Technology for modifying BT toxins for transgenic control of aphids and the steps required to instigate a program for ACP

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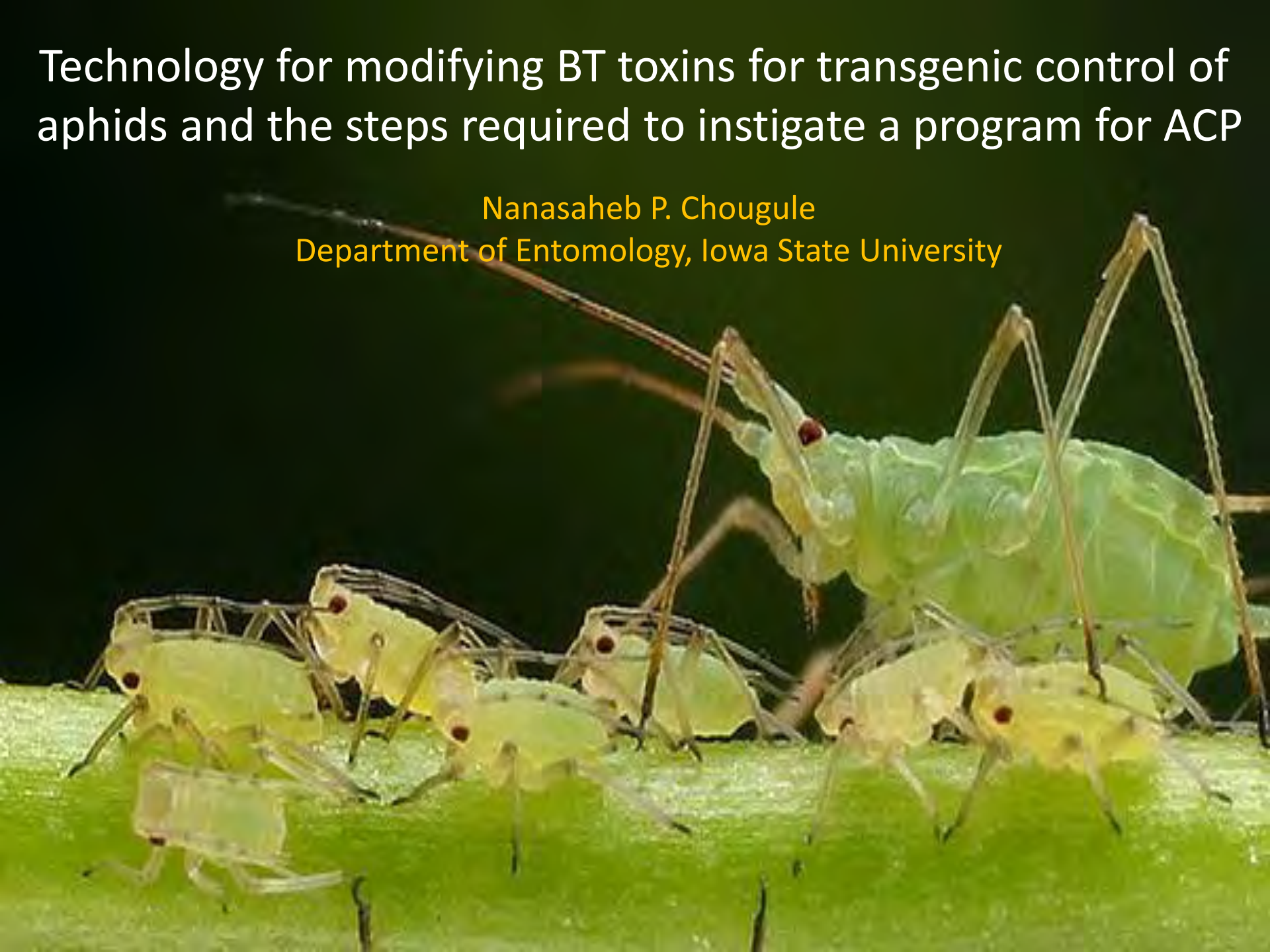
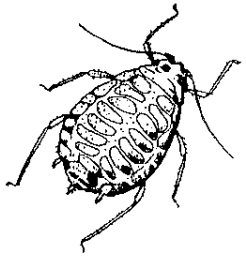
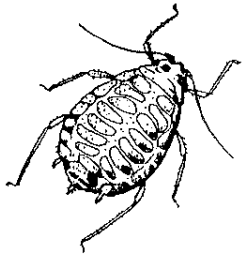




Aphids

- among most pervasive pests of temperate agriculture
- losses of > $1 billion p.a. in the U.S.
  - aphid feeding
  - transmission of plant viruses
- impact of aphids on Bt cotton is compromising the success of Bt technology

Greene et al., 1999. J. Econ. Entomol 92:941;
Greene et al., 2001 J. Econ. Entomol. 94: 403 2001.

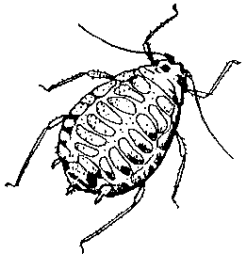


Aphids

The soybean aphid, *Aphis glycines* in the U.S. alone has cost an estimated $1.6 billion over the last decade

(Kim CH et al., 2008. *Agric. Res. Econ. Rev.* 37: 227-42)

Aphid management
-Primarily classical chemical insecticides
-Aphid resistant cultivars have met with some success
-Aphid resistant transgenic plants expressing lectins have not been adopted



Aphids are not particularly susceptible to the effects of Cry toxins derived from *Bacillus thuringiensis*

Toxin	Toxicity	Species	Reference
Cry2 Cry3A Cry4	Some	Potato aphid, *Macrosiphum euphorbiae*	F. S. Walters et al., *Ent. Exp. & Appl.* 77: 211-6 F. S. Walters et al. *Insect Biochem. Mol. Biol.* 24: 963-8
Cry3A Cry4Aa Cry11Aa	Low to moderate at 125 to 500 µg/ml	Pea aphid, *Acyrthosiphon* pisum	M. Porcar et al., 2009. *Appl Environ Microbiol* 75: 4897-900
For comparison:			
Cry1Ac	LC_{50} 1 µg/ml	Tobacco budworm, *Heliothis virescens*	M.I. Ali et al 2006. *J. Econ. Entomol.* 99: 164-75
Cry3Aa	LC_{50} 3.56 µg/ml	Colorado potato beetle, *Leptinotarsa decemlineata*	Y. Park et al 2009. *Appl. Environ. Microbiol.* 75: 3086-92

A pea aphid gut binding peptide was isolated from phage peptide display library screen

Virology 401 (2010) 107–116

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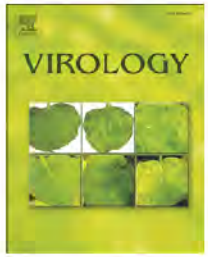


A peptide that binds the pea aphid gut impedes entry of Pea enation mosaic virus into the aphid hemocoel

Sijun Liu [a], S. Sivakumar [a], Wendy O. Sparks [a,c], W. Allen Miller [b], Bryony C. Bonning [a,*]

Will addition of the pea aphid gut binding peptide to a Bt toxin increase gut binding and enhance toxicity against aphids?

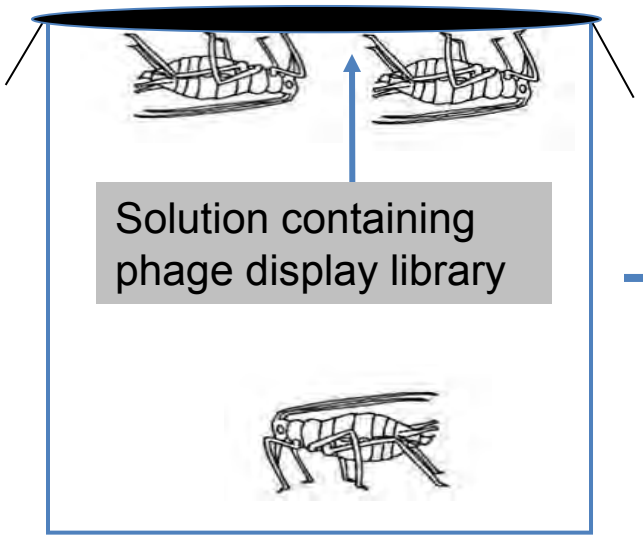
Bio-panning for aphid gut binding proteins

Filamentous phage F88.4 phage display library: 12 aa peptide (XCXXXXXXXXCX)

Membrane feeding

Isolation of mid- and hind guts

Elution of bound phage



3 rounds, 2 replicate experiments

Amplification of phage

Gut binding peptide (GBP3.1) impedes binding of a plant virus to the aphid gut

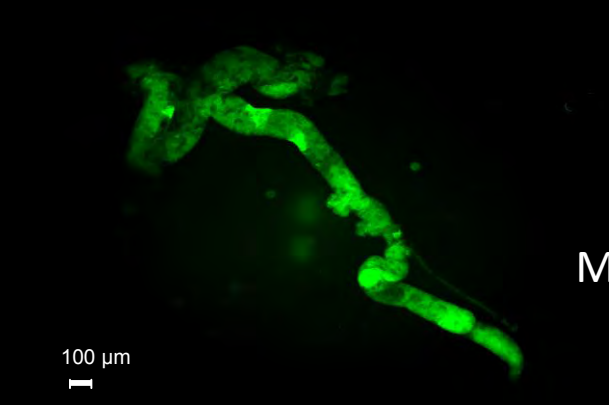
Liu et al., (2010) Virology 401: 107-116

Confirmation of GBP3.1 binding to the pea aphid gut

C6-EGFP

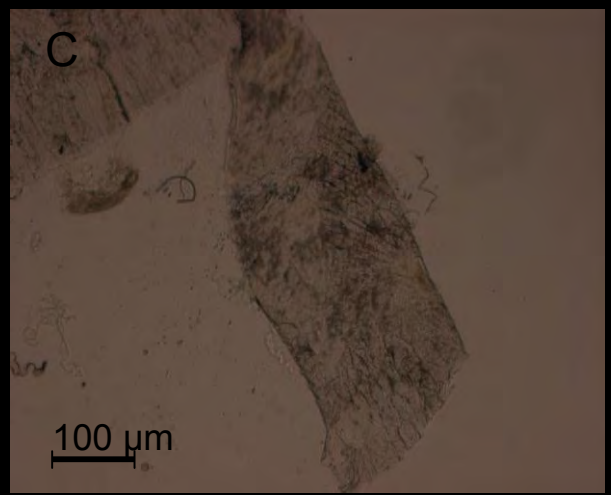
Midgut

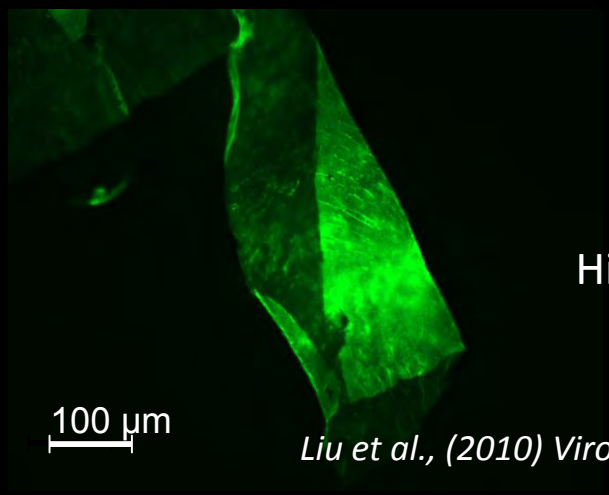
GBP3.1-EGFP



Midgut

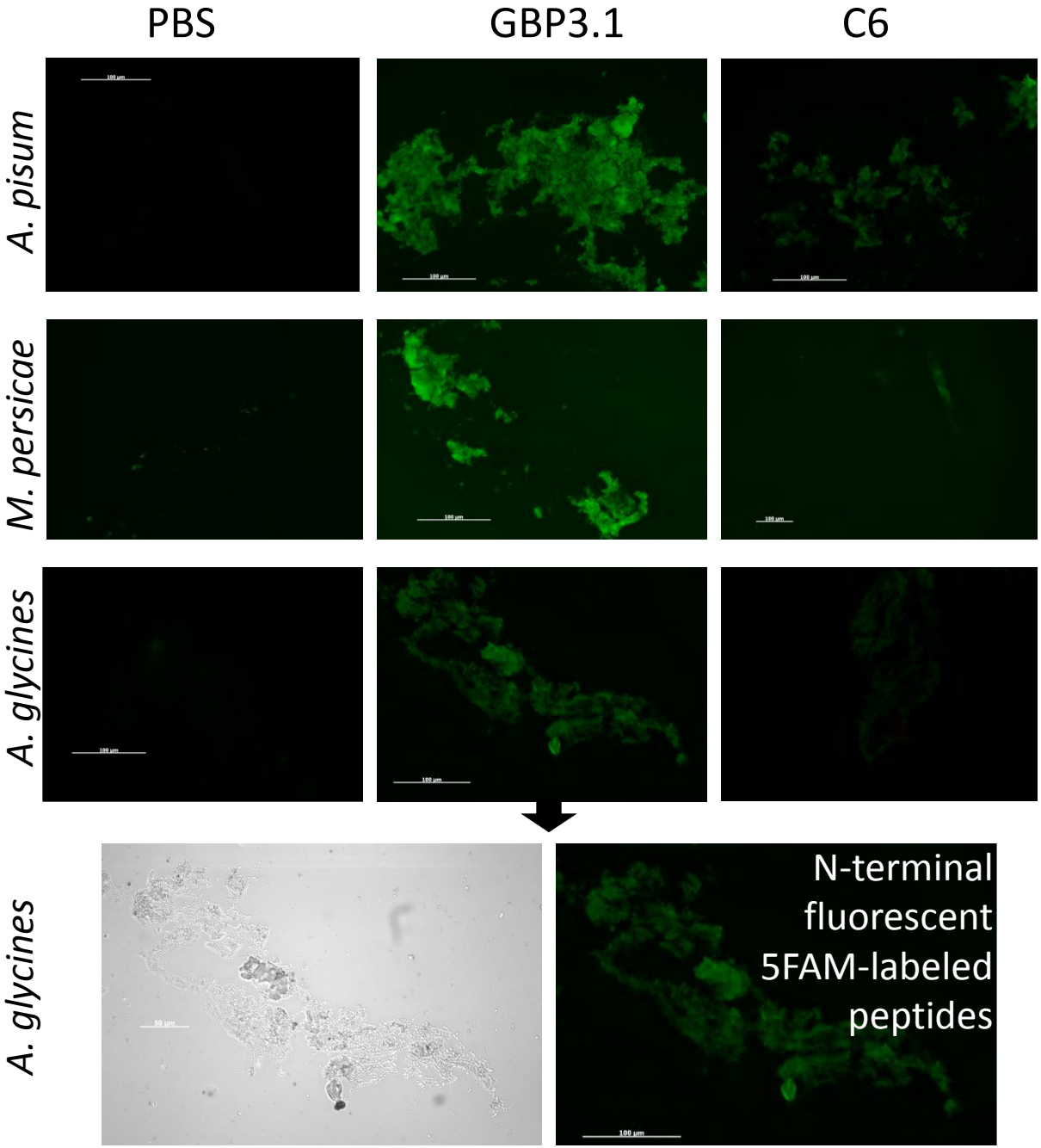
GBP3.1-EGFP





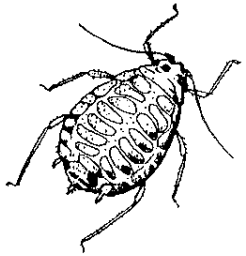
Hindgut

Liu et al., (2010) Virology 401: 107-116



GBP3.1 binds to the guts of other aphids

Liu et al., (2010) Virology 401: 107-116

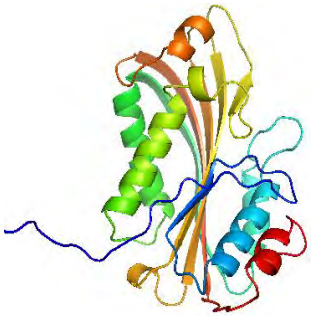


Will addition of GBP3.1 to a Bt toxin increase gut binding and enhance toxicity against aphids?

Cyt2Aa selected as a model toxin

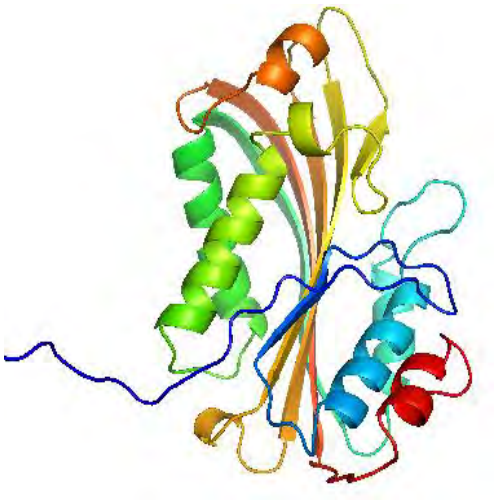
(i) Mode of action of cytolytic (Cyt) toxins is less complex than that of Cry toxins
(ii) Cyt2Aa is toxic to mosquito larvae
(iii) Cyt2Aa shows low toxicity against the pea aphid and the green peach aphid, *Myzus persicae*

LC50 >> 150 µg/ml



Cyt2Aa

- Produced as 29 kDa protoxin dimer
- Activated to 21-23 kDa monomer



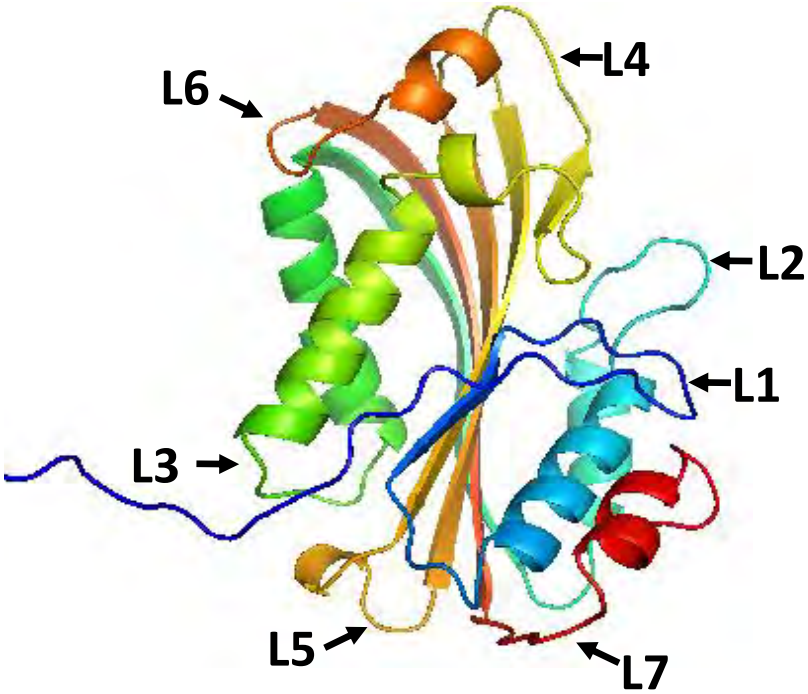
Cyt2Aa:1CBY

```
                                                  ↓    ↓
MYTKNFSNSRMEVKGNNGCSAPIIRKPFKHIVLTVPSSDLDNF
                                  β1        L1
NTVFYVQPQYINQALHLANAFQGAIDPLNLNFNFEKALQIANG
β2              αA                L2      αB
IPNSAIVKTLNQSVIQQTVEISVMVEQLKKIIQEVLGLVINST
          β3                    αC          L3
SFWNSVEATIKGTFTNLDTQIDEAWIFWHSLSAHNTSYYYNIL
    αD                        β4   L4        β5
FSIQNEDTGAVMAVLPLAFEVSVDVEKQKVLFFTIKDSARYEV
     L5          β6            αE     L6
KMKALTLVQALHSSDAPIVDIFNVNNYNLYHSNHKIIQNLNLSN
   β7        L7      αF ↑
              ↑
```

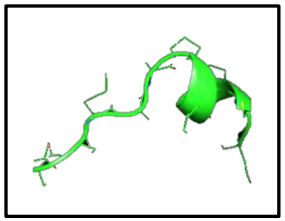
α−Helix (6) β−Sheet (7) Loop (7)

↑ Proteinase K processing sites

Construction of Cyt2Aa-GBP3.1 fusions



Cyt2Aa
PDB Code:1CBY



Predicted GBP3.1

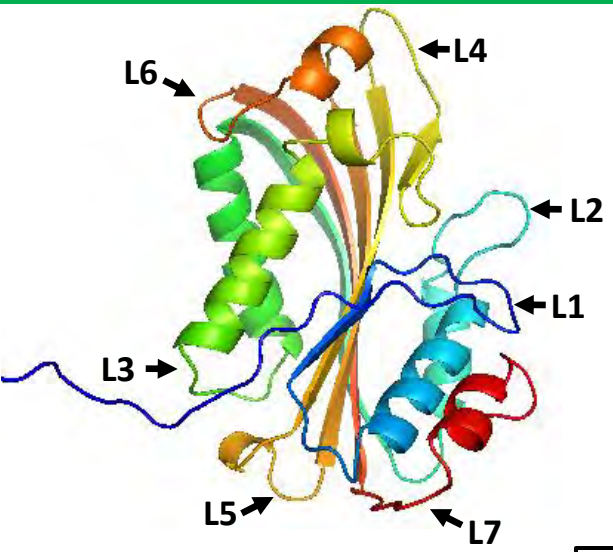
GBP3.1 : TCSKKYPRSPCM

Exposed loops for addition of GBP3.1 identified using PyMol

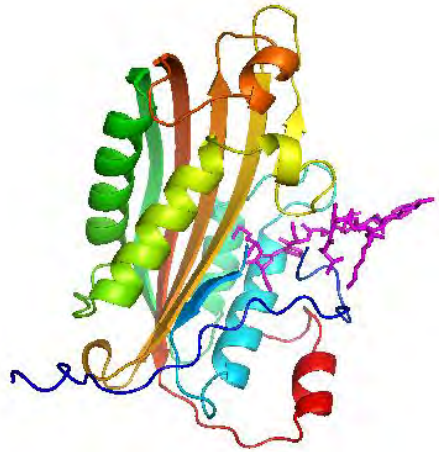
Two sets of mutants developed:

1. Six addition mutants
2. Six substitution mutants

Models of Cyt2Aa-GBP3.1 Addition Loopn (CGALn)



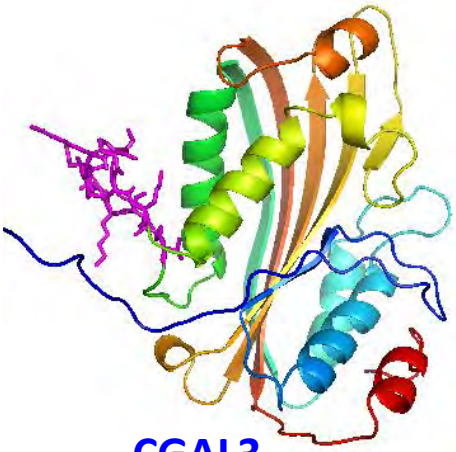
WT Cyt2Aa



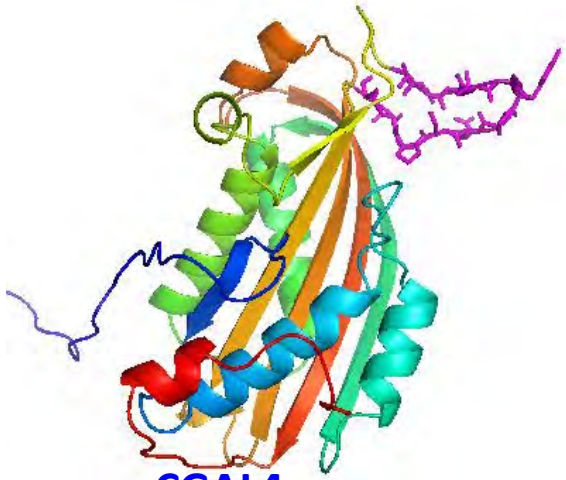
CGAL1

CGAL2

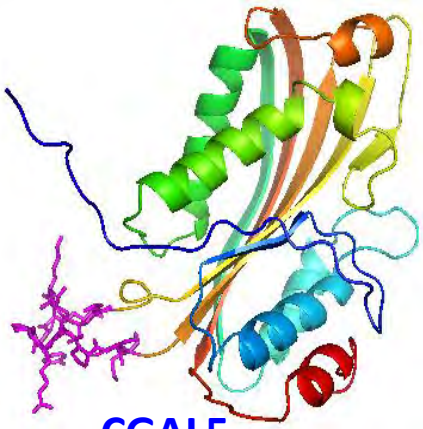
Models predict extension of Cyt2Aa loops with exposed GBP3.1



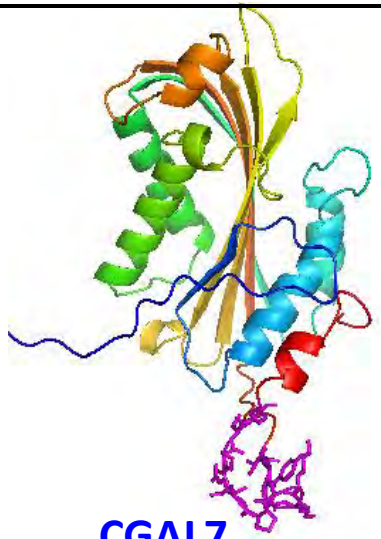
CGAL3



CGAL4



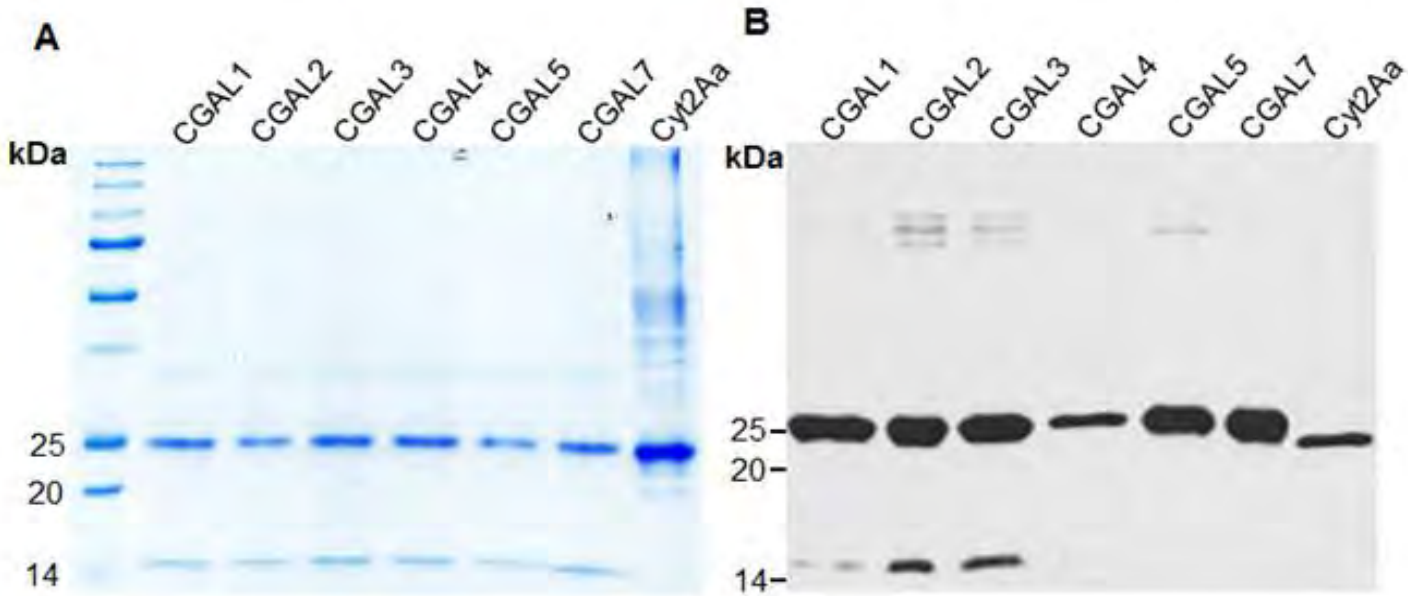
CGAL5



CGAL7

Relative expression of Wt Cyt2Aa and CGALn

Expressed in pGEM-Teasy vector in *E. coli* (BL21 DE3 pLysE),
Purified according to Promdonkoy B and Ellar DJ. 2000 (*Biochem J* 350: 275-82)



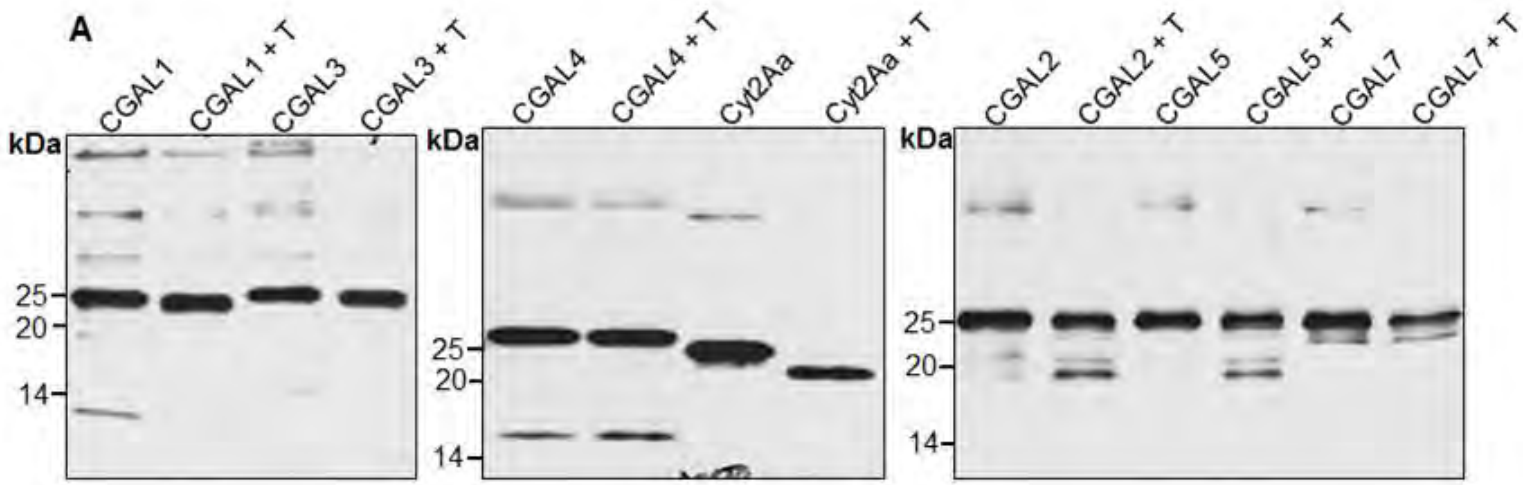
CBB stained gel

Western blot

- CGALn expression was lower relative to Wt Cyt2Aa

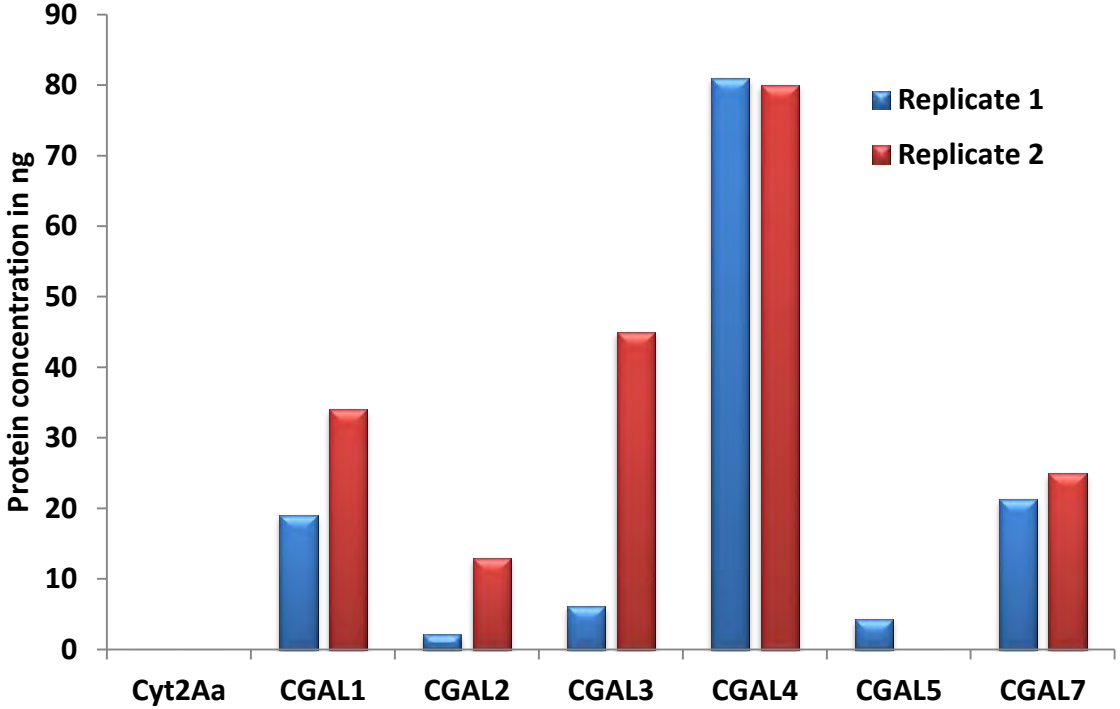
CGALn were proteolytically stable against trypsin

- Toxin pH adjusted to 8.0 with 1 M HCl
- Toxins treated with 1% trypsin at 37^0C for 60 min
- Control toxins incubated with buffer



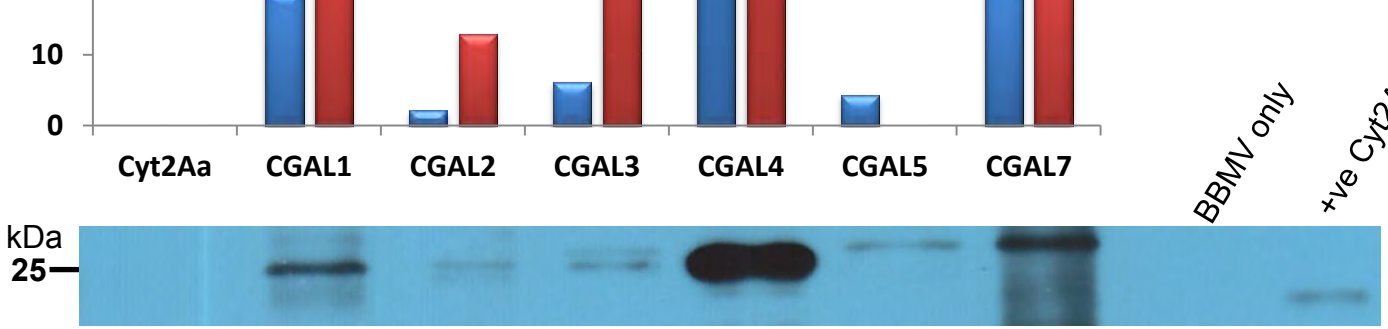
- CGAL2 and CGAL5 show two degradation fragments in small amount

Addition of peptide GBP3.1 increased toxin binding to aphid gut BBMV



Cyt2Aa-GBP3.1-AdditionLoop n

Relative binding determined by pull down assay



Western blot
Replicate 1

CGAL1, CGAL3, CGAL4, CGAL7 binds more strongly to aphid Gut BBMV than other constructs

Toxicity of wild type Cyt2Aa and CGALn against *Aedes aegypti*

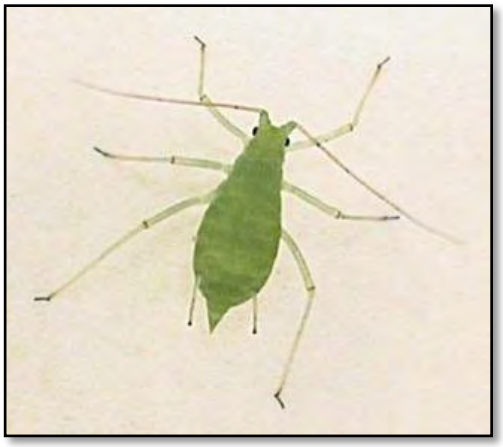
Toxin	*Aedes aegypti*		
	LC_{50} (µg/ml)	CL95%	Relative LC_{50}
Cyt2Aa	**0.368**	0.210 - 0.903	1
CGAL1	**0.217**	0.058-0.432	0.58
CGAL3	**0.621**	0.238-1.301	1.68
CGAL4	**0.181**	0.008 - 0.822	0.49

Toxic action of CGAL2, 5 and 7 was reduced by addition of GBP3.1

- LC50 could not be determined at concentrations tested (0.195 to 100 µg/ml)
- Loops 2, 5 and 7 implicated in Cyt2Aa pore formation

Toxicity of wild type Cyt2Aa and CGALn against *Ae. aegypti*, *A. pisum*

Toxin	*Ae. aegypti*			*A. pisum*	
	LC_{50} (µg/ml)	CL95%	Relative LC_{50}	LC_{50} (µg/ml)	CL95%
Cyt2Aa	**0.368**	0.210 - 0.903	1	>>150	ND
CGAL1	**0.217**	0.058-0.432	0.58	**12.809** [a]	2.51 - 21.00
CGAL3	**0.621**	0.238-1.301	1.68	**5.807** [b]	0.65 - 12.23
CGAL4	**0.181**	0.008 - 0.822	0.49	[a] **8.93** (Day3)	0.83 - 22.43



LC50 values for mortality at day 4, except where indicated

CGAL1, 3, 4 significantly more toxic than Cyt2Aa against the pea aphid

- LC50 could not be determined for Cyt2Aa at concentrations tested (0.195 to 100 µg/ml)
-Toxicity did not reflect relative binding of toxins to aphid gut BBMV

Toxicity of wild type Cyt2Aa, and CGALn against *Aedes aegypti*, *A. pisum* and *M. persciae*

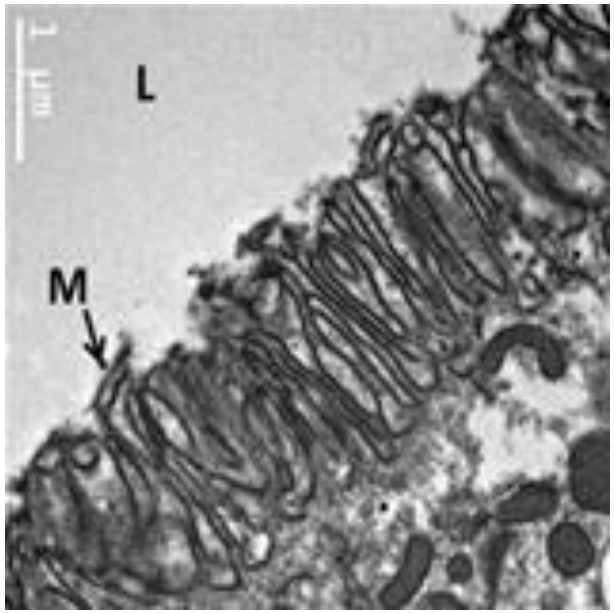
Toxin	*Ae. aegypti*			*A. pisum*		*M. persicae*	
	LC_{50} (µg/ml)	**CL95%**	**Relative LC_{50}**	**LC_{50} (µg/ml)**	**CL95%**	**LC_{50} (µg/ml)**	**CL95%**
Cyt2Aa	**0.368**	0.210 - 0.903	1	>>150	ND	>>150	ND
CGAL1	**0.217**	0.058-0.432	0.58	**12.809** [a]	2.51 - 21.00	**55.96** [a]	35.01 - 65.73
CGAL3	**0.621**	0.238-1.301	1.68	**5.807** [b]	0.65 - 12.23	**43.17** [a]	17.18 - 83.04
CGAL4	**0.181**	0.008 - 0.822	0.49	**8.93** [a] (Day3)	0.83 - 22.43	**95.29** [b]	34.67 - 152.96

CGAL1, 3, 4 more toxic against A. pisum than M. persicae

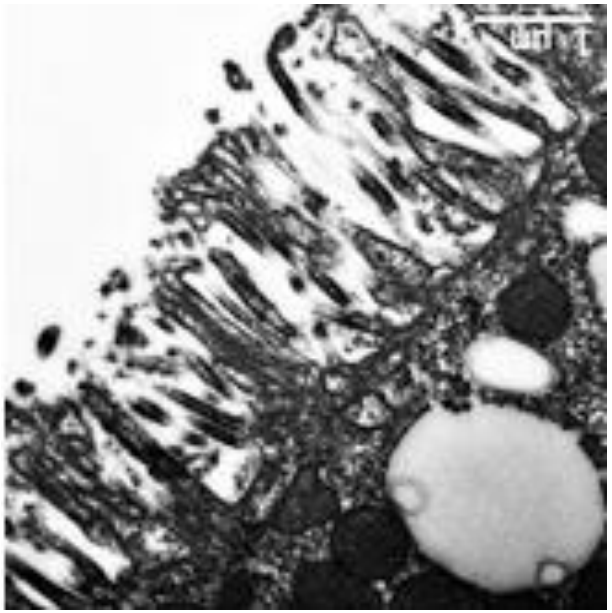
-GBP3.1 selected for binding to the pea aphid gut

Impact of Cyt2Aa and CGAL1 on the pea aphid gut membrane

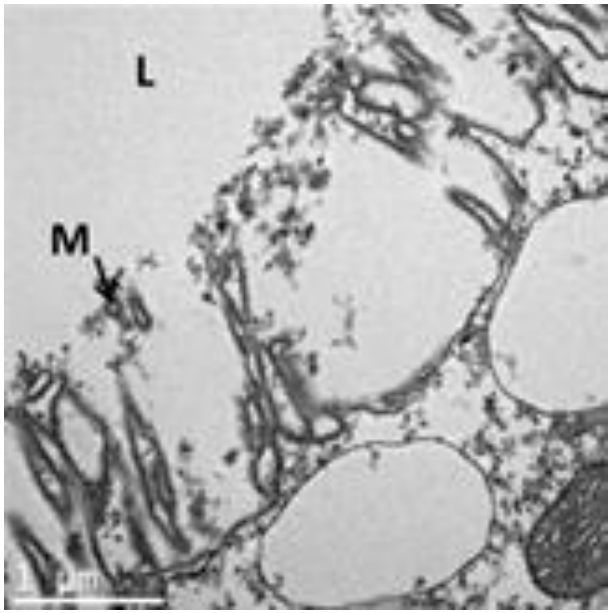
Control



Wt Cyt2Aa



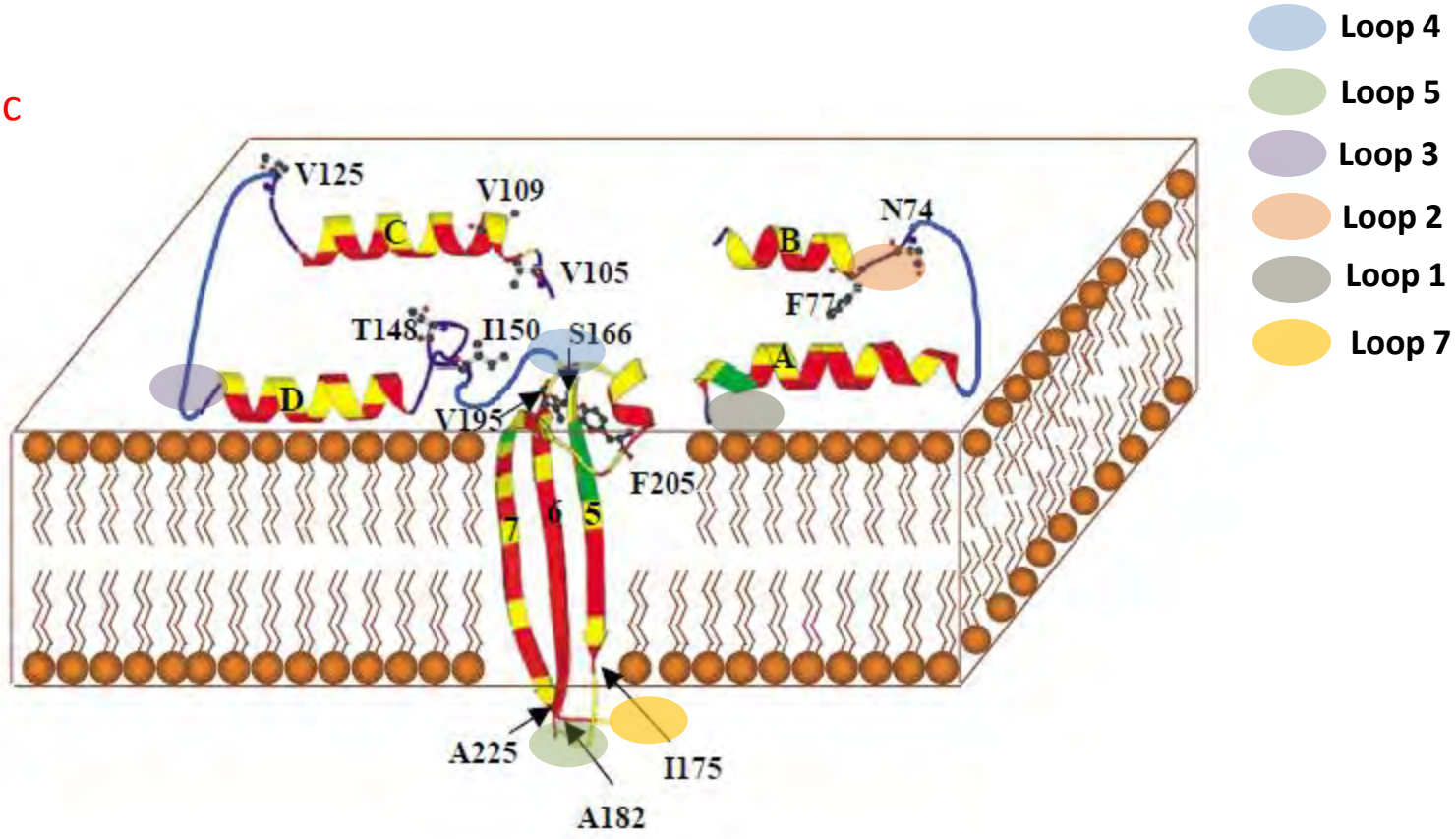
CGAL1



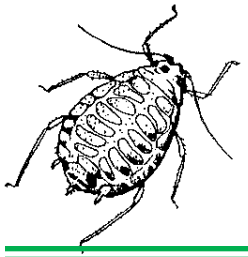
Second instar pea aphids fed on diet only, 100 µg/ml Cyt2Aa or CGAL1 for 72 hr

Possible model for insertion of Cyt2Aa into the cell membrane

Red: Hydrophobic
Yellow: Charged
Green: Tyrosine



Promdonkoy and Ellar (2005) Molecular Membrane Biology 22(4) 327-337



CGALn:Summary

1. Cyt2Aa shows only low toxicity against the pea aphid
2. Peptide GBP3.1 was isolated by screening a phage display library for peptides that bind to the pea aphid gut (*A. pisum*)
3. Addition of GBP3.1 to Cyt2Aa at loops 1, 3 and 4 did not affect the mosquitocial activity but enhanced the aphicidal activity of Cyt2Aa
   - Addition of GBP3.1 to Cyt2Aa at loops 2, 5 and 7 resulted in reduced Cyt2Aa toxicity against mosquito larvae
4. CGALn showed increased binding to pea aphid gut BBMV
5. Aphid toxicity was associated with damage to the gut epithelial membrane
6. CGALn proteins were more toxic to *A. pisum* than to *M. persicae*

Bt technology for Asian Citrus Psyllid (ACP)

What we need to know:

Cry toxins with some toxicity against ACP?

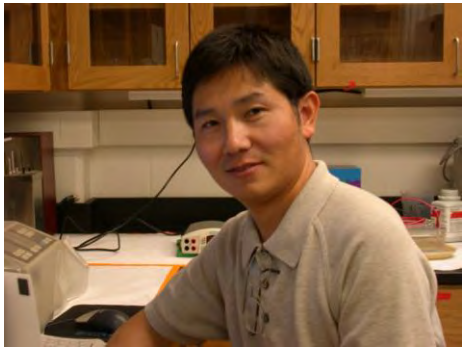
Putative Cry toxin receptors in the ACP gut epithelial cell membrane?

Digestive gut physiology of ACP?

Acknowledgements

Dr. Bryony Bonning

Dr. Sijun Liu



Dr. Huarong Li

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