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A CRISPR/Cas9 mediated point mutation in the alpha 6 subunit of the nicotinic acetylcholine receptor confers resistance to spinosad in *Drosophila melanogaster*

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ABSTRACT

Spinosad, a widely used and economically important insecticide, targets the nicotinic acetylcholine receptor (nAChRs) of the insect nervous system. Several studies have associated loss of function mutations in the insect nAChR α6 subunit with resistance to spinosad, and in the process identified this particular subunit as the specific target site. More recently a single non-synonymous point mutation, that does not result in loss of function, was identified in spinosad resistant strains of three insect species that results in an amino acid substitution (G275E) of the nAChR α6 subunit. The causal role of this mutation has been called into question as, to date, functional evidence proving its involvement in resistance has been limited to the study of vertebrate receptors. Here we use the CRISPR/Cas9 gene editing platform to introduce the G275E mutation into the nAChR α6 subunit of *Drosophila melanogaster*. Reverse transcriptase-PCR and sequencing confirmed the presence of the mutation in *Dα6* transcripts of mutant flies and verified that it does not disrupt the normal splicing of the two exons in close vicinity to the mutation site. A marked decrease in sensitivity to spinosad (66-fold) was observed in flies with the mutation compared to flies of the same genetic background minus the mutation, clearly demonstrating the functional role of this amino acid substitution in resistance to spinosad. Although the resistance levels observed are 4.7-fold lower than exhibited by a fly strain with a null mutation of *Dα6*, they are nevertheless predicated to be sufficient to result in resistance to spinosad at recommended field rates. Reciprocal crossings with susceptible fly strains followed by spinosad bioassays revealed G275E is inherited as an incompletely recessive trait, thus resembling the mode of inheritance described for this mutation in the western flower thrips, *Frankliniella occidentalis*. This study both resolves a debate on the functional significance of a target-site mutation and provides an example of how recent advances in genome editing can be harnessed to study insecticide resistance.

1. Introduction

Insecticide resistance is an exceptional example of rapid adaptive evolution and has provided a range of insights into the diversity of genetic alterations that occur in response to novel selective pressures. A common mechanism of insect resistance to insecticides, termed 'target-site resistance' involves alterations (mutations) in the insecticide target protein that reduce its sensitivity to insecticide. Target-site resistance most frequently involves point mutations at select positions in the target receptor as small changes to proteins are least likely to disrupt their, usually important, native function (ffrench-Constant et al., 1998).

The nicotinic acetylcholine receptor (nAChR) α6 subunit is a rare example of an insecticide target-site that can tolerate more radical alterations as it appears to be a redundant target (Perry et al., 2007). Insect α6-containing receptors are the target of spinosad, a macrocyclic lactone bio-insecticide derived from secondary

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metabolites of the soil bacteria *Saccharopolyspora spinosa*. Several lines of research indicate that spinosad binds at a site distinct from the neonicotinoid insecticides one exerting its effect through an allosteric mechanism (Orr et al., 2009; Puinean et al., 2013; Salgado and Saar, 2004).

The first resistance-conferring mutation described in the gene encoding this subunit was a null mutation of *Dα6*, in the fruit fly, *Drosophila melanogaster*, which was found to result in >1000-fold resistance to spinosad (Perry et al., 2007). Significantly, mutant flies were viable and displayed no obvious fitness deficit. Based on this finding Perry et al. predicted that loss of function mutations in *Dα6* orthologues may lead to spinosad resistance in field populations of insect pests (Perry et al., 2007). This prediction has held true with a range of genetic alterations in nAChR *α6* now described in several insect crop pests that result in truncated non-functional proteins. For example, several mutations resulting in mis-splicing and premature stop codons in nAChR α6 transcripts are associated with spinosad resistance in the diamondback moth, *Plutella xylostella*, and the oriental fruit fly *Bactrocera dorsalis* (Baxter et al., 2010; Hsu et al., 2012; Rinkevich et al., 2010).

Recently, however, spinosad resistance in several insect pest species has been associated with the same non-synonymous point mutation in exon 9 of the α6 nAChR that does not result in loss of function (Bao et al., 2014; Puinean et al., 2013; Silva et al., 2016). This mutation was initially described in western flower thrips, *Frankliniella occidentalis* (Puinean et al., 2013), and results in the replacement of a glycine (G) residue at position 275 observed in susceptible strains with a glutamic acid (E) in resistant strains. The same substitution was subsequently described in spinosad resistant melon thrips, *Thrips palmi* (Bao et al., 2014), and, very recently, tomato leafminer, *Tuta absoluta* (Silva et al., 2016).

The causal role of this mutation in resistance was recently questioned by Hou et al., after these authors characterized the nAChR α6 from three susceptible and two spinosad resistant strains of *F. occidentalis* from China and the USA and observed no difference in the cDNA sequences of resistant and susceptible thrips (Hou et al., 2014).

Functional validation of mutations in insect nAChRs, such as G275E, has been hampered by difficulties encountered in their expression in heterologous systems. Indeed, as a surrogate, Puinean et al. examined the potential effects of the G275E using human nAChR α7, a model receptor that readily forms functional homomeric receptors when expressed in heterologous systems (Puinean et al., 2013). Expression of the analogous mutation (A275E) in human α7 in *Xenopus oocytes* was found to abolish the modulatory effects of spinosad but had no significant effect upon activation by the natural ligand acetylcholine (Puinean et al., 2013). Although this evidence supports a causal role for the mutation, no functional validation of G275E in an insect system has been performed to date.

The type II clustered regular interspersed short palindromic repeat (CRISPR)/associated protein-9 nuclease (Cas9) system has recently emerged as an efficient tool to introduce precise, targeted changes to the genome of living cells. The CRISPR/Cas9 system exploits the RNA-guided endonuclease function of Cas9 to introduce double-stand breaks (DSBs) at defined loci that are then repaired by either nonhomologous end joining (NHEJ) or homology-directed repair (HDR). To introduce single nucleotide replacements in target genes HDR is exploited to repair DSBs by providing homologous sequence from a donor template such as a ssOligo or plasmid. CRISPR/Cas9-mediated editing of the genome of *D. melanogaster* has now been reported (Port et al., 2014) and the first use of this technology to introduce a resistant mutation into a controlled genetic background has also recently been described (Somers et al., 2015).

Here we describe the use of the CRISPR/Cas9 system to introduce the G275E mutation into *D. melanogaster* and demonstrate the causal role of this amino acid replacement in resistance to spinosad.

2. Material and methods

2.1. D. melanogaster strains

Fly strains described in this study were maintained on standard food (Bloomington formulation) at 24 °C. Fly strains deficient for DNA ligase 4 (#28877, genotype w^{1118} $Lig4^{169}$), expressing endonuclease Cas9 (#51324, genotype w^{1118}; PBac{y[+mDint2] = vas-Cas9}VK00027), deficient for the alpha6 subunit of the nAChR (#556, genotype w*; Df(2L)s1402, P{w[+mC] = lacW}s1402/CyO) and throughout the manuscript referred to as 'Dα6 KO', as well as the wildtype strain Canton-S (#1, wildtype) were sourced from the Bloomington Drosophila Stock Center at Indiana University, USA. The lig4 deficient strain and the Cas9 expressing strain were crossed and consecutive PCR assisted sibling mating allowed the rescue of a strain homozygous for both traits (genotype w^{1118} $Lig4^{169}$; Bac{y[+mDint2] = vas-Cas9}VK00027), hereafter called 'lig4 KO Cas9'.

2.2. gRNA design, template oligo and plasmid construction

The gRNA was designed using the online platform http://www.flyrnai.org/crispr2/ (Housden et al., 2015). A region spanning ~250 bp either side (>2L:9798031-978511) of the position of the desired point mutation was specified for the design. Based on the number of predicted off-targets a gRNA (>2L 2L:9798305.9798324 (- strand) AATTTCGCACCTAAATCCTT) was chosen as this was the only gRNA predicted to have no off-targets in combination with the predicted cutting site in close proximity to the nt position of the desired mutation (2L:9798305/9798306) (Fig. 1A). A gRNA expression plasmid was generated by cloning annealed gRNA oligonucleotides (Table 1) into the pCFD3: U6:3-gRNA plasmid (addgene #49410) as described elsewhere (Port et al., 2014). A single stranded oligonucleotide of 110 nt in size (template ssOligo) was designed to serve as a template for HDR following the Cas9 induced double strand break. The template ssOligo was designed with a dinucleotide polymorphism (Fig. 1B) which when incorporated into the genome would result in an alternate codon encoding glutamatic acid (E) instead of the native glycine (G) at AA position 275 of Dα6 (accession number NT_033779, AA count differs by 26 AA as position 275 refers to the protein after cleavage of the signal peptide). The template also contained a single nucleotide polymorphism (SNP) corresponding to intronic sequence just upstream of the above mutation site to prevent re-cleavage from Cas9 after incorporation (Fig. 1B).

2.3. Embryo injections and rescue of CRISPR mediated mutations

Embryos were collected from lig4 KO Cas9 flies and injections were carried out on an inverted microscope (eclipse Ti–U Nikon, Japan) equipped with a 10×/0.25 lens, 10×/22 eyepiece and fluorescence illumination. The injection mix comprised 0.5× phosphate buffer (pH 6.8, 0.05 mM sodium phosphate, 2.5 mM KCL) containing 200 ng μl^{-1} gRNA expression plasmid, 1 μg μl^{-1} template ssOligo and 200 mg L^{-1} fluorescein sodium salt to improve the monitoring of injections. The mix was delivered by a micromanipulation set-up consisting of a motorised micromanipulator TransferMan NK2 (Eppendorf, Hamburg, Germany) and a Femto Jet express microinjector (Eppendorf, Hamburg, Germany). Injection needles were prepared according to Miller et al. (2002) and injections into non-dechorionated embryos was carried out