Mylne working in a collaborator's lab in Texas. Central America is the centre of origin of sunflowers, and has since been a hotbed of sunflower evolution.

Prickly

Joshua Mylne has shown how two distantly related plants have landed on the same solution to defend their seeds. And now this solution could open new avenues in cancer treatment.

By Graeme O'Neill

AT MATURITY, THE BRIGHT orange fruit of Southern-Asia's gac, or spiny bitter gourd, *Momordica cochinchinensis*, harbours a cluster of large three centimetre diameter seeds, an alluring high-protein meal for a hungry ruminant. However, according to molecular geneticist Dr Joshua Mylne, if it indulges too much the animal might end up only with a case of severe dyspepsia.

Mylne is an Australian Research Council QE2 Fellow at the Institute of Molecular Biosciences at the University of Queensland, and will be speaking at ComBio 2012. He has shown that the tropical liana's seeds are laced with an extremely potent inhibitor of bovine trypsin, a major gut enzyme involved in digesting proteins. Even the inhibitor's name verges on indigestible: *Momordica cochinchinensis* trypsin inhibitor II (MCoTI-II).

Gac is a member of the Cucurbitaceae family, and Mylne's investigation of the genes encoding the trypsin inhibitor in its seeds has added to an emerging tale of evolutionary parallelism spanning the plant kingdom. Cancer-drug developers are following developments with keen interest, because proteases play a key role

in the development of cancer in mammals, particularly cancers of epithelial tissues.

According to Mylne, the human body is tightly packed, leaving few voids. As such, tumours have to make room to accommodate their rapid growth. "Commonly, one of the earliest indications of cancer is the activation of latent protease genes," he says.

"For a tumour to expand it needs to invade the space occupied by healthy neighbouring cells. The tumour activates otherwise silent proteases, to kill and digest healthy tissues and create growing room for itself. Different types of tumours express various proteases, for example, prostate cancers commonly over-express a trypsin-like protease called KLK4, and metastasising breast tumours over-express a protease called matriptase."

Between 2001 and 2005, Mylne worked as a plant developmental biologist at the renowned John Innes Centre for plant research in Norwich, United Kingdom, studying the genes involved in the effect that winter has on the timing of flowering. He returned to Australia in 2006 to pursue a different career path in biomedical research at the IMB, where he joined the lab of a renowned nuclear magnetic resonance spectroscopist and drug designer, Professor David Craik.

CYCLES

The foundation for his most interesting discovery was laid in the late 1990s by a Russian biochemist, Dr Alexander Konarev, who was studying protease inhibitors in plants, and discovered an extraordinarily potent inhibitor in an extract of sunflower (*Helianthus annuus*) seeds. Konarev later purified the molecule – a tiny peptide – during a collaborative research visit to the UK's Rothamstead Research Station.

Bristol University PhD researcher Sue Luckett crystallised the purified molecule, both in its native state and bound to trypsin. X-ray crystallography revealed it to be 14-amino acids long. Surprisingly, it turned out to be ring-shaped, a cyclic peptide. Luckett suggested that the exceptional potency of the cyclic peptide, called Sunflower Trypsin Inhibitor 1 (SFTI-1), was due to its structural rigidity, which derives both from its ring structure and the fact that it is braced by an internal disulphide bond between cysteine residues at positions 3 and 11 in the peptide ring.

As a trypsin inhibitor, SFTI-1 is thought to act as an insect anti-feedant in sunflower seed, similar to the role of more recently discovered MCoTI-II in *M. cochinchinensis*. UK researchers confirmed it was a potent trypsin inhibitor at extremely low – sub-nanomolar – concentrations.

Mylne became intrigued that such evolutionary distant taxa as sunflower (Asteraceae) and gac (Cucurbitae) had arrived at essentially the same solution to deter insects from attacking their seeds. Both inhibit trypsin, but the two cyclic peptides differ markedly in mass. Sunflower's 14-amino acid SFTI-1 is the smallest known cyclic peptide; gac's 34-amino acid MCoTI-II is more than twice as large.

At IMB, he set out to determine how the cyclic peptide is synthesised

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in sunflower. In a paper published in *Nature Chemical Biology* in March last year, the IMB team and a colleague from CSIRO described how the DNA sequence encoding the 14 amino acids of the ring is embedded within a larger gene, *Preproalbumin with SFTI-1*, or *PawS1*.

The *PawS1* gene codes for a 151-amino acid protein, which includes a signal sequence that targets it to the endoplasmic reticulum (ER), and a region typical of seed albumin precursor proteins – plant albumins that form a store of nitrogen and sulfur for the seedling during germination.

Using the *PawS1* DNA sequence to search GenBank, Mylne and his colleagues found a similar sequence that codes for a 137-amino acid protein they named *PawS2*. Based on the amino-acid sequence for the cyclic peptide in *PawS1*, they predicted it would code for a 12-residue cyclic peptide, smaller than SFTI-1, which makes it the smallest cyclic peptide encoded by a gene yet discovered in any higher organism. The new cyclic peptide has been called SFTI-Like1, or SFT-L1.

SFT-L1 lacks a lysine residue that is key to SFTI-1's trypsin-inhibiting activity, and the molecule's function has yet to be identified. In a subsequent analysis of 123 daisy-family species, spanning some of the most divergent lineages within the Asteraceae, the IMB team found SFTI-1 in sunflower cousins including Jerusalem artichoke, and silverleaf and serpentine sunflowers. These species are also likely to be producing SFTI-1 in their seeds, as well as mature albumins from the preproalbumin precursor.

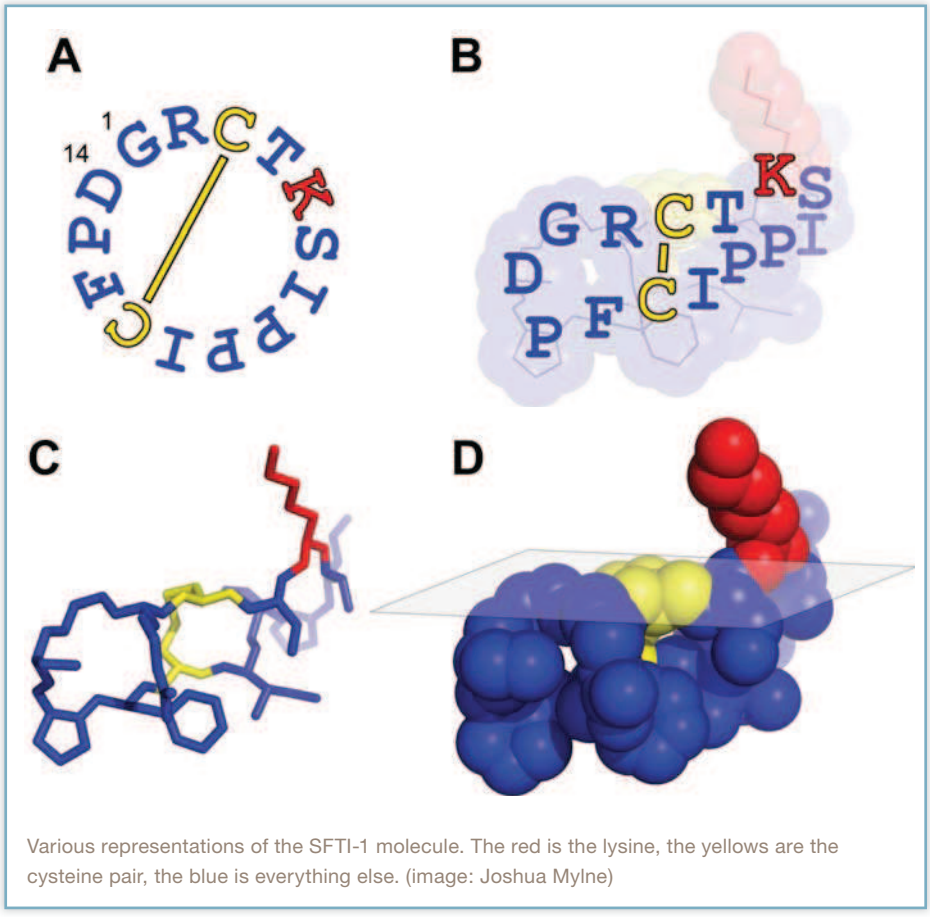
Mylne and colleagues demonstrated the feasibility of producing the trypsin-inhibiting cyclic peptide in commercial crops by developing a transgenic *Arabidopsis* line in which the *PawS1* cDNA is expressed under the control of an *Arabidopsis* seed-specific promoter. The transgenic plants were able to produce the sunflower SFTI-1 peptide in their seeds.

CONSERVED MACHINERY

In another paper published in *The Plant Cell* this year, Mylne and his colleagues describe how the biosynthetic pathway in plants that produces SFTI-1 in sunflowers is in fact conserved, resembling the one used to make MCoTI-II in the cucurbit, gac. The fact that *Arabidopsis*, a member of the Brassica family, is capable of producing the SFTI-1 cyclic peptide from transgenic *PawS1* points to all dicotyledonous plants possessing the necessary machinery to produce cyclic trypsin-inhibitor peptides.

The biosynthesis of cyclic peptides both in sunflower and gac is mediated by the enzyme asparaginyl endopeptidase. But where the sunflower *PawS1* gene codes for only a single copy, the gac's MCoTI-II gene codes for up to seven copies of the larger ring peptide, which Mylne attributes to a series of gene-duplication events, probably resulting from unequal linking and crossover during meiotic recombination in the past.

He believes that, as the asparaginyl endopeptidase chops the linear peptides out of the precursor proteins, one end spontaneously reacts with the other during cleavage to create the ring structure. The mature peptides lack the amino and carboxyl 'tails' found on the vast majority of proteins. Peptide ligation



Various representations of the SFTI-1 molecule. The red is the lysine, the yellows are the cysteine pair, the blue is everything else. (image: Joshua Mylne)

Dr Joshua Mylne is a molecular geneticist at the Institute of Molecular Biosciences at the Univesity of Queenland. He is also a Queen Elizabeth II Research Fellow, and is currently the IMB's John Mattick Fellow. He is studying the biosynthesis and evolution of cyclic peptides in sunflowers, and the potential use of sunflowers to synthesise peptide drugs in bulk.

is normally achieved by hydrolysis – the free ends of the peptide fragments "grab" a water molecule to join together, but Mylne believes that the ring peptides self-ligate when they come close together, in the absence of water.

"The thing that makes SFTI-1 so potent is that, in front of the site that is cut, it has a nice, flat beta strand," Mylne says. "My IMB colleague David Fairlie, an expert on protease inhibitors, says the best inhibitors always have a preceding beta strand."

Trypsin homes in on lysine residues in target proteins. Mylne says the reaction centre can be imagined as a set of 'Pac-Man' jaws that clamp down on the projecting lysine residue, severing the protein strand and releasing the pieces.

LYSINE RESIDUE

A lysine residue projects from the flat surface of the ring-shaped SFTI-1 molecule, which is perfectly shaped according to Mylne, to inhibit trypsin's reactive centre. The trypsin 'jaws' clamp down around SFTI-1's lysine residue, but can neither cut nor release it.

"Trypsin cuts peptides at lysine (K) and arginine (R) residues. The cyclic peptide works by blocking the enzyme in a way that allows it to recognise and clamp down on its target, but prevents it releasing it. To do its job, trypsin's active site has to be flexible, and the beta strand prevents that.

"I'm writing a paper about how this structural motif came to be, but it's not the first time it has arisen. Legume seeds contain variants of a family of serine protease inhibitors called Bowman-Birk inhibitors. These protease inhibitors contain a loop that looks identical to SFTI-1, although its amino acid sequence is different.

Mylne said that, in 2001, US cancer-drug researchers showed that SFTI-1 in its natural form inhibits the breast-cancer protease matriptase. But it did not reach the clinic because it blocks other proteases in the body. "Specificity is always the challenge for developing biomedically useful protease inhibitors," he says.

More recently, researchers at Queensland University of Technology showed than an experimental variant of SFTI-1 with three amino acid substitutions at residues flanking the trypsin-binding site inhibits the trypsin-like KLK4 involved in prostate cancer, but has no effect on several closely related trypsin-like proteases.

"So it looks feasible to design selective trypsin inhibitors for particular types of cancer, and there's no question that they can be synthesised in plants, using the same protein-synthesising machinery that plants already use to make them."

Mylne says seeds are a very practical solution for making cyclic peptide-based drugs in commercial quantities, given that plants like sunflower and bitter gourd already produce the compounds naturally.

"But as drugs, peptides have inherent problems. Like all proteins, they get broken down by enzymes in the digestive tract, and few can cross the blood-brain barrier. A lot of researchers pooh-pooh the idea of peptide drugs, but the big pharmaceutical companies are showing keen interest.

"My motivation for trying to understand these cyclic peptides lies not in changing their specificity, but in working out how plants make them. There's a lot of interest around the world in developing these inhibitors as drugs. But making peptide drugs by chemical synthesis, even in microgram quantities, is very expensive. If we can understand how plants make cyclic peptides, we can modify plants to make them using their own cellular machinery, circumventing the need to synthesise them." ALS

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