Neuronal signalling molecules as targets for green peach aphid (*Myzus persicae*) control *via* RNA interference

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Insect pests: a threat to crop production

• **Economic costs of pesticides:**

  Globally, per year about 3 billion kg of pesticides are applied at a cost of around 40 billion USD (Pan-UK, 2003).

• **Other impacts of insecticides:**

  I. Toxic to non-target organisms

  II. Pollution and residual effects on environment

  III. Most alarmingly, insect resistance has been reported for many of these
Neuronal signalling molecules as efficient target for insecticides

Neuronal signalling molecules (NSMs)

- Neuropeptide (NP)
- Neuropeptide Receptor (NPR)
- Biogenic Amine Receptor (BAR)

**Common Features of NSMs:**
- Present in both interneurons and neuro-secretory or endocrine cells
- Act as neuromodulators, neurotransmitters or neurohormones
- Master regulators and multifunctional
Neuronal Signalling Cascade in organisms

Basic mechanism

1. Receptor-ligand binding
2. Signal transduction (via second messengers)
3. Cellular responses
4. Changes in gene expression

Different ways of signal integration

1. One receptor activates multiple pathways
2. Different receptors activate the same pathway
3. Different receptors activate different pathways; one pathway affects the other

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Mechanism of pesticide resistance in insects

i. Susceptible insect

Insecticides

Environment → Insect body

penetration → degradation → interaction

excretion

ii. Resistant insect

Insecticides

Environment → Insect body

Reduced penetration → Increased detoxification

Increased excretion

Behavioural modification

Target site modification

(Source: Modified from Lapied, Pennetier et al. 2009)
What is the alternative to insecticide use?

Insecticide:
- Toxic
- Costly, Artificial

RNAi:
- Safe
- Natural

Target primary endogenous molecule, not foreign particle

It will be introduced to insects as a natural process

Vital to complete insect life cycle and reproduction

Neuronal Signalling Molecule (NSM)

Insect gene rather than protein

RNA interference (RNAi)
RNAi mechanism
How NSMs will work to control insect via RNAi

Basic mechanism

1. Receptor-ligand binding
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Different ways of signal integration

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3. Different receptors activate different pathways; one pathway affects the other

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RNAi application to control NSMs gene in insects

<table>
<thead>
<tr>
<th>Insect Order</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Lepidoptera</td>
<td>✓</td>
</tr>
<tr>
<td>Coleoptera</td>
<td>✓</td>
</tr>
<tr>
<td>Diptera</td>
<td>?</td>
</tr>
<tr>
<td>Hemiptera</td>
<td>?</td>
</tr>
<tr>
<td>Hymenoptera</td>
<td>?</td>
</tr>
<tr>
<td>Isoptera</td>
<td>?</td>
</tr>
<tr>
<td>Orthoptera</td>
<td>✓</td>
</tr>
<tr>
<td>Blattaria</td>
<td>✓</td>
</tr>
</tbody>
</table>
Polyphenism: make GPA smarter to rule the world

**Colour Polyphenism**
- Temperature
- Diet quality
- Adaptation to avoid natural enemies

**Wing Polyphenism**
- Crowding
- Nutritional status

Source: Aphid base
GPA damage

(i) Direct damage

Underside of Canola leaf
Curled peach tree leaves
Crinkled and blistered Sunflower Leaf

(ii) Indirect damage

CMV (Infested fruit on right)
Sooty mould on leaves
Photosynthesis interruption

INRA, DAFWA, GRDC, OMAFRA
## Economic cost of pesticide control of aphids in Australia

<table>
<thead>
<tr>
<th>Crop</th>
<th>Aphid spp.</th>
<th>Total cost of pesticides treatment ($million)</th>
<th>Present Annual loss ($million)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canola</td>
<td>Cabbage Aphid, GPA</td>
<td>$3.950</td>
<td>$5.110</td>
</tr>
<tr>
<td>Wheat</td>
<td>Cereal Aphid</td>
<td>$17.860</td>
<td>$14.812</td>
</tr>
<tr>
<td>Barley</td>
<td>Cereal Aphid</td>
<td>$4.166</td>
<td>$3.576</td>
</tr>
<tr>
<td>Lupin</td>
<td>Various Aphid</td>
<td>$1.597</td>
<td>$1.962</td>
</tr>
</tbody>
</table>

> In **WA alone, $3.55 million** is spent annually on the control of **aphids** in canola crops

Sources: GRDC, Murray et al. 2013
Subject of this presentation

To assess the potential of neuronal signalling molecules as targets for insect control using RNA interference
Results
In silico identification of target genes

Compilation of genes of NSMs identified in other insects (e.g. red flour beetle, fruitfly, pea aphid)

GPA transcriptome analysis (Illumina HiSeq 2000)

In silico analysis (BLAST search) against GPA EST databases (e.g. NCBI, Aphidbase, Wormbase, Flybase)

Select ESTs most related to GPA for further characterisation using RT-PCR, cloning and sequencing

Re-analysis of sequenced products to check for off-target effects using sequences of other organisms
Characterization and validation of target genes

Gene amplification from GPA cDNA

Sequencing for confirmation

Sequence Alignment with target sequence

Re-analysis of sequence using blast program

Selection of target genes
### In silico identification of target genes

<table>
<thead>
<tr>
<th>Sources</th>
<th>NP</th>
<th>NPR</th>
<th>BAR</th>
<th>Total identified</th>
<th>Total Characterised</th>
<th>Query Coverage (%)</th>
<th>E-Value threshold</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESTs from databases</td>
<td>19</td>
<td>4</td>
<td>1</td>
<td>24</td>
<td>16</td>
<td>43-99</td>
<td>&gt;1E-05</td>
</tr>
<tr>
<td>ESTs from transcriptome</td>
<td>4</td>
<td>5</td>
<td>2</td>
<td>11</td>
<td>8</td>
<td>85-100</td>
<td>&gt;1E-05</td>
</tr>
</tbody>
</table>

### Functional group

<table>
<thead>
<tr>
<th>Functional group</th>
<th>Number of genes identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth and development</td>
<td>6</td>
</tr>
<tr>
<td>Moulting and reproduction</td>
<td>5</td>
</tr>
<tr>
<td>Muscle contraction</td>
<td>5</td>
</tr>
<tr>
<td>Feeding behaviour and water homeostasis</td>
<td>2</td>
</tr>
<tr>
<td>Receptor signalling pathway</td>
<td>4</td>
</tr>
<tr>
<td>Visibility</td>
<td>2</td>
</tr>
</tbody>
</table>
**In vitro** feeding trial to assess silencing effects on GPA

3rd/4th Instar 5 GPAs Collection

- 2ug/uL dsRNA with vital dye
- Control: 30% Sucrose & dsGFP

After 24 hours

Phenotypic observation

Gene expression analysis by q RT-PCR

Determine effect on aphid survival for 13 days
### RNAi phenotypes of six GPA NSM genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Phenotypic observation after 24 hours</th>
<th>% of dead GPA within 72 hr after putting on plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP-1</td>
<td>100% extremely paralysed</td>
<td>100</td>
</tr>
<tr>
<td>NP-2</td>
<td>No mortality observed but aphids stressed</td>
<td>0</td>
</tr>
<tr>
<td>NP-3</td>
<td>60% becomes less active</td>
<td>0</td>
</tr>
<tr>
<td>IP-4</td>
<td>No obvious phenotype</td>
<td>0</td>
</tr>
<tr>
<td>NPR-5</td>
<td>20% couldn’t moult and died</td>
<td>0</td>
</tr>
<tr>
<td>NP-6</td>
<td>20% couldn’t moult and died</td>
<td>0</td>
</tr>
<tr>
<td>sFP</td>
<td>100% alive and active</td>
<td>0</td>
</tr>
<tr>
<td>No dsRNA</td>
<td>100% alive and active</td>
<td>0</td>
</tr>
</tbody>
</table>
RNAi phenotypes of dsRNA-fed GPA after 24 hours

- NP-1 (Paralysed)
- NP-2 (Stressed)
- NPR-5 (could not moult)
- NP-6 (could not moult)
- dsGFP (normal)
- No dsRNA (normal)
Movement assay for dsNP-2 fed GPA after 24 hr

<table>
<thead>
<tr>
<th></th>
<th>% length travelled</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP-2</td>
<td>1</td>
</tr>
<tr>
<td>GFP</td>
<td>20</td>
</tr>
<tr>
<td>Sucrose</td>
<td>25</td>
</tr>
</tbody>
</table>

Tray used for movement assay

Stable dsRNA in diet after 24 hours of feeding
Mean reproduction of dsRNA-fed GPA after 13 days on plant

* indicates significant differences as determined by Student’s t-test (P< 0.05)
Mean longevity of dsRNA-fed GPA on plants

* indicates significant differences as determined by Student’s t-test (P< 0.05)
24 genes of GPA encoding NSMs have been identified and characterized

For some genes, *in vitro* feeding of dsRNA to nymphs induce gene silencing

For one neuropeptide gene, effect was lethal after only for 24 hours

For most genes, RNAi affected survival or reproduction of aphids or both

This work demonstrates that RNAi is an effective tool for analysis of gene function and for selection of a target gene for GPA control
Further Work

• *In vitro* RNAi is a first step for selecting a suitable target for a RNAi-based GPA control strategy

• Gene knockdown in aphids fed double stranded RNA and the persistence of such in subsequent generations will be assessed

• It is expected that plants engineered to produce dsRNA provide a constant source of dsRNA

• This theory will be tested by generating a series of transgenic plants producing hairpin dsRNA on which aphids will be allowed to feed
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Thank You