The use of anti-nematode and anti-feeding site factors to obtain resistance against potato cyst nematodes (PCN).

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Introduction

Figure 1: Potato field infested with PCN to which two strips of nematicide have been applied

- Potato Cyst Nematodes (*Globodera pallida* and *G. rostochiensis*) are damaging pests of potato (Figure 1). They cause damage estimated at £50 million each year in the UK. An absence of effective resistance to the most damaging species (*G. pallida*) results in the application of nematicides to 28,000 ha of infested fields each year (costing a further £8 million). However, many of these nematicides are currently being phased out, meaning that alternative control strategies are required.
- After invasion, PCN induces a large, multinucleate and metabolically active syncytium (Figure 2). Once the syncytium is established, the juvenile loses all body wall muscle and is unable to move or re-infect plants, becoming dependent on this feeding structure for the rest of its life. Therefore, disrupting the nematode or the feeding site at this stage inevitably results in nematode death.
- Here we present data comparing the efficiency of different silencing approaches, which will be used to target essential plant genes that are involved in post-transcriptional gene regulation or nematode genes involved in metabolic processes. The long-term aim of this work is to deliver resistance when such silencing constructs are linked to a feeding site-specific promoter.

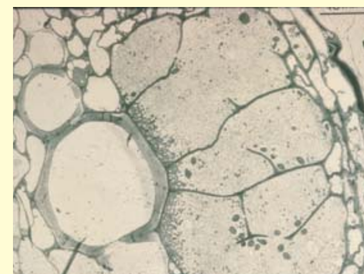


Figure 2: Cross section through a PCN syncytium showing enriched cytoplasm and degraded cell walls

Gene silencing with short dsRNAs

- The first step in this project was to develop an effective system for silencing plant and nematode genes using MicroRNAs
- MicroRNAs are short regulatory RNAs that are processed from double stranded RNA that direct cleavage of endogenous mRNAs. MicroRNAs control expression of developmentally important genes in many organisms
- We adapted miRNA 39/171from *Arabidopsis* which targets 3 scarecrow-like genes (Figure 3A).

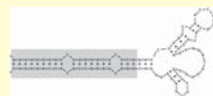


Figure 3A: miRNA 39. Silencing sequence is in grey, structured loop is to the right hand side.

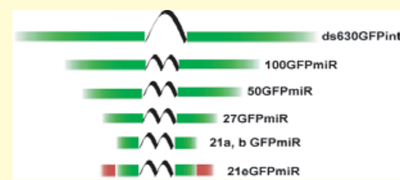
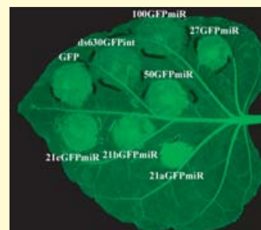


Figure 3B: Diagram of constructs. Large arch represents large intron, "M" arches represent structured miRNA loop. Green areas represent lengths of silencing sequence.

- We analysed the ability of the small miRNA39 structured loop to replace a large intron in a "conventional" dsRNA and the minmum sequence length required for effective silencing using green fluorescent protein (GFP) as a model system.
- Co-inoculation of the various constructs (Figure 3B) with a GFP-expressing construct in *Nicotiana benthamiana* showed that GFP silencing levels varied depending on the length of silencing sequence used. The miRNA loop worked as effectively as the much larger intron in this silencing assay (Figure 4A).

Figure 4A: GFP fluoresence following co-inoculation of various constructs with a GFP-expressing construct in N. benthamiana. Both ds630GFPint and 100GFPmiR greatly reduce GFP expression, 50GFPmiR shows some reduction in GFP and the remaining constructs show no visible reduction in GFP expression.



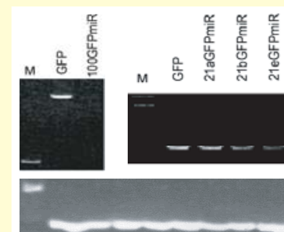


Figure 4B: RT-PCR analysis of GFP expression levels after exposure to various GFP silencing constructs. 100nt silencing sequence causes complete suppression of GFP mRNA levels (upper left panel) while varying levels of suppression are seen with the 21nt constructs (right upper). Control gene (ubiquitin) expression levels are constant throughout (lower panel).

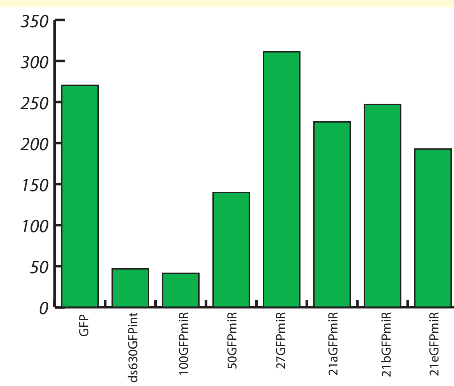


Figure 4C: GFP protein levels in leaves exposed to various silencing constructs

- RT-PCR showed that some reduction in GFP transcripts was achieved using the 21nt constructs, even where no reduction in GFP expression was visually detectable. The 21nt silencing sequences varied in the level of transcript reduction that they caused (Fig 4B).
- Quantitative analysis of GFP protein levels present after silencing confirmed that protein levels were reduced as a result of the silencing procedures and that the efficiency of the 21nt constructs is dependent on the sequence being used (Figure 4C).

Targeting plant post-transcriptional processing

- Two plant genes encoding important proteins involved in post-transcriptional processing - RNA Guanyl Transferase (RGT) and Like-Sm protein 4 (LSm4) were selected for silencing. Yeast mutants of these proteins are lethal, suggesting that targeting these proteins in the feeding site could provide nematode control.
- Constructs containing 500 or 21nt of silencing sequence were generated that targeted the plant genes.
- The ability of these constructs to silence the plant genes was assessed using Agroinoculation of *N. benthamiana* leaves followed by analysis of the mRNA levels of the two targeted genes.
- In both cases the constructs were able to cause silencing of the plant target genes with no effect on the expression of a control gene (ubiquitin - Figure 5).
- The constructs varied in their ability to silence the plant genes.
- The most efficient constructs have been selected for transformation into plants under the control of a feeding site specific promoter. Replicated potato lines will be screened for nematode resistance.

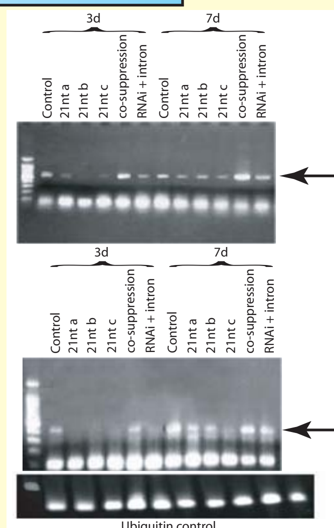


Figure 5: RT-PCR analysis of expression levels of RGT (upper panel) and LSm4 (lower panel) after exposure to silencing constructs. Arrows indicate expected product size

Targeting nematode genes

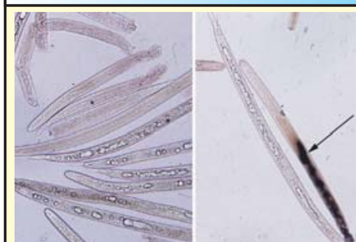


Figure 6A: in situ hybridisation shows the nematode gene is expressed in the intestine. Site of expression is indicated by a purple staining (arrow) while controls (left) show no stain.

- Several silencing constructs targeting the nematode gene have been made and introduced into potato cv Désirée.
- The first trials of these transgenic plants are promising, with three lines supporting significantly reduced nematode replication (Figure 6B).
- These constructs will be combined with the best of the plant targeting constructs in a stacking approach.

- A nematode gene encoding a protein involved in food transport was selected for silencing.
- As expected, the gene was expressed in the nematode digestive system (Figure 6A). It will therefore be exposed to dsRNA taken up by the nematode from the syncytium.

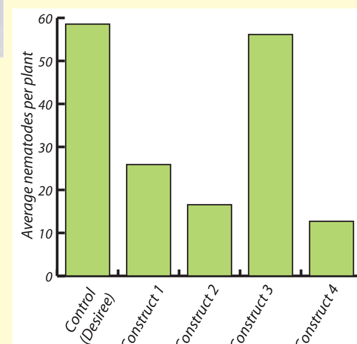


Figure 6B: Replication of G. pallida on control and transgenic plants containing silencing constructs. Figures are means of 5 replicates.