4*, *cyp6a18* and *cyp6a23*.

In preferred embodiments the P450 gene is selected from the group consisting of
 25 *cyp6g1*, *cyp6a2*, *cyp6a8* and homologues thereof, and it is particularly preferred that the P450 gene is either *cyp6g1* or *cyp6a2*.

The term "homologue" as used herein with respect to a P450 or *cyp* gene refers to any gene which exhibits substantial identity to a named P450 or *cyp* gene. Preferably a homologue exhibits at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% identity with
 30 a named P450 or *cyp* gene. Most preferably a homologue exhibits at least 96%, 97%, 98% or 99% identity with a named P450 or *cyp* gene.

Homologues include P450 or *cyp* genes found in the same genus and species as the named P450 or *cyp* gene, as well as P450 or *cyp* genes from insects of other genera and/or species. Thus the homologue may come from the group of insects consisting of:

Drosophila sp., *Bombyx mori*, *Tribolium castaneum*, *Aedes aegyptii*, *Anopheles gambiae*, *Anopheles albimanus*, *Anopheles stephensi*, *Ceratitis capitata*, *Pectinophora gossypiella*, *Helicoverpa zea*, *Bactrocera dorsalis*, *Anastrepha suspense*, *Musca domestica*, *Stomoxys calcitrans*, *Heliothis virescens*, *Manduca sexta* and *Lucilia cuprina*. Preferably the

5 homologue will come from a known insect pest species, including in particular (but not limited to) *Heliothis virescens* and *Manduca sexta*.

The transgenic insect or transgenic insect cell may be any insect or insect cell which is capable of being transformed. In particular the transgenic insect may be, or the transgenic insect cell may come from, any one of the following insects: *Drosophila sp.*

10 *Bombyx mori*, *Tribolium castaneum*, *Aedes aegyptii*, *Anopheles gambiae*, *Anopheles albimanus*, *Anopheles stephensi*, *Ceratitis capitata*, *Pectinophora gossypiella*, *Helicoverpa zea*, *Bactrocera dorsalis*, *Anastrepha suspense*, *Musca domestica*, *Stomoxys calcitrans*, and *Lucilia cuprina*. In addition, a transgenic insect cell of the invention may also be derived from *Spodoptera frugiperda* or *Trichoplusia ni*. In one embodiment it is

15 particularly preferred that the transgenic insect cell is derived from a *D. melanogaster* S2 cell.

Insects from the genera *Drosophila*, in particular *Drosophila melanogaster*, *Drosophila simulans* and *Drosophila virilis* are considered as particularly suitable for use in the invention. In one preferred embodiment, transgenic insects or insect cells of the

20 invention are derived from *D. melanogaster*.

Transgenic insects and insect cells according to this aspect of the invention exhibit a reduced level of expression of a P450 gene in comparison to the level of expression of said P450 gene in a wild-type non-transgenic insect or insect cell. Where the transgenic insect is *D. melanogaster*, *D. melanogaster* Canton S is particularly suitable as a wild-

25 type non-transgenic insect and where the transgenic insect cell is derived from a *Drosophila* cell line, such as the *Drosophila* S2 cell line, the untransformed cell line may act as a suitable non-transgenic insect cell for the purposes of comparison.

For the avoidance of doubt, a reduced level of expression of a P450 gene includes any level of expression that is less than the level of expression of the P450 gene in the

30 non-transgenic insect or insect cell and includes no detectable expression of said P450 gene in the transgenic insect or insect cell.

The reduced level of expression of the P450 gene in the transgenic insect or insect cell may be effected through gene disruption at the locus for the P450 gene in the insect concerned. This may be achieved through homologous recombination (see Rong & Golic

2000 Science 288(5473):2013-2018 for methodology relating to homologous recombination in *Drosophila*) or through insertional mutagenesis e.g. transposon insertion.

Alternatively the reduced level of P450 expression may be effected using an RNA-interference (RNAi) mediated knockdown or knockout of P450 or *cyp* gene function, via a
5 either a transient RNAi approach, or via the generation of a stable transgenic insect line (Kennerdell & Carthew 2000, Nature Biotech. 17:896-898. Where a stable *Drosophila* line is produced, it is preferred that the reduction in P450 gene expression level is inducible. This may be achieved by employing the GAL4-UAS system (Brand &
10 Perrimon, 1993, Development 118:401-415) as described herein in the Examples.

In one embodiment of the invention where the reduction in the level of P450 gene expression is mediated through an RNAi approach, an insect or insect cell is transformed with a DNA construct comprising a first DNA sequence encoding a fragment of an insect P450 gene and a second DNA sequence which is the reverse complement of the first DNA
15 sequence, wherein the first and second DNA sequences are present on the same strand of DNA. Upon transcription a mRNA molecule will be produced that is capable of forming a hairpin or stem-loop structure by virtue of the complementarity of the regions in the mRNA which correspond to the first and second DNA sequences. This double stranded mRNA region acts as a source of short interfering RNA molecules and thus mediates the
20 knockdown or knockout of P450 gene function, which is manifested in transgenic insects or insect cells as a reduced level of expression of the P450 or *cyp* gene.

Thus in a further aspect the invention provides a DNA construct comprising a first DNA sequence encoding a fragment of an insect P450 gene and a second DNA sequence which is the reverse complement of the first DNA sequence, wherein the first and second
25 DNA sequences are present on the same strand of DNA and are operably linked to a promoter region and optionally a terminator region.

It is thought that in order for the level of gene expression to be reduced through an RNAi mediated mechanism, the minimum size of a short interfering RNA is between 21 and 23 nucleotides (Zamore *et al.* 2000 Cell 101:25-33). Accordingly for just a single
30 short interfering RNA to be produced from the transcript of a DNA construct of this aspect of the invention, the minimum length of the first and second DNA sequences in said DNA construct is 23 nucleotides. However, it is preferred that the length of the first and second DNA sequences is considerably longer than 23 nucleotides. Preferably the

first and second DNA sequences will be greater than or equal to 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, or 1500 nucleotides in length.

In one embodiment a DNA construct of the invention will additionally comprise a region of DNA which acts as a spacer region between the first and second DNA sequences. Such a DNA sequence may be incorporated to facilitate cloning or to facilitate the formation of a double-stranded mRNA structure, such as a hairpin or stem-loop structure, when the DNA sequence of the DNA construct is transcribed.

Sequences that are known to be introns in a gene of the insect that is to be transformed with a DNA construct of the invention are particularly suitable for this purpose.

The first and second DNA sequences that are present in a DNA construct of the invention are operably linked to a promoter region. In general the promoter region will be any promoter region capable of driving expression of the first and second DNA sequences in the insect or insect cell into which a DNA construct of the invention is to be introduced. The promoter region may comprise a constitutive promoter, a tissue- or developmentally-specific promoter, or an inducible promoter. Preferably the promoter region comprises an inducible promoter so that the reduction in the level of expression of the P450 gene is controllable. Preferably the inducible promoter comprises UAS hsp70 TATA promoter sequences responsive to the GAL4 protein.

DNA constructs of the invention optionally comprise a transcriptional terminator region. Typically, such a transcriptional terminator region will be one that is suitable for the termination transcription in the insect/insect cell into which the DNA construct is to be introduced. Transcriptional terminator regions suitable for this purpose are described in the art.

DNA constructs of the invention are introduced into insect cells to form transgenic insects and insect cells using any suitable method available in the art. In particular, *D. melanogaster* embryos may be transformed using the methodology described by Spradling & Rubin 1982 (Science 218:341-347), for example, as described herein in the Examples.

Where the promoter region of a DNA construct of the invention comprises a constitutive promoter region, transformation of the DNA construct into an insect or insect cell will yield a transgenic insect or insect cell of the invention. Thus, a transgenic insect or insect cell of the invention may be produced merely by transforming an appropriate insect or insect cell with a DNA construct as described hereinbefore.

However, where the promoter region comprises an inducible promoter an inducing agent must be applied to (or be present within) the transformed insect or insect cell.

In *Drosophila* this may be achieved through the use of a binary system as described by Brand & Perrimon (1993, Development 118:401-415) and as used herein in the Examples. Essentially a DNA construct of the invention is introduced into a first insect and a second DNA construct, which encodes a protein capable of regulating expression from the inducible promoter in the DNA construct of the invention, is introduced into a second insect. The first and second insects are then genetically crossed to yield progeny, a proportion of which will comprise both the first and second DNA constructs. Where expression of the protein encoded by the second DNA construct is under the control of a constitutive promoter, the population of progeny comprising both the first and second DNA constructs will comprise not only a DNA construct of the invention but also the inducing agent necessary to effect induction from the promoter region in the first DNA construct. Consequently the population of progeny, which comprises both the first and second DNA constructs, consists of transgenic insects of the invention.

Thus in a further aspect of the invention there is provided a method of producing a transgenic insect of the invention, which comprises: (i) transforming a first insect with a first DNA construct as described hereinbefore wherein the promoter region comprises an inducible promoter; (ii) transforming a second insect with a second DNA construct encoding a protein that is capable of inducing expression from the inducible promoter in the first DNA construct; (iii) crossing the transformed insects resulting from steps (i) and (ii) to obtain progeny; wherein a population of the progeny resulting from step (iii) comprises both the first and the second DNA constructs and when the protein encoded in the second DNA construct is expressed, the level of expression of the P450 gene is reduced in said population of the progeny relative to the level of expression of the P450 gene in a non-transgenic insect.

Insects containing the second DNA construct (e.g. insects already transformed with a DNA construct encoding a protein that is capable of inducing expression from the inducible promoter in the first DNA construct are also readily available in the art (see Example 3 hereinafter). Accordingly the invention also provides a method of producing a transgenic insect of the invention, which comprises: (i) transforming a first insect with a first DNA construct as described hereinbefore wherein the promoter region comprises an inducible promoter; (ii) crossing the transformed insect resulting from step (i) with an

insect that expresses an inducing protein that is capable of inducing expression from the inducible promoter in the first DNA construct, to obtain progeny; wherein a population of the progeny resulting from step (ii) comprise the first DNA construct and express the inducing protein so that the level of expression of the P450 gene is reduced in said
5 population of the progeny relative to the level of expression of the P450 gene in a non-transgenic insect.

In a similar manner, a transgenic insect cell may be made by co-transforming a suitable insect cell with a DNA construct of the invention and a second DNA construct that encodes a protein capable of regulating expression from the inducible promoter in the
10 DNA construct of the invention.

The transgenic insects and transgenic insect cells of the invention are more susceptible to compounds that are subject to metabolism by the product of the P450 gene whose expression level has been reduced therein. Thus the transgenic insects and insect cells of the invention are useful in identifying compounds which are potential insecticides
15 and which would not have been identified, or would have only been identified as being weakly active, in a conventional screen using a wild-type and/or non-transgenic insect or insect cell.

Thus in a further aspect of the invention there is provided a method of identifying or verifying the ability of a compound to act as an insecticide, which comprises: (i)
20 placing a transgenic insect or insect cell of the invention in contact with the compound; (ii) assessing the transgenic insect or insect cell that has been placed in contact with the compound in step (i) for any deleterious effect on the insect or insect cell; wherein the presence of a deleterious effect is indicative that said compound is capable of acting as an insecticide.

25 A deleterious effect may be manifested as the death of the insect or insect cells, or by slow growth of the insect or insect cell in comparison to insect or insect cells that have not been treated with the compound, or by some other clear phenotypic change in treated insects or insect cells in comparison to untreated insects or insect cells.

The present invention also provides assays for identifying insecticidal compounds
30 that are resistant to degradation by one or more insect P450 gene product, in particular compounds that are resistant to degradation by the products of insect *cyp* genes. Particular *cyp* genes of interest are the *cyp6* genes as described above. The *cyp6* genes, include the *cyp6g1*, *cyp6a2* and *cyp6A8* genes, and the use of these genes is considered as

particularly desirable. Furthermore the invention provides a method of reducing the susceptibility of a compound to degradation by one or more *cyp* gene products.

These assays utilise transgenic insect and insect cells as described hereinbefore as well as cells and/or whole organisms that over-express an insect P450 or *cyp* gene, or
5 which express an insect P450 or *cyp* genes in a heterologous manner.

Where these assays involve the use of cells and/or whole organisms that over-express an insect P450 or *cyp* gene, or which express an insect P450 or *cyp* gene in a heterologous manner such cells and/or whole organisms may comprise a DNA construct comprising a *cyp* gene of *Drosophila melanogaster*, in particular one of the 90 known *cyp*
10 genes listed hereinbefore, operably linked to a promoter region and optionally a transcriptional terminator. Such DNA constructs are referred to herein as "expression constructs". It is preferred that the *cyp* gene will be selected from the group consisting of *cyp6g1*, *cyp6a2*, *cyp6a8* and homologues thereof. It is particularly preferred that the *cyp* gene is *cyp6g1* or *cyp6a2*.

15 In general the promoter region to which the *cyp* gene is operably linked will be any promoter region capable of driving expression of the *cyp* gene in the host cell into which an expression construct comprising the *cyp* gene is to be introduced. Thus, if it is desired that the expression construct be introduced into a bacterial cell, the promoter will be operable in that bacterial cell. Similarly if it is desired that the expression construct be
20 introduced into a yeast cell, the promoter will be operable in that yeast cell and the same logic prevails if the construct is to be introduced into an insect cell or a mammalian cell. Accordingly the promoter region may thus be the native promoter that is associated with the individual *cyp* gene in nature, provided that the promoter is capable of driving expression in the host cell into which the construct is to be introduced. Alternatively the
25 promoter region may be any one of those with which the skilled man is familiar with for mediating expression in the host of choice. Thus, the promoter region may comprise a constitutive promoter, a tissue- or developmentally-specific promoter, or an inducible promoter. In a particularly preferred embodiment, where the baculovirus expression system and *Sf9* or *Sf21* cells are to be employed the promoter region will comprise the
30 polyhedrin promoter from the *Autographa californica* nuclear polyhedrosis virus.

Expression constructs according to this aspect of the invention optionally comprise a transcriptional terminator region. Typically, such a transcriptional terminator region will be one that is suitable for termination transcription in the host cell into which

the expression construct is to be introduced. Transcriptional terminator regions suitable for this purpose are described in the art.

The present invention also provides a cell transformed with an expression construct as described hereinbefore. Suitable host cells for transformation with a DNA construct of the invention include bacterial cells (such as *Escherichia coli*), yeast cells (for example, *Saccharomyces cerevisiae*, *Pichia* species, *Schizosaccharomyces pombe*), insect cells (for example, *Drosophila* S2 cells, *Spodoptera frugiperda* Sf9 or Sf21 cells, *Trichoplusia ni* High Five™ cells), and mammalian cells. In a particularly preferred embodiment, an expression construct of the invention is transformed into insect cells, in particular Sf9 cells.

Introduction of the expression construct into a host cell may employ any suitable technique. In eukaryotic cells such techniques include calcium phosphate transfection, DEAE-Dextran, electroporation, particle bombardment, liposome-mediated transfection or transduction using retrovirus, adenovirus or other viruses, such as vaccinia or, for insect cells, baculovirus. In bacterial cells, suitable techniques may include calcium chloride transformation, electroporation or transfection using bacteriophage. In particularly preferred embodiment, Sf9 cells are transformed using baculovirus, using for example, the protocols supplied with the MaxBac®2.0 –Complete Baculovirus Expression System (Invitrogen).

The expression construct may also be introduced into a whole organism i.e. an insect, and a stable insect line generated. In one embodiment it is preferred that the expression construct is introduced into *Drosophila*, using standard transformation techniques (see for example Spradling & Rubin 1982, Science 218:341-347). Thus transgenic *Drosophila* insects may be produced wherein the level of expression of the P450 gene present in the expression construct is amplified over the level of expression observed in wild-type non-transformed *Drosophila*.

Cells or insects transformed with an expression construct may be employed in a method of assaying the susceptibility of a compound to degradation specifically by the product of the *cyp* gene present in the expression construct. Thus the invention also provides a method of assaying the susceptibility of a compound to degradation specifically by the P450 or *cyp* gene encoded on the expression construct which comprises: a) culturing in a suitable growth medium a population of cells or an insect transformed with a DNA construct of the invention b) incubating said compound with said population of cells or an insect; and c) determining after said incubation the level of

one or more degradation products of said compound in said population of cells or said insect; wherein the presence of a measurable level of one or more degradation products is indicative that said compound is susceptible to degradation by the product of the *cyp* gene present in the expression construct.

5 The suitable growth medium will be dependent upon the type of transformed cells of the invention. For example, if the transformed cells of the invention are *Saccharomyces cerevisiae* yeast cells, YPD is one such suitable medium. Where the transformed cells are Sf9 or Sf21 cells, Grace's Insect Cell Culture Medium is one such medium. Where the transformed cells are Drosophila S2 cells, suitable culture medium is
10 Schneider's insect medium, optionally supplemented with foetal calf serum. The skilled man will be familiar with alternative media that are suitable for growth of the particular host cells chosen.

 Degradation products of the compound being assayed may be measured using any suitable analytical technique. In a preferred embodiment LC-MS is the technique
15 employed to detect and measure metabolites. In order to facilitate the measurement of metabolites, the cells or insects treated with a compound may be lysed and the cell contents released into a suitable buffer. The skilled man will be familiar with suitable methods for cell lysis which are well-documented in the art.

 By comparing the effect of a compound on an insect or insect cell under-
20 expressing a *cyp* gene with the effect of the same compound on an insect or cell over-expressing the same *cyp* gene (and optionally also comparing the effect of the compound on a wild type and/or non-transformed insect or insect cell), the susceptibility of that compound to degradation by that *cyp* gene may be assessed. Such a test allows better detection of weak insecticidally active compounds that may otherwise not have been
25 identified, since they are susceptible to degradation by P450s and also provides confirmation that the degradation to which they are susceptible is mediated by the gene product of the under- or over-expressed *cyp* gene. The *in vivo* metabolism of the compound may then be examined using any suitable analytical technique (for example, an LC-MS technique) and solutions proposed for the enhancement of stability of the
30 compound by altering the chemistry of those parts of the molecule that are subject to degradation. The use of transgenic insects, insect and other cells in this way forms yet another aspect of the invention.

 Thus a compound that has been identified or verified as being capable of acting as an insecticide using a transgenic insect or insect cell as described in the first aspect of the

invention, may also be tested as described above to assess whether it is susceptible to degradation by the product of the *cyp* gene whose expression level was reduced in the transgenic insect or insect cell against which it has been tested. It will be appreciated by the skilled man that where a compound is tested in this manner, the same *cyp* gene will be used in each assay. It will also be appreciated that the assay for the susceptibility to degradation may be performed initially, and the assay using a transgenic insect or insect cell as described in the first aspect of the invention may be carried out subsequently. Where this is the order in which the assays are carried out, the presence of degradation products in the first assay should correlate with the observation of insecticidal activity in the second assay. However, the absence of degradation products in the first assay need not necessarily correlate with an absence of insecticidal activity in the second assay. In such a situation a likely explanation is provided by the *cyp* gene or gene product being involved in the indirect mediation of metabolism.

Where it is found that a compound being assayed is susceptible to degradation, the assay (or assays) may be used in an iterative manner to aid the design of compounds that are less susceptible to degradation. For example, if a potential insecticide comprises a backbone chemical structure to which one or more sub-groups are attached, it may be found that by substituting one or more of such sub groups for a different sub-group and re-testing the modified compound, the modified compound may be found to have an improved resistance to degradation. If this is the case, the process may be repeated either altering the same, or a different, sub-group and then re-testing the compound in the assay(s) of the invention. In this way compounds may be modified to produce insecticides that are resistant to degradation by whichever *cyp* gene is being either under- or over-expressed.

In a further aspect of the invention there is provided a method of identifying or verifying the ability of a compound to act as an insecticide, which comprises: a) culturing in a suitable growth medium a population of insect cells transformed with an expression construct of the invention; b) incubating said compound with said population of cells; and c) determining after said incubation the viability of said cells; wherein a lack of viability of said cells is indicative that said compound is capable of acting as an insecticide.

A lack of viability of cells is preferably indicated by cell death. However, the slow growth of cells treated with the compound in comparison to cells that have not been treated with the compound, or some other clear phenotypic change in treated cells in comparison to untreated cells, may also be used as a measure of cell viability.

This aspect of the invention may be modified to identify or verify the ability of a compound to act as a pro-insecticide. Where this is the desired intention, cells transformed with an expression construct will be treated with a test compound, cell viability will be monitored post-treatment and after a period of incubation with the test compound, a sample of cells will be assayed to determine the presence or absence of metabolites of the test compound. In conjunction with this assay, control cells (non-transformed cells or cells transformed with a control DNA construct lacking the *cyp* gene) will be treated with the same test compound and cell viability determined post treatment. Compounds that have no effect on the viability of the control cells, but cause a loss in viability of cells of the invention and which are shown to be degraded by the product of the *cyp* gene expressed in the cells are thus identified or verified as pro-insecticidal compounds.

A still further aspect of the invention a method of identifying a compound that regulates expression of an insect P450 gene, which comprises: (i) culturing in a suitable growth medium a population of cells transformed with a DNA construct comprising a reporter gene operably linked to the promoter region of the P450 gene; (ii) incubating said compound with a population of cells according to step (i); (iii) determining the level of expression of said reporter gene in cells that have been incubated with said compound; and (iv) determining the level of expression of said reporter gene in a population of cells according to step (i); wherein, a difference in the levels of reporter gene expression determined at (iii) and (iv) is indicative that said compound is capable of acting as a regulator of expression of said P450 gene.

DNA constructs for use in this aspect of the invention are referred to herein as "reporter gene constructs" and will comprise any suitable reporter gene available in the art, for example the reporter gene may be a gene encoding : β -galactosidase, luciferase, or a fluorescent protein, such as for example green fluorescent protein. operably. The reporter gene will be operably linked to the promoter region of an insect P450 gene.

Preferably the promoter region will comprise the promoter of one of the 90 known *cyp* genes from *D. melanogaster* as listed above. Even more preferably the promoter region will comprise the promoter of the *cyp6g1* gene or the *cyp6a2* gene. In a preferred embodiment, the promoter region will lack the consensus sequences for phenobarbital induction (i.e. the promoter will lack so called "barbie-box" regions as defined by Shaw and Fulco 1993, J. Biol. Chem. 268:2997-3004). The promoter regions of insect P450

genes for use in this aspect of the invention may be isolated and cloned using standard molecular biological techniques.

A reporter gene construct for use in this aspect of the invention may be transformed into *Drosophila* S2 cells using any suitable method available in the art.

- 5 Where the reporter gene is luciferase the level of reporter gene expression may be assayed using the Luciferase Assay System from Promega.

Compounds that are identified according to this aspect of the invention, in particular those that down-regulate expression of *cyp6g1* or *cyp6a2* may be useful as additives to existing insecticides that are subject to degradation by the products of these
10 two genes, or they may find utility as insecticides in their own right.

For the avoidance of doubt, where a compound is to be “identified” as having the ability to act as an insecticide or to act as a regulator of the expression of an insect P450 gene, that compound has not previously been shown to act as an insecticide or to act as a regulator of expression of an insect P450 gene. Thus any compound that has previously
15 been shown to act in such a way is excluded from the scope of any method of “identifying” a compound, e.g. phenobarbital which has previously been shown to regulate the expression of some *cyp* genes, is not considered as a compound that is capable of being “identified” as a regulator of expression of a P450 gene.

Where a compound is to be “verified” as having the ability to act as an insecticide,
20 the compound will already have been shown to have insecticidal activity (although such activity may be weak) through some other means.

Various aspects and embodiments of the present invention will now be illustrated in more detail by way of example. It will be appreciated that modification of detail may be made with out departing from the scope of the invention.

25

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 Shows a schematic representation of DNA construct
“cyp6g1_hp_construct_1”

Figure 2 Shows a schematic representation of DNA construct
30 “cyp6g1_hp_construct_2”

Figure 3 Graphical representation of the bioassay data from the treatment of transgenic *Drosophila* that under express *cyp6g1* (lines *act-gal4/hp_1* and *act-gal4/+; hp_2/+*), wild-type *Drosophila* (*Canton-S* – control) and mutant *Drosophila* that over express *cyp6g1* (*Hikone-R*).

EXAMPLES

Example 1 Construction of vectors for use in the production of insects wherein the level of expression of a P450 gene is reduced relative to wild type insects

5 Constructs were generated that encode inverted repeats of *cyp* gene fragments, which on expression fold to form an RNA duplex or as referred to herein a hairpin (hp) RNA molecule, which is capable of mediating a RNA interference (RNAi) effect. The constructs generated also include a spacer sequence, which serves to facilitate cloning, between the inverted repeats. The expression of the inverted repeats is under control of an
10 inducible promoter system.

 The constructs were generated using restriction-enzyme mediated cloning (1.1) and also site-specific recombinase mediated cloning (1.2).

 To provide *Drosophila* DNA template for the cloning, genomic DNA from a wild-type lab strain, *Canton-S* (Bloomington stock centre, stock number 1;
15 <http://flystocks.bio.Indiana.edu>, Drosophila Stock Center, Department of Biology, Indiana University, 1001 E. 3rd St., Bloomington, IN 47405-3700, USA) was isolated using standard molecular biology techniques (Sambrook, *et al.* 1989, Molecular Cloning, A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory, New York; Bender *et al.* 1983 J. Mol. Biol. 168: 17-33). This was then used as the DNA template for PCR
20 amplification in various steps as described below.

1.1 Restriction-enzyme mediated cloning

 First the spacer sequence was cloned into the parent vector (pB-UAS w⁺, described in 1.1.1 below). Then the two inverted repeats of *cyp* gene fragments were
25 inserted as described in 1.1.2. Two different hairpin constructs were made, these differed from each other in the size of the inverted repeat; one comprised a long gene fragment (~1700 bp) (1.1.2a), and the other comprised from a shorter gene fragment (~600 bp) (1.1.2b).

 A similar approach was taken for *cyp6a2* construct generation, and this is briefly
30 described in 1.1.3.

1.1.1 Cloning of a spacer sequence into the vector pB-UAS w⁺:

 An intron sequence from the *cyp4e2* gene was first cloned into the vector pB-UAS w⁺ (Genbank AY196823) to provide the spacer sequence to facilitate cloning of inverted

repeats. This intron was PCR-amplified from the *Canton-S* genomic DNA preparation using primers *cyp4e2_intron_F* and *cyp4e2_intron_R* which contain introduced XbaI and BamHI sites, respectively, into their 5' termini. The sequences of these primers are:

cyp4e2_intron_F 5' GCTCTAGAGCAGATCTGTCAGGTGGGTTTTTCATAG 3' (SEQ ID NO 1).

cyp4e2_intron_R 5' CGGGATCCCGCATATGTATTTCTATATAGACGTGAG 3' (SEQ ID NO 2).

The XbaI-BamHI fragment from this PCR product was then cloned into the corresponding XbaI-BamHI sites of the vector pB-UAS w+, using standard molecular biology techniques.

The sequence of the spacer region (derived from the first intron in the *cyp4e2* gene) thus introduced into pB-UAS w+ is (given in the 5' to 3' orientation):

GTCAGGTGGGTTTTTCATAGGAGCCGGAAAAGTCGTAGCGTTAGCCACGCAG
 15 AGGACACACCTGAAATTACCCATCTCGAATAGCGTTGTGACTGAGGTAAGTGC
 CGCATCGAATCTACTCAGCGGCGAGGCTATTCAAGTAAGGTTATTTTGGGCCA
 GCGACTTTACGACCGGAAAACAAGGGTGTAACTATCAGATAATGGGGCTAC
 TGGCACTTGTAGAAATGGGCTAATCGTAGCCTAGGTCGAGTATCGCAAAAAT
 AATGACGAGGCTTTGATTTGTGTTGTGTAATATACGATGTATGTATGTATGTA
 20 TTATACTGCACTAATGAATACTGAAAATCGCGTGCCAAAAACAAGCAATGAT
 TATCTACGGAATACCGATATTGGTACGCGATCCGCCTACTAATGGCATTCTTCT
 CACGTCTATATAGAAATAC (SEQ ID NO 3).

25 1.1.2 Generation of the *cyp6g1* hairpin constructs:

For cloning of the inverted repeat *cyp6g1* sequences, two constructs were generated: one that encodes a long inverted repeat (the '*cyp6g1_long*' construct), and one that encodes a shorter inverted repeat ('*cyp6g1_short*' construct). The *cyp6g1* gene sequence from EMBL:AE003823 is shown below. The *cyp6g1* gene fragments chosen for
 30 cloning and consequent expression as hairpin RNA molecules were selected to maximise the probability that only *cyp6g1* mRNA was degraded via an RNA interference mechanism. Other suitable gene fragments may be used, and the skilled man would recognise that these can be selected by comparing the sequence of the gene of interest (in this case *cyp6g1*) with the sequences of other *cyp* genes and choosing fragments of the

gene of interest that are ill-conserved amongst the series of *cyp* genes compared. Preferably the fragments chosen will be unique to the gene of interest.

The *cyp6g1* genomic DNA sequence may be found in the EMBL database under the reference:AE003823. The region encoding *cyp6g1* is shown below (in the 5' to 3' orientation), with the positions of introns underlined and in lower case and the position of the start codon and termination codon of the *cyp6g1* coding sequence are shown in bold and underlined.

SEQ ID NO 4:

GCCCCAAGTGCGGTGCGTAGAGCTTTAATTGTCGGTTGTGTACGCGGGTGCTC
10 AGAATTTATAGATCCAATAAAAGTTTCCTTGAAATTGCTGGACAACTTGTTG
CGAATTAGGCCAGTTGCAAATAAATTGTGTGACTAAAAAACCTGTATATTTT
CAAAGTAAGTTATGAAATTCGGTATACAAAATTGTATATTTTAATTTGAAAAA
GTGTGATATCCTTGAAAAAACCACTTAATATGGAATTGAGGGCAACTGTG
AAAATTAAGATTACTTGGTTACTGTTACTGTTACTAAAATAAATAGAGAACTG
15 GTTTCTAAGAAATATATTTTGGACATTTGCTCTATAGAAATAGTAGTCTCATG
TATAATTAATAAAGCTTAGGACGAAATCCTTGCTGGCAAATTTTATAGTGTGAT
ATTTGCTGCCCCGAAAAGATTTTCTATTTAGCTTTACAAGCAGCTTAATTGGGT
GTAGATATGGCGGGAATTACTATAAGCGTATCATAAACACTCATTGTAATGCT
CCCTATCATAACTAATTGTAATTCGCGTTGGAATGGAAAAGATAATGCCAGCT
20 AAAAATAATTAAGACGGCTGATTTAATAAAATCTAGGTAAACATGGGTAGCA
AAACACTCTAGATTGCATTTGAAACCATCGACCGTTTCAAATGCGATCGAGAT
AAGATAATGCTGAGGGCACTGGCATTGATTAATTGTTTTAGCGATTAATTTT
AGAGTGGCGATACCCATTACATCGACATCCCCAAAATGGTGTGACCGAGGT
CCTCTTTGTGGTGGTCGCCGCACTGGTGGCGCTCTACACTTGGTTCCAGCGCA
25 ACCATAGCTACTGGCAACGCAAGGGCATACCTATATTCCGCCCACGCCGATC
ATTGGCAACACCAAGGTGGTCTTCAAGATGGAGAACTCCTTTGGGATGCACCT
ATCGGAGATATACAATGATCCGCGGCTGAAGGACGAGGCTGTGGTGGGCATC
TACTCCATGAACAAGCCCGGCTTGATAATACGCGACATAGAGCTGATCAAAT
CCATTCTGATCAAGGACTTCAATCGGTTCCACAACCGATACGCCCGCTGCGAT
30 CCCCATGGCGATCCATTGGGCTATAATAACCTGTTCTTCGTCAGGGATGCCCA
TTGGAAGGGAATTCGCACCAAGCTGACTCCCGTTTTTCACCAGCGGCAAGGTC
AAGCAGATGTACACCCTTATGCAGGAaggtaaggattgtgtgaaactatttatccttatgaggatttaa
catacatatgctgtgcagATTGGAAAGGATCTGGAGCTGGCACTGCAGAGGCGTGGAG
AGAAGAACTCTGGGAGTTTCATTACGGAGATTAAGGAGATCTGCGCTCAGTT

CTCCACGGACAGCATAGCCACGATTGCATTTGGCATTTCGTGCTAACAGCCTAG
 AGAATCCCAACGCAGAGTTCCGTAACCTATGGACGCAAGATGTTACCTTCAC
 CGTAGCGCGTGCCAAGGACTTCTTTGTGGCCTTCTTCCTGCCCAAGCTGGTGT
 CGCTGATGCGCATCCAGTTCTTCACGGCGGACTTTTCCCACTTTATGCGCAGC
 5 ACCATTGGTCACGTTATGGAGGAGCGAGAGCGATCGGGCCTGCTCCGCAATG
 ATCTCATAGATGTCTTGGTGAGTCTGCGCAAAGAGGCGGCTGCCGAGCCTTCG
 AAGCCTCACTATGCCAAGAACCAGGACTTCCTGGTGGCTCAGGCGGGCGTGT
 TTTTACGGCGGGTTTCGAGACCTCCTCCTCGACCATGTCTTTTGCCCTGTACG
 AGATGGCCAAGCATCCAGAGATGCAGAAACGCCTGCGCGACGAGATCAACG
 10 AAGCTTTGGTGGAGGGCGGTGGGTCATTGAGCTACGAGAAAATCCAGTCCCT
 GGAGTATCTGGCCATGGTGGTGGACGAGGTGCTGCGCATGTATCCGGTGCTG
 CCGTTCCTGGACCGCGAGTACGAGAGCGTGGAGGGACAGCCAGACTTGAGCC
 TGAAGCCGTTCTACGACTACACTCTCGAGAACGGAACCCCTGTGTTTCATACCC
 ATCTATGCACTGCATCATGATCCAAAGgtattccatcacattgagaagttcataagccccctcctctaact
 15 atctggttattcctagTACTGGACCAATCCCAGCCAATTCGATCCGGAGCGTTTCTCACC
 CGCGAACCGCAAGAACATAGTGGCCATGGCATATCAACCCTTCGGATCTGGG
 CCGCACAACTGCATTGGCAGCCGGATTGGCCTGCTACAGAGCAAACCTGGGAC
 TGGTCAGCCTGCTGAAGAATCACTCAGTGCGCAACTGCGAGGCCACCATGAA
 GGACATGAAATTCGATCCCAAGGGTTTCGTGCTCCAGGCAGATGGTGGCATA
 20 CATTTGGAGATAGTCAACGATCGCCTCTACGATCAGAGCGCTCCATCGCTCCCA
ATGAATTTGAATCGCATGAACTGTGTGATCTGTATGGATACACATATGGATAC
 ATATATGTACATATATGGAATAAGTGATTAGTTGATGGGGCAATCGCCTTCTG
 ACAGCTGGCATTTCCTGACTTATGATGCAGATTATATAATGATAATCCGTGT
 GTTAGAAGCGGCGCCTAGAGTCTACTAAATAAAAACAAAATCATTATCAAAT
 25 TACATTTAATCTGCGTGCTGAGCAGCTAGCAGCTACTCAATTGCGCCACCAAT
 AAATAAATGCAATAAATGCTATCTTTACGATT

1.1.2a Generation of the "cyp6g1_long" "hairpin construct":

The cyp6g1_long construct contains inverted repeats of bases 743-2501 of the
 30 cyp6g1 gene sequence from EMBL:AE003823. This sequence from bases 743-2501 is
 shown below (again, intron sequences are shown in lowercase and underlined).

cyp6g1_long sequence (SEQ ID NO 5):

5' AGTGGCGATACCCATTACATCGACATCCCCAAAATGGTGTGACCGA
 GGTCCTCTTTGTGGTGGTCGCCGCACTGGTGGCGCTCTACACTTGTTCCAGC

GCAACCATAGCTACTGGCAACGCAAGGGCATACCCTATATTCCGCCCACGCC
GATCATTGGCAACACCAAGGTGGTCTTCAAGATGGAGAACTCCTTTGGGATG
CACCTATCGGAGATATACAATGATCCGCGGCTGAAGGACGAGGCTGTGGTGG
GCATCTACTCCATGAACAAGCCCGGCTTGATAATACGCGACATAGAGCTGAT
5 CAAATCCATTCTGATCAAGGACTTCAATCGGTTCCACAACCGATACGCCCCGCT
GCGATCCCCATGGCGATCCATTGGGCTATAATAACCTGTTCTTCGTCAGGGAT
GCCCATTTGGAAGGGAATTCGCACCAAGCTGACTCCCGTTTTACACGCGGCA
AGGTCAAGCAGATGTACACCCTTATGCAGGAggtaaggatttggttgaaactatttatccttatgag
ggatttaacatacatatatgctgtgcagATTGGAAAGGATCTGGAGCTGGCACTGCAGAGGCGT
10 GGAGAGAAGAACTCTGGGAGTTTCATTACGGAGATTAAGGAGATCTGCGCTC
AGTTCTCCACGGACAGCATAGCCACGATTGCATTTGGCATTTCGTGCTAACAGC
TAGAGAATCCCAACGCAGAGTTCCGTAACCTATGGACGCAAGATGTTACCTTC
ACCGTAGCGCGTGCCAAGGACTTCTTTGTGGCCTTCTTCCTGCCCAAGCTGGT
GTCGCTGATGCGCATCCAGTTCTTCACGGCGGACTTTTCCCACTTTATGCGCA
15 GCACCATTTGGTCACGTTATGGAGGAGCGAGAGCGATCGGGCCTGCTCCGCAA
TGATCTCATAGATGTCTTGGTGAGTCTGCGCAAAGAGGGCGGCTGCCGAGCCTT
CGAAGCCTCACTATGCCAAGAACCAGGACTTCCTGGTGGCTCAGGCGGGCGT
GTTTTTTACGGCGGGTTTCGAGACCTCCTCCTCGACCATGTCTTTTGCCCTGTA
CGAGATGGCCAAGCATCCAGAGATGCAGAAACGCCTGCGCGACGAGATCAAC
20 GAAGCTTTGGTGGAGGGCGGTGGGTCATTGAGCTACGAGAAAATCCAGTCCC
TGGAGTATCTGGCCATGGTGGTGGACGAGGTGCTGCGCATGTATCCGGTGCTG
CCGTTCTGACCGCGAGTACGAGAGCGTGGAGGGACAGCCAGACTTGAGCC
TGAAGCCGTTCTACGACTACACTCTCGAGAACGGAACCCCTGTGTTCATACCC
ATCTATGCACTGCATCATGATCCAAAGgtattccatcacattgagaaggttcataagccccctcctctaact
25 atctgggtattcctagTACTGGACCAATCCCAGCCAATTTCGATCCGGAGCGTTTCTCACC
CGCGAACCGCAAGAACATAGTGGCCATGGCATATCAACCCTTCGGATCTGGG
CCGCACAACCTGCATTGGCAGCCGGATTGGCCTGCTACAGAGCAAACCTGGGAC
TGGTCAGCCTGCTGAAGAATCACTCAGTGCGCAACTGCGAGGCCACCATGAA
GGACATGAAATTCGATCCCAAGGGTTTCGTGCTCCAGGCAGATGGTGGCATA
30 CATTTGGAGATAGTCAACGATCGCCTCTACGATCAGAGCGCTCCATCGCTCCA
ATGAATTTGAATCGCATGAACTGTGTGATC 3'

The *Cyp6g1_FWD* primer (containing a BamH1 and a XbaI restriction site introduced at the 5' terminus of the primer: 5' GCGGATCCATTCTAGAAAGTGG

CGATACCCAT TACATCG 3'-SEQ ID NO 6) and the *Cyp6g1_REV* primer (containing a NotI and an EcoRI restriction sites introduced at the 5' terminus: 5' TAGAATTCGCGGCCGCGATC ACACAGTTCATGCGATTC 3' -SEQ ID NO 7) were used to PCR-amplify these *cyp6g1* sequences from the same *Canton-S* genomic DNA template described earlier. A XbaI-NotI fragment from this PCR product was cloned into the corresponding XbaI-NotI sites in the plasmid generated in 1.1.1 above and a BamHI-EcoRI fragment from the PCR product was then cloned into the BamHI-EcoRI sites of the resulting plasmid to give *cyp6g1_hp_construct 1* (Figure 1).

1.1.2b Generation of the *cyp6g1* short hairpin construct:

The *cyp6g1_short* construct contains inverted repeats of two *cyp6g1* sequences that are fused together. Bases 743-1162 are fused to bases 2028-2192 of the *cyp6g1* gene sequence from EMBL:AE003823. This fused sequence is shown below (SEQ ID NO 8):

5' AGTGGCGATACCCATTACATCGACATCCCCAAAATGGTGTGACCGAG
 15 GTCCTCTTTGTGGTGGTCGCCGCACTGGTGGCGCTCTACACTTGGTTCCAGCG
 CAACCATAGCTACTGGCAACGCAAGGGCATACCCTATATTCCGCCCACGCCG
 ATCATTGGCAACACCAAGGTGGTCTTCAAGATGGAGAACTCCTTTGGGATGC
 ACCTATCGGAGATATACAATGATCCGCGGCTGAAGGACGAGGCTGTGGTGGG
 CATCTACTCCATGAACAAGCCCGGCTTGATAATACGCGACATAGAGCTGATC
 20 AAATCCATTCTGATCAAGGACTTCAATCGGTTCCACAACCGATAACGCCGCTG
 CGATCCCCATGGCGATCCATTGGGCTATAATAACCTGTTCTTCGTCAGGGATG
 CCCATGAAGCCGTTCTACGACTACACTCTCGAGAACGGAACCCCTGTGTTTCAT
 ACCCATCTATGCACTGCATCATGATCCAAAGGTATTCCATCACATTGAGAAGT
 TCATAAGCCCCCTCCTCTAACTATCTGGTTATTCCTAGTACTGGACCAATCCCA
 25 GCCAATTTCGA 3'

The fusion sequence was generated by a 2-step PCR method. First the two sequences were separately PCR-amplified from the *Canton-S* template DNA. The primer pairs used for this reaction are *Cyp6g1_FWD* and *Bridge_REV* (5'

30 CGTAGAACGGCTTCATGGGCATCCCTGACGAAGAACA 3' - SEQ ID NO 9), which PCR-amplified bases 743-1162 of the *cyp6g1* gene sequence from EMBL:AE003823. The primer pairs used for the other reaction are *Cyp6g1_REV* and *Bridge_FWD* (5' CGTCAGGGATGCCCATGAAGCCGTTCTACGACTACAC 3' - SEQ ID NO 10), which PCR-amplified bases 2028-2192 of the *cyp6g1* gene sequence

from EMBL:AE003823. In the next step, equimolar amounts of the two PCR products from these reactions were mixed together and used as the template DNA for a further PCR amplification reaction. PCR amplification from this mixed template, using primers *Cyp6g1_FWD* and *Cyp6g1_REV*, generated a fusion DNA sequence: bases 743-1162
5 were fused to bases 2028-2192 of the *cyp6g1* gene. This occurred because the primers *Bridge_FWD* and *Bridge_REV* contain complementary sequences at their 5' ends, thus permitting annealing of the two original PCR products.

From the resulting fusion PCR product, the Xba1-Not1 fragment was isolated and cloned into the corresponding Xba1-Not1 sites in the plasmid generated in 1.1.1 above, using standard restriction enzyme mediated cloning methods. The BamH1-EcoR1
10 fragment from this PCR product was then cloned into the BamH1-EcoR1 sites of the resulting plasmid.

1.1.3. Generation of the *cyp6a2* hairpin constructs:

15 Constructs for *cyp6a2* (*cyp6a2_long* and *cyp6a2_short*) were generated, using the same cloning strategy as described for the *cyp6g1* constructs above.

The genomic *cyp6a2* gene sequence shown below was generated from sequences in the EMBL database: sequence from EMBL Accession No:AE003790 and cDNA sequence from EMBL Accession No. DMU78088. Intron sequences are shown in lower
20 case and are underlined and the start codon and termination codon of the *cyp6a2* coding sequence are shown in bold and underlined.

SEQ ID NO 11:

5' GATCTTGTGTTGTACATTAGCACTGATTTCTGAAAATAAAACGAGATA
AGGCAACAACTAAATTTATTAGTCATTTTAAATGTTGGTGTGACATGTAGAT
25 TTAACACGCGACATTGCTTTTGAATGAATACATTTTAAATCACATATATGCTA
AATATAATTTAACACAGCACAGTTGATGTGTTGTTTCCAAATGAGCAATAAAA
CCCGATGCGATTATAATGTTTAAACCAACCGCGACCAGTTTAAACGACAATCA
AGTTATCTACTGTTTATATTTTGATCGTCATTCAAACAGTGAAGCGTTTAAATA
GCTCTTAGAAAGAGTTATTGGTTCTCCCAGCGAATTCATTTCGTTTTATCGCCGT
30 GGAACACCAAACATTAACGTTGTAGTGGGGTGCTAATTACTGAAATAAATGG
GTGACATTTTAAATGTGTATGTCCGTGTATGTATTTATCTACGGGAAATTATGT
CATTACAATTTTCATGCGATAATGTTTCGCTCCGATTCCCAAGGCTTAAAACT
CATTTAATTATTTGCTCTGTTTTCTAATAAATATTGAATCGCATAGGAGAA
AAACCTTTATGAGCCTTAACCAGCTTGTTTAAATAACAATCTTGTAATCGTTTGT

TTGCATGTAGATCCACTGCAATAGAACAGTAGAACATCAAGAGAATATCTCTT
ATGTCAAATCTTGAATCTCTCATAAAAAGCAAAAGAGCTTGGTGTGTCGCTG
ATAATGAAAGCTAGATGGAACGCTCGGCTATCAAATACCCATTACTTAAACA
CCTTTACCTTTAGTTACAAGTGGGATCGTCCTGTACATTTGAAGCTACTTTGAA
5 TGACCGTGTCAATTGCGTTTTTATAGTAATACAATGATTTGATACATTTTCCGCA
TATCGATGTGTATTTACCATTTAATGCTACCTCTACTTTTCGCACATGCCGAGC
TTATAAATGAGTGATAAAACCTGATGGGACACTTTTTACCCAAGCACCAACCG
GTTTGTTTAACAACAATCTCGTTATCATTTGTTTATATTTTGATTCGCGAGCAT
TTTTGCAGTCAAGCGTATGAGTATCTCTTTGAAAGTGCTCTTAAATCGGTTTG
10 AAAAGTGCCTGCATATTATCGCCGAGTCTGTAATCATGACAACAACCTTAAAA
GTAGTAGTCATGGTGATAGAAATATTTAGCTAGCTAGCTCACATGCTGTCATG
CCTGTGCGTCGCAGGGGAATCTTATAAAAAGTGTGCGAACATATTGTGGTGAT
CAGTAATTCGTCGTAGGTCGAGCACGACGATTGCGAAAAGGGAGCAGCTACG
CAAAATGTTTTGTTCTAATATACCTGTTGATCGCGATCTCCTCGCTTTTGGCCTA
15 CTTGTACCACCGCAACTTCAACTACTGGAATCGCCGCGGCGTGCCACACGAtgc
tcctcaccactgtatggcaacatggtcgggttcggaagaaccgggtgatgcacgacttctctacGACTACTACAA
CAAGTACCGGAAGAGCGGCTTTCCCTTCGTGGGCTTTTACTTTCTGCACAAGC
CGGCCGCTTCATCGTGGACACCCAGCTGGCCAAGAACATCCTGATCAAGGA
TTTCTCGAACTTTGCCGATCGTGGCCAGTTTCACAACGGGCGCGACGACCCGC
20 TCACGCAGCACCTGTTCAACCTGGACGGAAGAAGTGGAAGGACATGCGCCA
GAGGCTGACGCCGACTTTCACCTCGGGCAAGATGAAGTTCATGTTCCCGACG
GTGATCAAGGTGTCTGAGGAGTTCGTCAAGGTGATCACGGAGCAGGTGCCCG
CCGCCCAGAACGGCGCTGTGCTCGAGATCAAGGAGCTGATGGCCAGGTTTAC
CACCGATGTGATTGGCACCTGTGCTTCGGCATTGAGTGTAACACGCTGCGCA
25 CCCCTGTCAGTGATTTCCGCACCATGGGACAGAAGGTGTTACCGATATGCGC
CACGGGAAACTGCTGACCATGTTTCGTGTTTACGCTTTCCCAAGCTGGCCAGCAG
GTTGAGAATGCGCATGATGCCCCGAGGACGTCCACCAGTTCTTCATGCGCCTGG
TCAACGACACGATTGCCCTCAGGGAGCGGGAGAACTTCAAGAGGAACGACTT
CATGAACCTGCTGATTGAACTGAAGCAGAAGGGGCGCGTCACCCTGGACAAC
30 GGAGAGGTGATCGAGGGCATGGACATCGGCCGAACCTGGCCGCCAGGTGTTTCG
TCTTTTATGTGGCCGGATTTGAGACCTCCTCCTCGACAATGAGTTACTGCCTGT
ATGAGTTGGCTCAGAATCAGGACATTCAGGACAGGCTGCGCAACGAGATCCA
AACGGTGCTGGAGGAACAGGAGGGGCAGCTAACGTACGAATCCATCAAAGC
CATGACCTACTTGAACCAGGTCATCTCAGGTAGGTGTTCTGTCTGAAGCACAG

CCCCAAGAGGGATTGTAACCTGGTTTTACCCAACATTGCTCTTCAGAAACCCTG
AGGCTCTACACACTGGTGCCCCACCTCGAACGGAAGGCCCTCAACGACTACG
TGGTGCCGGGCCATGAAAAGCTTGTGATTGAGAAGGGGCACACAGGTCATAAT
CCCCGCTTGCGCCTACCACCGCGACGAGGATCTTTATCCGAATCCGGAGACCT
5 TTGATCCGGAGCGCTTCTCGCCGGAGAAAGTGGCCGCCCGGGAGTCCGTGGA
GTGGCTGCCCTTCGGCGACGGGGCCGCGGAACTGCATCGGGATGCGGTTTGGA
CAAATGCAGGCTCGCATCGGTTTGGCTCAGATCATCAGCCGGTTCAGGGTATC
CGTCTGCGATACGACAGAGATCCCACTGAAGTATAGTCCCATGTCCATAGTTT
TGGGCACCGTTGGGGGCATCTACTTGCGAGTGGAACGCATCTAACCTCCATAT
10 TCGTTGCTCCCATGTATATAGCTTAGGATCCAAAGCTAAAGTGATGTACATTT
TAGACTGTTCAATTATTAATAACCTTAACCTAAACAGCCATATTAACCTATT
GGCCTGTGATAAATCACTTATGTTTCACAGTTTAGAAGATCTAATTGTCTACCA
GTTAGATGCATTCAGGCAAATGTTGTTCCCATCAGATTTGCAGGTTTCATAAAT
TGCATTCGGCTTAAGTTGCAACATGCCACGCGAATCGCCACCTGACTAGTCAT
15 AATGTTGTTAGAGTCAATGACTTTTCTCTGTATAGCTGAGCGAAAATAATTAC
TATTCACAGACGCTCTAATGTTTTGCATACCTCATGCCACACTCGTTAGGTTGC
TCAACCGTAGCAGCGAAAACCCCAAATTAATAATATGGTGCTGAAGCATGCAA
CACGAGTCGGCTAATTGAGGTAAGCCCTACCTGAAAAGAAATAATGACTTTA
CCACGTCGAACTGTTTCGCAAATGTCAATGCCTCGGCCAGACTATGAGATAACT
20 TTTACGGATTAGTCACTGATTGGTTCGCCCGCTTATCATAGATTTTTTTTGGTGA
TATCGTATAATGTAGCTGAATTATTAATAACACTGCTTTTGTTCGTGTATTCA
AAAACAAGATAGTAATATACTTGAGGGCAGCTATTAGATTCTATTAAATACCT
GTTGTCAAGTCTCAACTTTCTAGCTTTTATGGTTCCCGGATATCTCGACTTTCA
CACGAACGAACGGACAAACGGACAAACGGACAAACGGACAGACGGACAGAC
25 GGACATAACGGACAGACAGACATCGAGACGGGCATGGCTAGATCGTCTGGGC
TAGTTAGCACGAACAAGAACTATAGTTGTCAGCACTTGACTTTTGTTTTATTT
ATTCTCAATGGCAAACGTAGTAGTATATTTCTTATTTTAGCTGGAACTGTTTT
CATTTCCAGAACTTTATTCGTTTAGTGTACGATTGAGTGATTAAAGTTTCCGGT
CAATGACGCTAAGCAGTCTGTCTAGTTCATTCTAAAGAATAATTTATATTAG
30 CAATGTGAGTACAATCGAACTTATTGAAGTTGAAAGCAAACAATAATTAAT
TCGAGAATTGAAAATTTCTTGTATAAATTTCAAACCTTTAAATACATTTTTATGT
ATGTATATTCTACTTTTTGGGCTGGCTTGTAACGCGGAAATTGGAACAGTCAT
TTAAAATGTATTTTATGCCCTCCGGATTAATAGTATCCCATCTTATTTTAATT
ATGCTGGTAGGGAAATGTTTATCTAACGTCAGCGGCGCATGCAACGCGTGGA

AATTGAGGCAAGTGTCTAAAGGTGGGAAAATCGGGGGCGTGTAATTGTGCT
CTGTAATGGGCGTGAATCTAACGGTTATGGGAACACAAAATGTAGTTTGATA
AAACTAATTATATACTCTCTTATTGGCACTAGCTCATCATCCGGTAATAGACA
CGTTTTATGAACCTATAGCCGGATTAAAAACCACAAACAAGTGAATATTTTCC
5 AATTAATAACTGATGGCCTAATTATCATAATTAATTAACAAATAACGTAGTC
AGTGATTTATTCAAACCGCGCGAATGCCGTAAGCAAATAGTTTTTCTGAATGA
AATATGTGATACTTGTATCTGGCGGATACACGCTGCCTGAGCATAACAACGCTC
GGTTTTCTAAGCATTTCGAGAATCTTGACTTGGCCTGGCACTTGGCCCTACCTTC
GCCGTGGGAGGGCGAACTAAATTTAATTACTAGCCCGCAAGAAACAAGTGCG
10 GCTAAACAAAAACAAGTGCAAAACCAACCGACATGAAGATAAAGCATAACT
GAAATAAAAGTGTCAAGGTTGTTCAACCCAAGCCACAAAGCATTTCGACTGT
TTAAAGCCGCAACTTTAAGGTTGTTTGGCCCGAAAACCTTTCAATTCCTCAACCA
GCAGCATCTCATCTGAAATTCCATTAGCCCCAGCACCATCAAAGCAAGAAGC
ACGCATCGCCATCGCCCAAAGTTAAGTCCACATCCTTGTTTGCCCAACTGAAT
15 TGGAACGAGTGCTGCAATGCGAGACCAACTGCAGCGGCATCAAATGTTGCAG
CAACAGCAGAAGCAGCAGCAATTGCAACTGCGACGGACGGAAGCGTGCTCAT
ATGTGCGTAAATAGTTTGTCAAGCGCGCCAGTGAACAAAAAGTCGACTTGGA
CGTGGGATACTGGACATTTGGCCAAAAACAATGCCGTGGGTGCATGTGGCAT
GGCCAGGGACAGGGCTTAGGTTTCACCGCCACTATCGCTATCGCTATTGCCTT
20 AGCTGTGGGCATGGATACGGGCATGTCCGCCGACATGGATATGCCCATGGAC
ATGGCCACGGTCTGCGCCCGGAACTTTTGCGCGGATTAATTGCATGCACCAAG
CGTCACCAAAGTCTGCCATGCCACCGTATCGGGATGAGCGCTGCCTCACTGCT
GCACACCGAATTTAAAATGGCCTTTGTATCACTTATGTTTAGTTTGCTAAAGTT
TAACGAAAGTTTATTTATTTTTGAACTCCCTTGAACGTTTCATTCCAAGTTCCGA
25 CCTATGAGTGTAAGAAAAGAGTAGTTCCGTCACCTCCCTGTTACATAAATCACT
CCCATTTTCGGATTCCATTCTAGATAGCTAATATTTGGCAGTCACTTATTTGAC
ACATGTGGTCAATTGAGCTGTCATCGCACAGAGCGTTCCAATTTAACCGTCCA
ACCTAGCTTGCTGCTGCTGCTGATGTTGCAGGTGTTGCTGTTGCTGTTGGCCAC
ACTCGAG 3'

30

1.1.3a Generation of the cyp6a2 long hairpin construct:

For generating the cyp6a2_long construct, primers were designed as for cyp6g1_long cloning, with the corresponding restriction sites added to the 5' termini of

the primers to facilitate cloning. Constructs were then generated for *cyp6a2* in the same way as described above for *cyp6g1*.

The *cyp6a2*_long sequence cloned from bases 1306 - 2952 of the *cyp6a2* gene sequence given above is:

```
5  5'    CGAAAAGGGAGCAGCTACGCAAAATGTTTGTCTAATATACCTGTTGA
TCGCGATCTCCTCGCTTTTGGCCTACTTGTACCACCGCAACTTCAACTACTGGA
ATCGCCGCGGCGTGCCACACGATGCTCCTCACCCACTGTATGGCAACATGGTC
GGGTTCCGGAAGAACCGGGTGATGCACGACTTCTTCTACGACTACTACAACA
AGTACCGGAAGAGCGGCTTTCCTTCGTGGGCTTTTACTTTCTGCACAAGCCG
10  GCCGCCTTCATCGTGGACACCCAGCTGGCCAAGAACATCCTGATCAAGGATTT
CTCGAACTTTGCCGATCGTGGCCAGTTTCACAACGGGGCGCGACGACCCGCTCA
CGCAGCACCTGTTCAACCTGGACGGAAAGAAGTGGAAGGACATGCGCCAGAG
GCTGACGCCGACTTTCACCTCGGGCAAGATGAAGTTCATGTTCCCGACGGTGA
TCAAGGTGTCTGAGGAGTTCGTCAAGGTGATCACGGAGCAGGTGCCCCGCCGC
15  CCAGAACGGCGCTGTGCTCGAGATCAAGGAGCTGATGGCCAGGTTCACCACC
GATGTGATTGGCACCTGTGCCTTCGGCATTGAGTGTAACACGCTGCGCACCCC
TGTCAGTGATTTCCGCACCATGGGACAGAAGGTGTTACCGGATATGCGCCACG
GGAAACTGCTGACCATGTTTCGTGTTTCAGCTTTCCCAAGCTGGCCAGCAGGTTG
AGAATGCGCATGATGCCCCGAGGACGTCCACCAGTTCTTCATGCGCCTGGTCAA
20  CGACACGATTGCCCTCAGGGAGCGGGAGAAGTTCAAGAGGAACGACTTCATG
AACCTGCTGATTGAACTGAAGCAGAAGGGGCGCGTCACCCTGGACAACGGAG
AGGTGATCGAGGGCATGGACATCGGCGAACTGGCCGCCAGGTGTTTCGTCTT
TTATGTGGCCGGATTTGAGACCTCCTCCTCGACAATGAGTTACTGCCTGTATG
AGTTGGCTCAGAATCAGGACATTCAGGACAGGCTGCGCAACGAGATCCAAAC
25  GGTGCTGGAGGAACAGGAGGGGCAGCTAACGTACGAATCCATCAAAGCCATG
ACCTACTTGAACCAGGTCATCTCAGGTAGGTGTTCTGTCTGAAGCACAGCTCC
AAGAGGGATTGTAAGTGGTTTTACCCAACATTGCTCTTCAGAAACCCTGAGGC
TCTACACACTGGTGCCCCACCTCGAACGGAAGGCCCTCAACGACTACGTGGT
GCCGGGCCATGAAAAGCTTGTGATTGAGAAGGGCACACAGGTCATAATCCCC
30  GCTTGCGCCTACCACCGCGACGAGGATCTTTATCCGAATCCGGAGACCTTTGA
TCCGGAGCGCTTCTCGCCGGAGAAAGTGGCCGCCCGGGAGTCCGTGGAGTGG
CTGCCCTTCGGCGACGGGCCGCGGAAGTGCATCGGGATGCGGTTTGGACAAA
TGCAGGCTCGCATCGGTTTGGCTCAGATCATCAGCCGGTTCAGGGTATCCGTC
TGCGATACGACAGAGATCCCCTGAAGTATAGTCCCATGTCCATAGTTTTGGG
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CACCGTTGGGGGCATCTACTTGCGAGTGGAACGCATCTAACCTCCATATTCGT
TGCTCCCATGTATATAGCTTA 3' (SEQ ID NO 12).

1.1.3b Generation of the *cyp6a2*_short hairpin construct:

5 For generation of the *cyp6a2*_short construct, primers were designed as for
*cyp6g1*_short cloning, with the corresponding restriction sites added to the 5' termini of
the FWD and REV primers to facilitate cloning. Internal 'BRIDGE' primers were also
used to facilitate fusion of the two primary PCR templates, thus creating the chimeric
template for the second PCR. Constructs were then generated as described above with
10 respect to the *cyp6g1*_short construct.

The *cyp6a2*_short sequence that was cloned comprises bases 1306 - 1718 fused to
bases 2780 -- 2952 of the *cyp6a2* gene sequence given above. The *cyp6a2*_short
sequence is:

5' CGAAAAGGGAGCAGCTACGCAAAATGTTTGTTCTAATATACCTGTTG
15 ATCGCGATCTCCTCGCTTTTGGCCTACTTGTACCACCGCAACTTCAACTACTGG
AATCGCCGCGGCGTGCCACACGATGCTCCTCACCCACTGTATGGCAACATGGT
CGGGTTCCGGAAGAACCGGGTGATGCACGACTTCTTCTACGACTACTACAAC
AAGTACCGGAAGAGCGGCTTTCCCTTCGTGGGCTTTTACTTTCTGCACAAGCC
GGCCGCCTTCATCGTGGACACCCAGCTGGCCAAGAACATCCTGATCAAGGAT
20 TTCTCGAACTTTGCCGATCGTGGCCAGTTTCACAACGGGCGCGACGACCCGCT
CACGCAGCACCTGTTCAACCTGGACGGAAAGAAGTGGAAGGACATGCGCTCG
CATCGGTTTGGCTCAGATCATCAGCCGGTTCAGGGTATCCGTCTGCGATACGA
CAGAGATCCCACTGAAGTATAGTCCCATGTCCATAGTTTTGGGCACCGTTGGG
GGCATCTACTTGCGAGTGGAACGCATCTAACCTCCATATTCGTTGCTCCCATG
25 TATATAGCTTA 3' (SEQ ID NO 13).

1.2 Site-specific recombinase mediated cloning

In an alternative cloning strategy, site-specific phage recombinase mediated
cloning (using Invitrogen's Gateway technology) was used. The pB-UGIR w+ vector
30 (Genbank AY196824) is particularly suitable for use in this method as it has been
engineered to contain recombinase target sites that allow recombination of inverted
repeats into the vector.

The insert sequences for the selected *cyp* genes were PCR amplified as described
above, using primers containing the appropriate recombinase target site sequences to

facilitate recombinase-mediated cloning into ENTRY vectors (Invitrogen). Then, in a single step, these ENTRY-derived plasmids were mixed with the pB-UGIR w+ vector, and the desired constructs were generated via recombinase-mediated cloning following the manufacturer's protocols.

- 5 Figure 2 shows how the *cyp* sequence inserts are cloned in the tail-to-tail orientation using the pB-UGIR w+ vector (the example shown is for the *cyp6g1* construct).

Example 2 Transformation of *D. melanogaster* and generation of transgenic flies

- 10 *Drosophila melanogaster* *w*¹¹⁸ embryos (Bloomington stock centre, stock No. 6326) were transformed according to Spradling, A.C. and Rubin, G.M (1982, Science 218:341-347). Briefly, the DNA plasmids for transformation were prepared using a Qiagen Midi kit. Each transformation construct was then co-precipitated and resuspended into injection buffer (5 mM KCl, 0.1 mM NaH₂PO₄, pH 6.8) at a concentration of 0.5-1
- 15 μg/μl, together with the piggyBac helper plasmid (pBlu-uTp, Genbank AY196821) at a concentration of 0.1 μg/μl. Embryos were injected prior to pole cell formation; thus 0-30 min old *w*¹¹⁸ embryos were collected, dechorionated and dessicated, and then lined up on glass slides covered with double-sided sticky tape to hold the embryos in place. The embryos were covered with a minimal amount of halocarbon oil, and microinjected
- 20 posteriorly with the transformation construct and helper plasmid mix. The injected embryos were then incubated at 25°C, and any hatching larvae were recovered and transferred to standard diet. Adults emerging from these larvae were then crossed to *w*¹¹⁸ adults, and stable transformants were selected from their progeny (expressing the *mini-w*+ marker for transformation). Several independent transgenic flies per injected construct
- 25 were isolated, and stocks of these transgenic flies were established. Transgenics were also confirmed by PCR amplification of sequences specific to the transformation plasmids.

For each *cyp6g1* construct, one transgenic line is described in further detail: line hp_1 which is a transgenic line derived from the 'cyp6g1_short' construct, line hp_2 which is a transgenic line deriving from the 'cyp6g1_long' construct.

Example 3 Induction of expression of the *cyp* hairpin RNA molecules using a binary component system

The transgenic flies generated in Example 2 comprise the *cyp6g1*- or *cyp6a2*-specific hairpin RNA molecules, and the expression of these hairpin molecules is under the control of an inducible promoter. Induction of hairpin expression mediates the degradation of the corresponding endogenous mRNAs through an RNA interference (RNAi) mechanism. As expression of the hairpin molecules is under control of an inducible promoter, an inducing agent is provided to effect the RNAi in these flies. This inducing agent is provided from another transgenic line by means of a genetic cross that brings together the inducing agent and the inducible transgene (i.e. in this case the hairpin molecule) into the same individual flies.

In this type of binary component system, the yeast GAL4 protein acts as a transcriptional regulator and thus as an inducing agent; it binds to UAS promoter sequences and activates the expression of any sequences encoded downstream of this promoter, thus rendering the expression of such sequences inducible by the GAL4 protein (Brand, A.H & Perrimon, N 1993, Development 118:401-415).

This binary system is applied by a genetic cross, between (a) a transgenic fly line that encodes for the transcription factor (GAL4), and (b) the transgenic fly line that contains the UAS promoter encoded upstream of the cloned *cyp* inverted repeat sequences (e.g. a *hp_1* or *hp_2* transgenic fly).

A proportion of the progeny from such a cross will express both the GAL4 protein as well as the hairpin molecule (via GAL4 regulation), and express double-stranded (ds) RNA corresponding to partial *cyp* sequences encoded by the hairpin (e.g. partial *cyp6g1* or *cyp6a2* sequences). These dsRNA molecules then mediate degradation of the endogenous mRNA transcripts for *cyp6g1* and *cyp6a2*, via an RNAi mechanism. The result is that the amount of endogenous mRNA transcripts for *cyp6g1* and *cyp6a2* are depleted. This effects a reduced level of expression of the specific *cyp* gene (e.g. *cyp6g1* or *cyp6a2*) and the product thereof, and a knockdown or knockout of *cyp* gene function is thus observed.

The GAL4-expressing transgenic fly line used herein was obtained from the Bloomington stock centre (*supra*) and is described by the genotype: *y[1] w[*]^{*}*; *P{w[+mC]=Act5C-GAL4}25FO1/CyO*, *y[+]* (Bloomington stock number 4414). The transgenic fly lines comprising *cyp* inverted repeat regions (line *hp_1*, which is a transgenic line derived from the 'cyp6g1_short' construct and line *hp_2*, which is a

transgenic line deriving from the 'cyp6g1_long' construct) were crossed with the GAL-4-expressing transgenic fly line.

The resulting F1 progeny were selected for those that carried both the GAL4 and the cyp6g1_long or cyp6g1_short transgenes. The F1 progeny from the cross between the GAL4 and hp_1 transgenic lines are referred to herein as *act-gal4/hp_1* flies, and similarly the F1 progeny from the cross between the GAL4 and hp_2 transgenic lines are referred to herein as *act-gal4/+; hp_2/+* flies. These *act-gal4/hp_1* and *act-gal4/+; hp_2/+* flies had no obvious morphological phenotype and were viable.

10 **Example 4 Validation of the mRNA depletion effect (due to RNAi) at the molecular level:**

The *cyp6g1* or *cyp6a2* mRNA depletion in these F1 flies was measured at the molecular level, using a Quantitative reverse transcription-polymerase chain reaction (Q-RT-PCR) to measure the amount of *cyp6g1* and *cyp6a2* mRNA present in these flies compared to wild-type or other suitable control flies.

Q-RT-PCR amplification of mRNA was carried out on pools of these *act-gal4/hp_1* or *act-gal4/+; hp_2/+* flies (between ten to twenty, 0-3 day old, adults were used). The control flies used were *Canton-S*. The expression of *cyp6g1* was also monitored in a population of *Hikone-R* flies (Bloomington stock number 4267). *Hikone-R* flies exhibit resistance to some insecticides and this resistance is thought to be due to overexpression of *cyp6g1* in this non-transgenic strain (Daborn P, Boundy S, Yen J, Pittendrigh B, ffrench-Constant R. *Mol Genet Genomics*. 2001 Dec; 266(4):556-63). The reference endogenous RNA sample used for relative quantitation was *RpL32*.

25 The primers and Taqman probes used for this mRNA detection assay were:

Cyp6g1 Q-PCR assay

Cyp6g1_fwd_primer	5' CCTGGACCGCGAGTACGA 3' (SEQ ID NO 14)
Cyp6g1_rev_primer	5' GCTGGGATTGGTCCAGTACTTT 3' (SEQ ID NO 15)
Cyp6g1_taqman_probe	5' CAGCCAGACTTGAGCCTGAAGCCG 3' (SEQ ID NO 16)

Cyp6a2 Q-PCR assay

Cyp6a2_fwd_primer	5' TCATCTCAGAAACCCTGAGGC 3' (SEQ ID NO 17)
Cyp6a2_rev_primer	5' GACCTGTGTGCCCTTCTCAATC 3' (SEQ ID NO 18)

Cyp6a2_taqman_probe 5' ACGACTACGTGGTGGCCGGGCC 3' (SEQ ID NO 19)

RpL32 Q-PCR assay

5 RpL32_fwd_primer 5' GATATGCTAAGCTGTCGCACAAAT 3' (SEQ ID NO 20)
 RpL32_rev_primer 5' GGCATCAGATACTGTCCCTTGAA 3' (SEQ ID NO 21)
 RpL32_taqman_probe 5' CGCAAGCCCAAGGGTATCGACAAC 3' (SEQ ID NO 22)

For the F1 progeny that were generated as described above, the assays showed that overall *cyp6g1* expression was reduced (in comparison to the *Canton-S* wild-type flies), ranging from approximately 5-fold in the *act-gal4;hp_1* flies, to approximately 100-fold in the *act-gal4/+; hp_2/+* flies. In comparison, *cyp6g1* expression in the population of *Hikone-R*, was found by this method to be approximately 16-fold higher than in the *Canton-S* control flies.

These results confirmed that there is indeed a knockdown of *cyp6g1* expression in the F1 progeny of *act-gal4;hp_1* flies and *act-gal4/+; hp_2/+* flies.

Similar genetic crosses and Q-RT-PCR assays were carried out to validate the *cyp6a2* knockdown by RNAi, using the transgenic fly lines that were generated to inducibly express hairpin molecules corresponding to *cyp6a2* sequences. These similarly showed successful reduction of *cyp6a2* mRNA levels.

Example 5 Susceptibility of transgenic lines to known insecticides

5.1 Bioassays:

The susceptibility of the transgenic *D. melanogaster* to known insecticides was assessed by bioassay as follows. A 5% sucrose: 1% agar mix was prepared and 0.75 mls poured into each well of a 24-well plate, and allowed to set. The test chemical (imidacloprid or fipronil) was dissolved in a suitable ethanol-acetone based solvent system to the dilutions required.

The dilutions used ranged from 0 to 20,000 ppm. 20 µl of a single dilution was spread uniformly on the surface of the sucrose-agar base in a well. By applying the

dilution series to consecutive wells in a plate, a dose range of the chemical was created. At least 3 replicate wells of each dose were made. The control wells contained the formulation solution only, applied similarly to the surface of the sucrose-agar base.

10 (0-3 day old) adult flies of the appropriate genotype were added to each well, after the applied chemical solution has dried. At least 3 replicates per genotype and per dose were included. The plates were sealed with a breatheable film, and incubated under normal culture conditions (25°C, 70-75% humidity).

The effects on the flies were monitored for up to 6 days by scoring for mortality of the flies in the wells. Any other phenotypic effects were also assessed. Comparisons between different genotypes were carried out on data from assessments that were performed contemporaneously and by the same assessor.

Bioassays were carried out as described above, using *Hikone-R* flies as the *cyp6g1* overexpressing strain, *Canton-S* as the wild-type flies expressing normal levels of *cyp6g1*, and *act-gal4/hp_1* and *act-gal4/+; hp_2/+* as the *cyp6g1* underexpressing strains.

15

5.2 Bioassay results

Overexpression of *cyp6g1* in *Drosophila* has been correlated to, or shown to confer, resistance to some insecticides (Daborn P, et al. *Mol Genet Genomics*. 2001 Dec; 266(4):556-63; Daborn,P.J. et al (2002) *Science* 297:2253-2256). The insecticide imidacloprid contains a potential P450-labile site, and has been used in the example here to demonstrate the model for how *cyp6g1* underexpressing strains can be used for chemical lead investigation. Fipronil was selected as the 'control' chemical for the experiment. Fipronil is also an insecticide but its activity is not reduced by P450-mediated metabolism. It was included to determine if any increased susceptibility to imidacloprid in the underexpressing strains could be due a non-imidacloprid-specific effect in these flies.

The bioassays were set up as described earlier, using the 4 fly strains listed above. The mortality and any other effects on the flies were assessed for up to 6 days, and the data recorded. The results demonstrate that the underexpressing strains show increased susceptibility to imidacloprid in comparison to wild-type and overexpressing strains (*Canton-S* and *Hikone-R* respectively) (see Tables 1 and 2 and Figure 3).

30

Table 1 LC₅₀ values for test compounds Results of two tests with Imidacloprid (A & B) are compared with Fipronil (C) as a control. The 95% confidence levels are given in brackets.

<i>Drosophila</i> Line	LC ₅₀		
	A	B	C
<i>Hikone-R</i>	5301 (4155-6791)	6799 (5444-8526)	0.557 (0.436-0.711)
<i>Canton-S</i>	732 (574-931)	554 (444-690)	0.567 (0.444-0.723)
<i>act-gal4/hp_1</i>	537 (422-684)	386 (310-480)	0.718 (0.564-0.916)
<i>act-gal4/+; hp_2/+</i>	264 (206-337)	217 (174-270)	0.477 (0.373-0.610)

5 **Table 2 Relative Tolerance of *Drosophila* lines to Imidacloprid (A & B) or Fipronil (C) in comparison to the tolerance of Canton S.** The 95% confidence levels are given in brackets.

	A	B	C
<i>Hikone-R</i>	7.25 (5.15-10.26)	12.27 (8.98-16.86)	0.98 (0.70-1.39)
<i>act-gal4/hp_1</i>	0.73 (0.52-1.04)	0.70 (0.51-0.95)	1.27 (0.90-1.79)
<i>act-gal4/+; hp_2/+</i>	0.36 (0.26-0.51)	0.39 (0.29-0.53)	0.84 (0.59-1.19)

CLAIMS

1. A transgenic insect or insect cell, wherein the level of expression of a P450 gene has been reduced relative to the level of expression of said P450 gene in a non-transgenic insect or insect cell,
5
2. A transgenic insect or insect cell according to claim 1 wherein the P450 gene is selected from the group consisting of the 90 known *cyp* genes from *D. melanogaster* and homologues thereof.
10
3. A transgenic insect or insect cell according to claim 1 or claim 2 wherein the P450 gene is selected from the group consisting of the *cyp6* genes from *D. melanogaster* or homologues thereof.
- 15 4. A transgenic insect or insect cell according to any one of the preceding claims wherein the P450 gene is *cyp6g1* or *cyp6a2*.
5. A transgenic insect or insect cell according to any one of the preceding claims wherein the transgenic insect or insect cell is selected from the group of insects
20 consisting of: *Drosophila sp.*, *Bombyx mori*, *Tribolium castaneum*, *Aedes aegyptii*, *Anopheles gambiae*, *Anopheles albimanus*, *Anopheles stephensi*, *Ceratitis capitata*, *Pectinophora gossypiella*, *Helicoverpa zea*, *Bactrocera dorsalis*, *Anastrepha suspense*, *Musca domestica*, *Stomoxys calcitrans*, and *Lucilia cuprina*.
- 25 6. A transgenic insect or insect cell according to claim 5 which is or comes from a species of *Drosophila*.
7. A transgenic insect or insect cell according to claim 6 which is or comes from *D. melanogaster*.
30
8. A transgenic insect or insect cell according to any one of the preceding claims wherein the level of expression of the P450 gene has been reduced through RNAi.

9. A transgenic insect or insect cell according to any one of claims 1 to 7 wherein the level of expression of the P450 gene has been reduced through insertional mutagenesis.
- 5 10. A transgenic insect or insect cell according to any one of claims 1-7 wherein the level of expression of the P450 gene has been reduced through homologous recombination.
- 10 11. A DNA construct comprising a first DNA sequence encoding a fragment of an insect P450 gene and a second DNA sequence which is the reverse complement of the first DNA sequence, wherein the first and second DNA sequences are present on the same strand of DNA and are operably linked to a promoter region and optionally a terminator region.
- 15 12. A DNA construct according to claim 11 wherein the first and second DNA sequences are separated by a third DNA sequence which acts a spacer sequence between the first and second DNA sequences.
- 20 13. A DNA construct according to claim 10 or claim 11 wherein the insect P450 gene is selected from the group consisting of the *cyp6* genes from *D. melanogaster* or homologues thereof.
- 25 14. A DNA construct according to claim 13 wherein the insect P450 gene is *cyp6g1* or *cyp6a2*.
15. A DNA construct according to claim 14 which comprises SEQ ID NO 5, SEQ ID NO 8, SEQ ID NO 12 or SEQ ID NO 13.
- 30 16. A method of producing a transgenic insect as defined in any one of claims 1-8, which comprises:
- (i) transforming a first insect with a first DNA construct according to any one of claims 11 to 15 wherein the promoter region comprises an inducible promoter;

- (ii) transforming a second insect with a second DNA construct encoding a protein that is capable of inducing expression from the inducible promoter in the first DNA construct;
- (iii) crossing the transformed insects resulting from steps (i) and (ii) to obtain progeny;

wherein a population of the progeny resulting from step (iii) comprises both the first and the second DNA constructs and when the protein encoded in the second DNA construct is expressed, the level of expression of the P450 gene is reduced in said population of the progeny relative to the level of expression of the P450 gene in a non-transgenic insect.

19. A method of producing a transgenic as defined in any one of claims 1-8, which comprises:

- (i) transforming a first insect with a first DNA construct according to any one of claims 11 to 15 wherein the promoter region comprises an inducible promoter; and,
- (ii) crossing the transformed insect resulting from step (i) with an insect that expresses an inducing protein that is capable of inducing expression from the inducible promoter in the first DNA construct, to obtain progeny;

wherein a population of the progeny resulting from step (ii) comprise the first DNA construct and express the inducing protein so that the level of expression of the P450 gene is reduced in said population of the progeny relative to the level of expression of the P450 gene in a non-transgenic insect.

18. A method of identifying or verifying the ability of a compound to act as an insecticide, which comprises:

- (i) placing a transgenic insect or insect cell according to any one claims 1 to 8 in contact with the compound
- (ii) assessing the transgenic insect or insect cell that has been placed in contact with the compound in step (i) for any deleterious effect on the insect or insect cell;

wherein the presence of a deleterious effect is indicative that said compound is capable of acting as an insecticide.

19. A method of identifying a compound other than phenobarbital that regulates expression of an insect P450 gene, which comprises:

(i) culturing in a suitable growth medium a population of cells transformed with a DNA construct comprising a reporter gene operably linked to the promoter region of the P450 gene

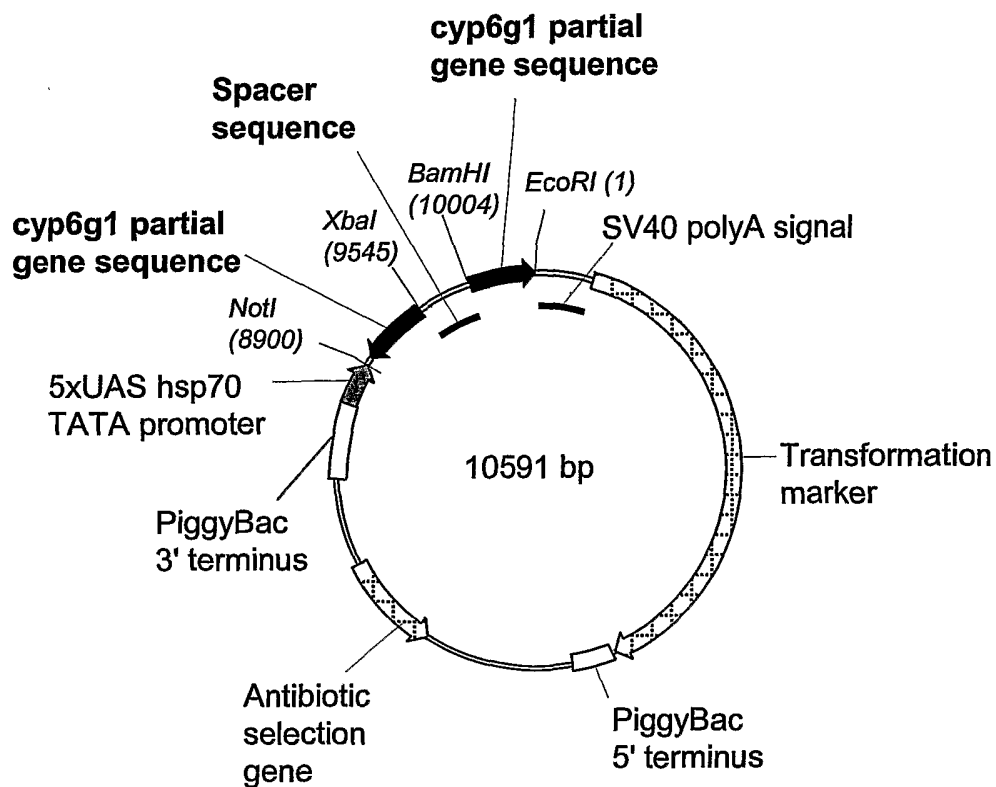
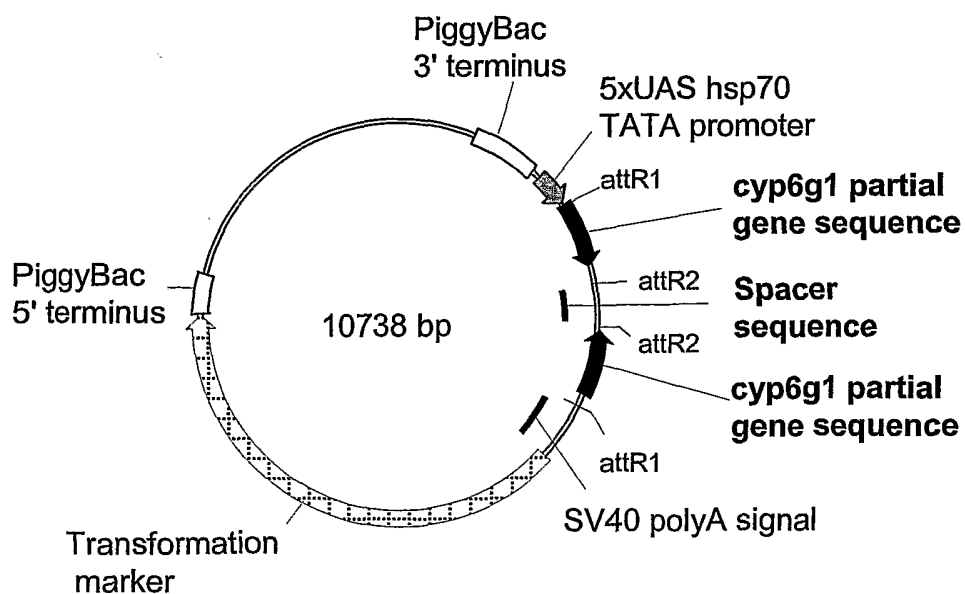
(ii) incubating said compound with a population of cells according to step (i);

(iii) determining the level of expression of said reporter gene in cells that have been incubated with said compound; and

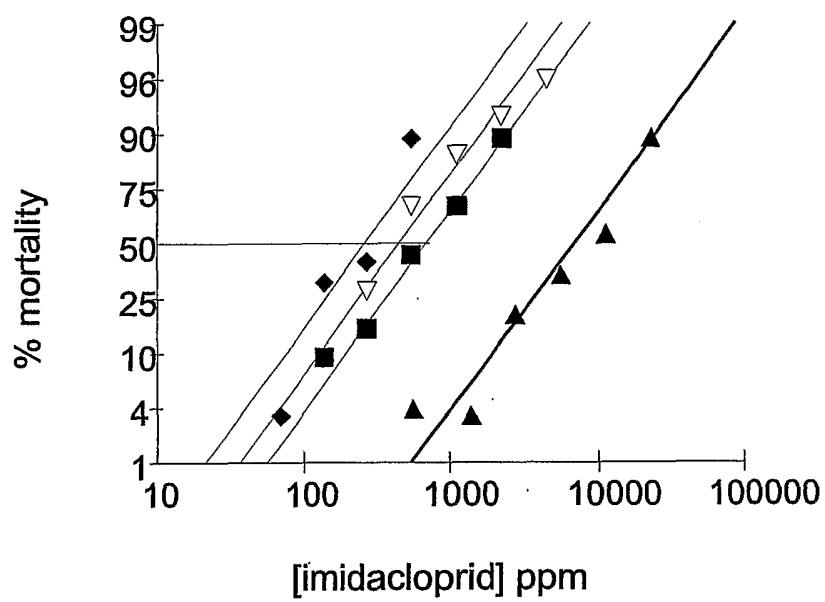
(iv) determining the level of expression of said reporter gene in a population of cells according to step (i);

wherein, a difference in the levels of reporter gene expression determined at (iii) and (iv) is indicative that said compound is capable of acting as a regulator of expression of said P450 gene.

1/2

FIGURE 1**FIGURE 2**

2/2

FIGURE 3

KEY:

▲ *Hikone-R*■ *Canton-S*▽ *act-gal4/hp_1*◆ *act-gal4/*;hp_2/**

SEQUENCE LISTING

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<120> METHODS

<130> PPD 70112/WO

<160> 22

<170> PatentIn version 3.1

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