Screening of new Bt proteins against regional cotton lepidopteran pests in India using *E. coli* direct bio-assay method

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Summary:

Entomology team, India has adopted the *E.coli* Direct Bio-assay to screen proteins expressed in plasmids against regional cotton lepidopteran pests. Plasmids expressing TIC868, TIC4763, TIC4760, TIC4248, TIC4247 and variants of TIC2160, TIC6757 were mobilized in to *E. coli*, expressed and tested against *Helicoverpa armigera, Spodoptera litura, Spodoptera exigua, Earias vittella* and *Pectinophora gossypiella* using a diet-overlay method. The assays were scored for mortality and growth retardation. Plasmids expressing variants of TIC2160 protein were found to be highly effective (% mortality ranging from 75 to 95) for all the insect species tested.

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Keywords: *E. coli*, Plasmids, competent cells, *Helicoverpa armigera*, *Spodoptera litura*, *Spodoptera exigua*, *Pectinophora gossypiella, Earias vittella,* Eco-direct, Bio-assay.

1. Introduction:

Entomology team, India is involved in screening novel Bt proteins in purified form against the regional pests for their efficacy. This involves importing of purified proteins from our St. Louis (STL), US counter parts ad testing these proteins for their efficacy against test insect species using a diet overlay method.

The team adopted the *E. coli* direct assays during FY 15 which involves the import of plasmids, mobilization into *E. coli* Agilent (NEB Cat # 71397) cells followed by protein induction. The expressed crude proteins after lysis with 50 mM NaCl were directly used for diet overlaying assay. This method is used as confirmation assays to test the effectiveness of the protein, and enables quick decision making for recommending the effective proteins for purification and follow up testing's. This method is also helpful in screening the effectiveness of proteins showing decreased stability when used in purified proteins.

A total of twenty one plasmids (17 test proteins, 3 positive controls and 5 negative controls) were tested using diet overlaying assay for their efficacy against cotton lepidopteran pests: *Helicoverpa armigera, Spodoptera litura, Spodoptera exigua, Pectinophora gossypiella* and *Earias vittella.*

2. Material and methods:

2.1 Mobilization of plasmid in to E.coli Agilent Cells:

Transformation of *E.coli* cells was carried out according to manufacturer's instructions.

All plasmid DNA and competent cells to be transformed were collected and thawed on ice. Manually operated multichannel pipettes were used for transformation.

a. Competent cells were diluted with TB buffer as 1:10.
b. 15µL of 1:10 Agilent cells were taken and mixed with 4 µL of diluted pDNA in a 96 well PCR conical plate (BioRad Cat# HSP-9601) at 4°C.
c. Plate was incubated on ice for 5 minutes
d. After 5 min plate was transferred on 42°C for 45 seconds.
e. The plate was transferred on ice for incubation for 5 minutes.
f. 50 µL of SOC media (New England BioLabs #B90205) was added to each well; plate was covered with Qiagen Air pore Strip (Cat No 19571) and incubated the plate at 37°C in an Eppendorff thermo mixer comfort with shaking at 900rpm for one hour.
g. 500 µL ofLB (Luria Bertani)+50ug/mL Kanamycin (Sigma- Aldrich #60615) and 25ug/mL Chloramphenicol (Sigma- Aldrich #C0378) was dispensed into a 48-well deep

well block (max volume 2.5 mL/well).

h. 20uL of each transformation recovery was taken and transferred to the 48-well block and the block was covered with Qiagen Air pore strips to cover the block and incubated the block overnight at 37°C with shaking at 900rpm in an Eppendorf thermomixer comfort.

i. After overnight incubation 100uL of 80% glycerol (Sigma-Aldrich #G5516) was added to each well and mixed.

j. 100uL of the glycerol culture was transferred to 3 or 4 96-well plates and the plates were stored at -80°C and considered "starter glycerol"and each plate was used only once.

k. Transformation protocol was repeated for the failed samples.

2.2 Culture Initiation on Auto Induction Medium (AIM) media:

a. A "starter glycerol" plate was thawed on ice.

1. 25uL of each "starter glycerol" was used to inoculate 2.5mL of AIM (Media prep recipe# 2006 Lynx Database) +100ug/mL Kanamycin (Sigma- Aldrich #60615) and 25ug/mL Chloramphenicol (Sigma- Aldrich #C0378) in 48-well deep-well blocks and Qiagen Air pore strips were used to cover the blocks.

b. 48-well blocks were incubated at 22° C for 2 nights (44 hours) in an Eppendorf Thermo-mixer comfort with shaking at 900 rpm.

c. After 2 days of incubation the cells were pelleted by centrifugation at 3,000 rpm for 10 minutes in swing bucket plate centrifuge and the supernatant was discarded.

The cells were re-suspended to final volume (10 ml) in filter sterilized 50mM NaCl.

d. The re-suspended cells were used for overlaying on insect assay diet. 100ul of re-suspended cells were overlaid on the diet. Final volume after cell lysis by 50mM NaCl was considered as 1X as per the protocol provided by St. Louis counter parts.

Table 1: Constructs Details with Gene Name:

S.No	PMON	Gene Details
1	pMON302669	eGFP control
2	pET-28a(+)	empty vector
3	pMON109268	Vip3A control
4	pMON101686	original TIC2161 protein
5	pMON101685	original TIC2160 protein
6	pMON324235	TIC5420_10
7	pMON330222	TIC2160_41
8	pMON320047	TIC2160_37
9	pMON324234	TIC5420_8
10	pMON324254	TIC5420_9
11	pMON330053	TIC2160_42
12	pMON324236	TIC5420_11
13	pMON320097	TIC2160_34
14	pMON319960	TIC2160_36
15	pMON306714	TIC4763
16	pMON306633	TIC4760
17	pMON322575	TIC4248
18	pMON322620	TIC4247
19	pMON339397	pMON101621 (T7-C His) with TIC6757
20	pMON339300	pMON101669 (T7-N His) with TIC6757
21	pMON329795	TIC868

2.3 Bioassay Method:

EcoDirect bio-assay was carried out for 7 pests at a given time using diet overlay assays with replications at various dilutions. Approximately 1ml assay diet (prepared as per BPD-ENT-MET-3238) was dispensed into each of the 128 wells of the bio-assay tray (CD International trays™, Massachusetts, USA) and allowed to solidify. Care was taken, so that the diet should not fall on the edges of the well. 100 µl of resuspended cells (1X) was dispensed over the solidified diet to a block of 16 wells.

Prepared plates were dried under laminar air flow (5-6 hrs). Individual healthy neonate larvae of lepidopteran insects were transferred to each well with the help of fine hair brush and covered with self-adhesive pull-n-peel tabs (CD International pull-n-peel tabs™). The trays were labeled and incubated in the environmental chamber where temperature of 25±2°C and relative humidity at 65 ±5% was maintained. Minimum of three replications were maintained for each construct; empty vector (pET28+) and eGFP was used as a negative control. Vip3A original TIC2161 and original TIC2160 protein was used as positive control. All 17 constructs were screened for the below mentioned insects (Table 2) and observations were recorded on 8th day after infestation for *S. litura, S. exigua, E. vitella, H. armigera* and 15 days for *P. gossypiella*.

The assays are scored when >90% of the larvae in control reach III instar. The assays are scored for mortality, efficacy and growth retardation. Additionally assays showing >10% contamination or mortality in controls were rejected. Assays were conducted in replications which gives us the confidence level for our experiments. Data was analyzed and reported for % mortality and % efficacy (dead + first, considered as functionally dead as they would not be causing any economic damage).

Gel Analysis: The protein expression in the *E. coli* cells was confirmed by using gel analysis. The proteins were induced with and without IPTG and the expression levels were compared in the AIM media and Luria Bertani Media after one day and two days growth period. Total protein and the supernatant were used for protein screen. (Figure 6).

Table 2: Details of Cotton Lepidopteran pests used for bio-assay-

Insect	Description	Details
Helicoverpa armigera	H. armigera	Lab population
Spodoptera exigua	S. exigua	Lab population
Spodoptera litura	S. litura	Lab population
Earias vitella	E. vitella	Lab population
Pectinophora gossypiella	P. gossypiella(Lab)	Lab population
Pectinophora gossypiella	P. gossypiella(Field)	Collected from field and reared in lab
Pectinophora gossypiella	P. gossypiella(Cry1Ac)	Collected from field and selected on Cry1Ac diet

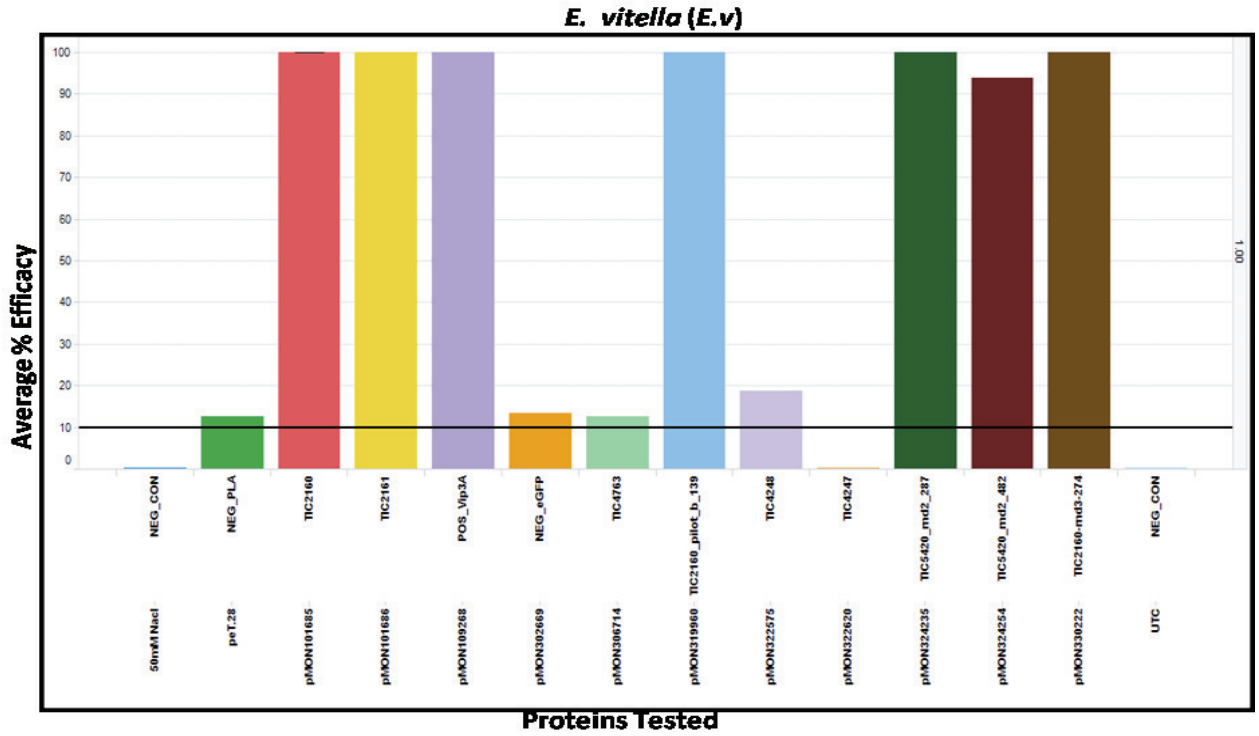
3. Results:

Table 3: Average Percentage Mortality of new proteins expressed in *E. coli* cells against cotton against Lepidopteran insects:

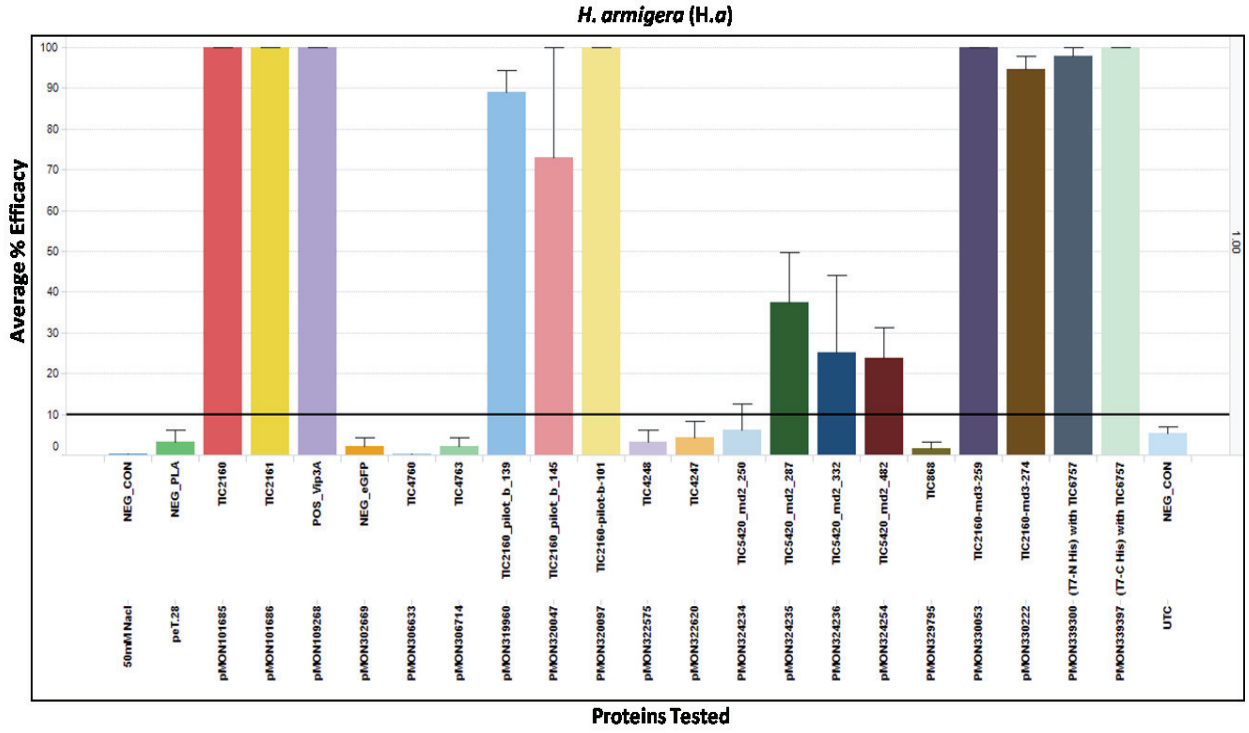
S.No.	pMON	E. vitella	H. armigera	P. gossypiella (Cry1Ac)	P. gossypiella (Field)	P. gossypiella (Lab)	S. exigua	S. litura
1	50mM Nacl	0	0	0	8	5	3	4
2	peT.28	0	3	0	4	9	6	11
3	pMON101685	100	87	42	54	50	32	100
4	pMON101686	100	96	96	98	100	24	100
5	pMON109268	100	100	100	100	100	98	100
6	pMON302669	7	2	2	0	8	0	17
7	PMON306633	#N/A	0	23	34	6	12	52
8	pMON306714	12	2	10	39	19	0	21
9	pMON319960	100	75	53	69	71	27	94
10	PMON320047	#N/A	73	63	85	94	31	98
11	PMON320097	#N/A	94	94	88	94	17	100
12	pMON322575	19	0	4	22	17	0	0
13	pMON322620	0	4	0	15	13	0	6
14	PMON324234	#N/A	6	25	32	69	6	69
15	pMON324235	100	25	24	50	60	28	76
16	PMON324236	#N/A	23	12	39	8	40	19
17	pMON324254	88	20	54	69	58	41	84
18	PMON329795	#N/A	2	100	100	100	100	98
19	PMON330053	#N/A	100	75	89	94	69	100
20	pMON330222	100	92	75	84	68	84	100
21	PMON339300	#N/A	98	23	4	8	100	100
22	PMON339397	#N/A	100	12	2	12	100	100
23	UTC	0	1	2	0	4	3	4

#N/A – data not available

Figure 1: Average percentage efficacy of new proteins expressed in *E. coli* cells tested against cotton pests *E. vitella* and *H. armigera*

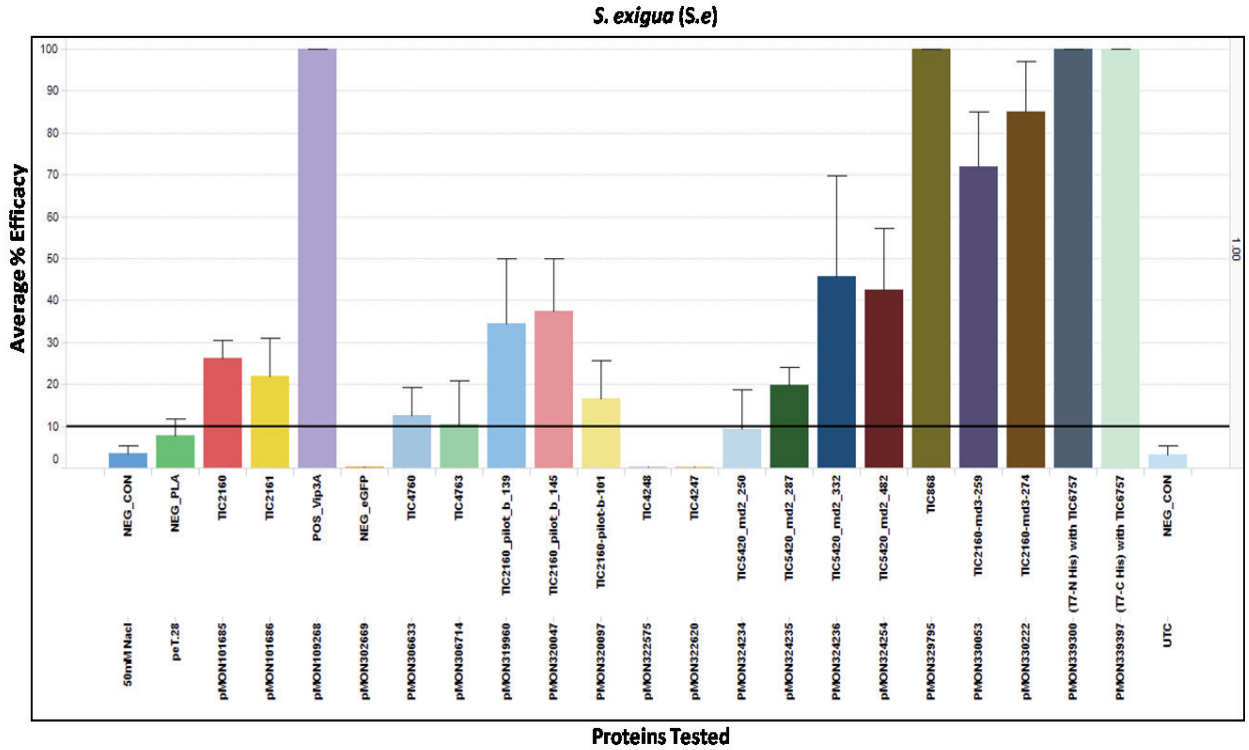


Note: The line across graph at 10% refers to the threshold for contamination. Assays discarded if contamination >10%.

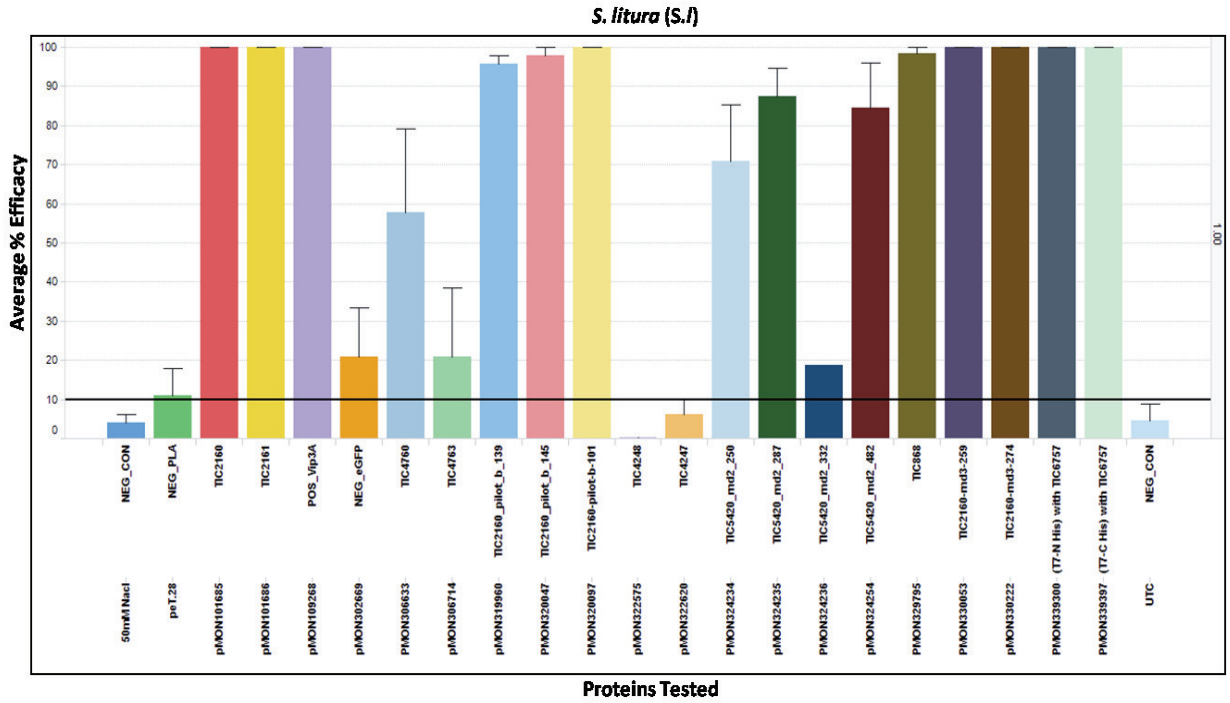


Note: The line across graph at 10% refers to the threshold for contamination. Assays discarded if contamination >10%.

Figure 2: Average percentage efficacy of new proteins expressed in *E. coli* cells tested against cotton pests *S. exigua* and *S. litura*

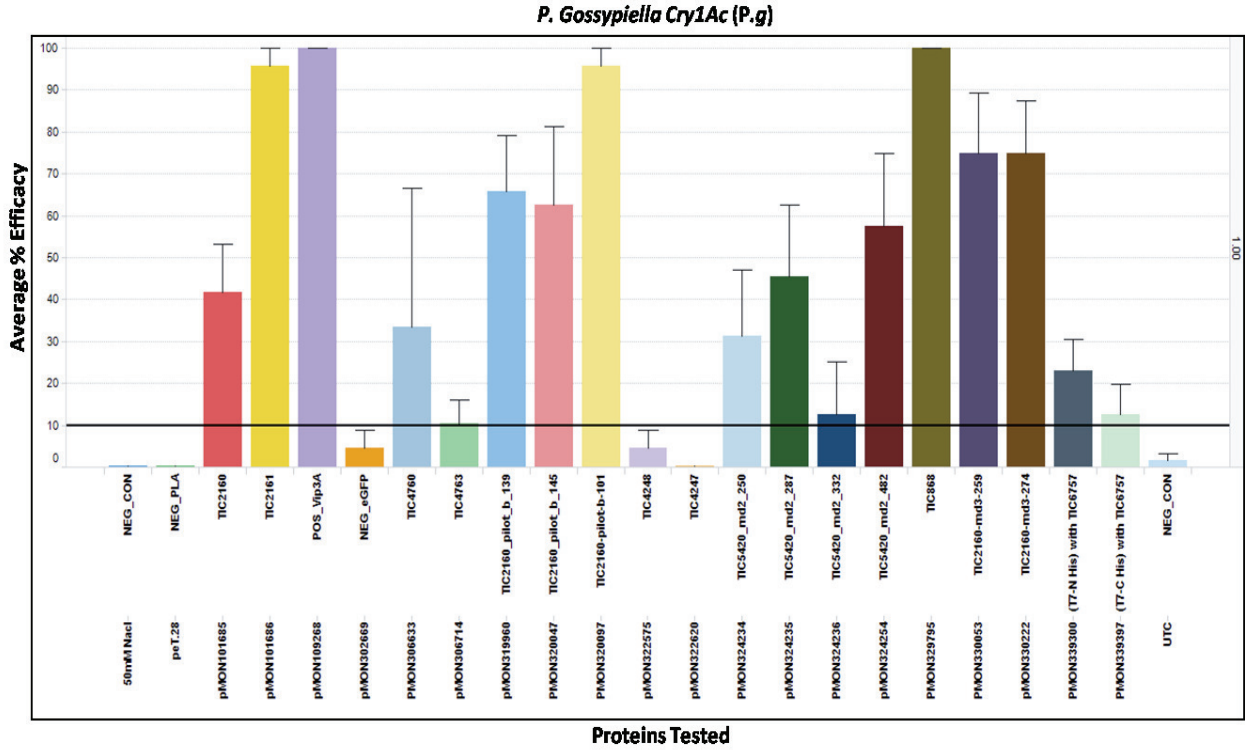


Note: The line across graph at 10% refers to the threshold for contamination. Assays discarded if contamination >10%.

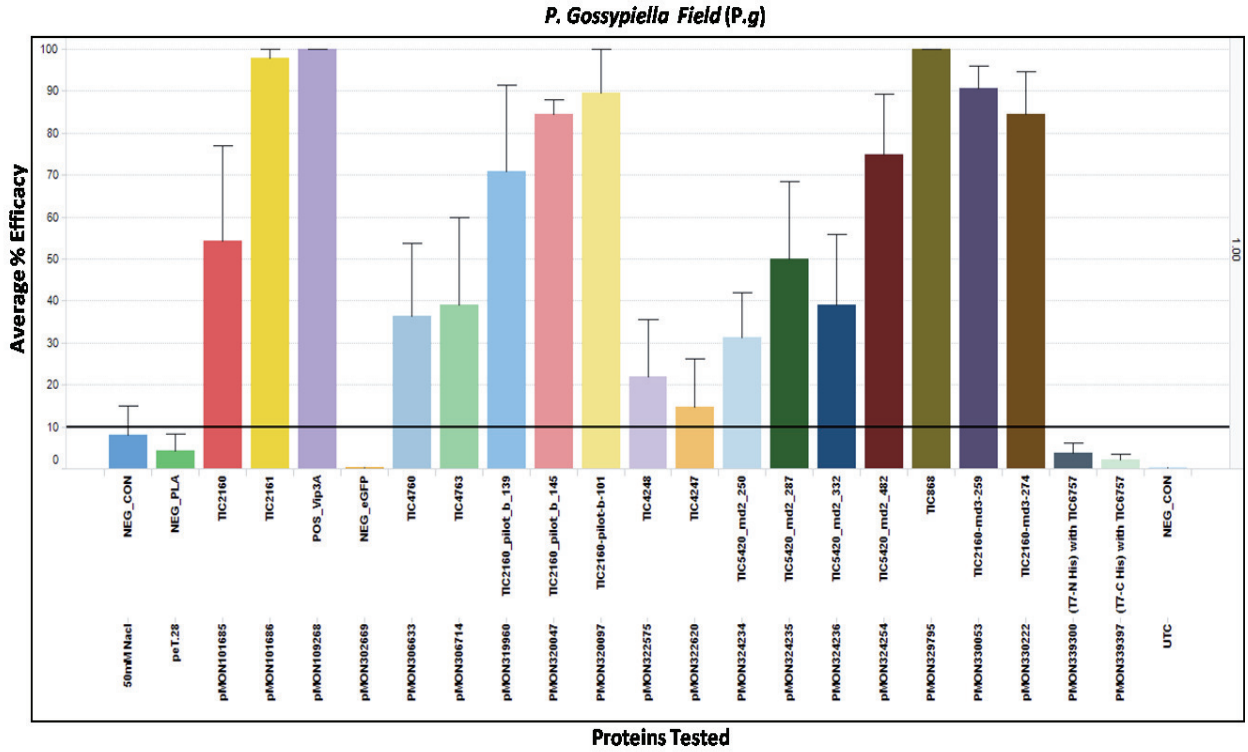


Note: The line across graph at 10% refers to the threshold for contamination. Assays discarded if contamination >10%.

Figure 3: Average percentage efficacy of new proteins expressed in *E. coli* cells tested against cotton pests *P. gossypiella* (Cry1Ac) and *P. gossypiella* (Field)

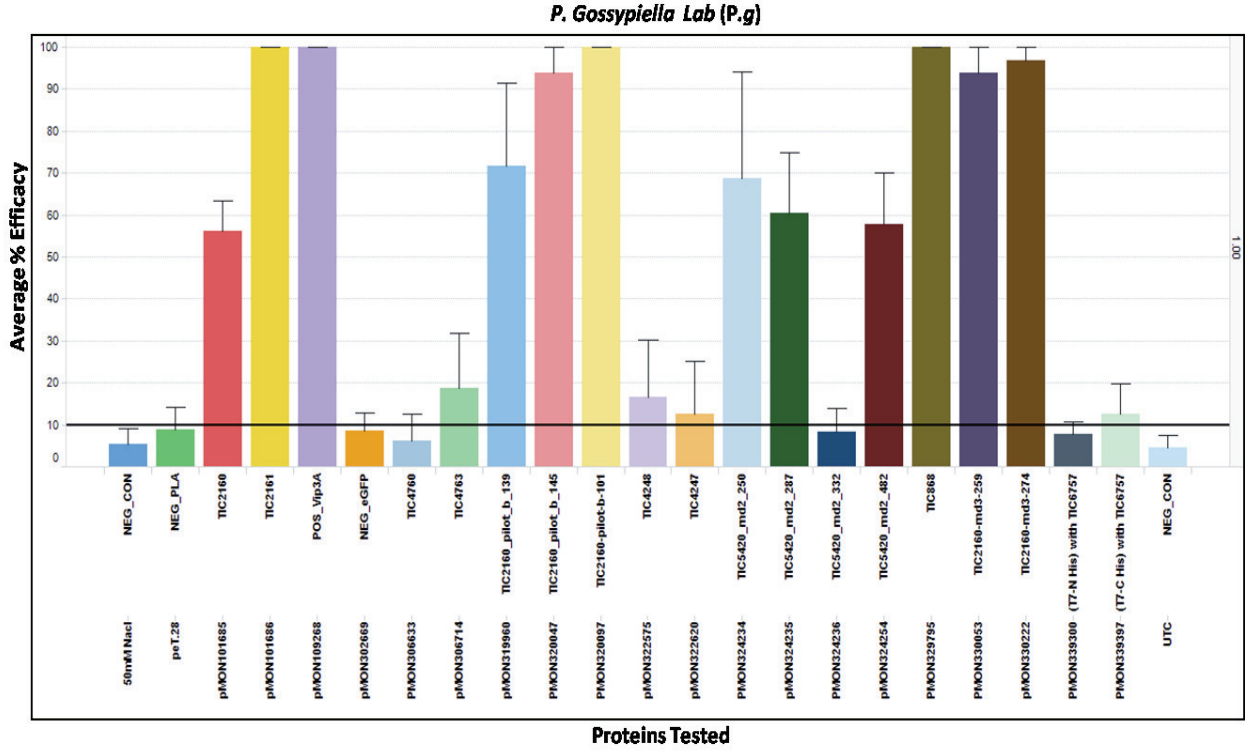


Note: The line across graph at 10% refers to the threshold for contamination. Assays discarded if contamination >10%.



Note: The line across graph at 10% refers to the threshold for contamination. Assays discarded if contamination >10%.

Figure 4: Average percentage efficacy of new proteins expressed in *E. coli* cells tested against cotton pests *P. gossypiella* (Lab)



Note: The line across graph at 10% refers to the threshold for contamination. Assays discarded if contamination >10%.

Figure 5: Heat map showing the average % efficacy of the various plasmids tested across different insect species

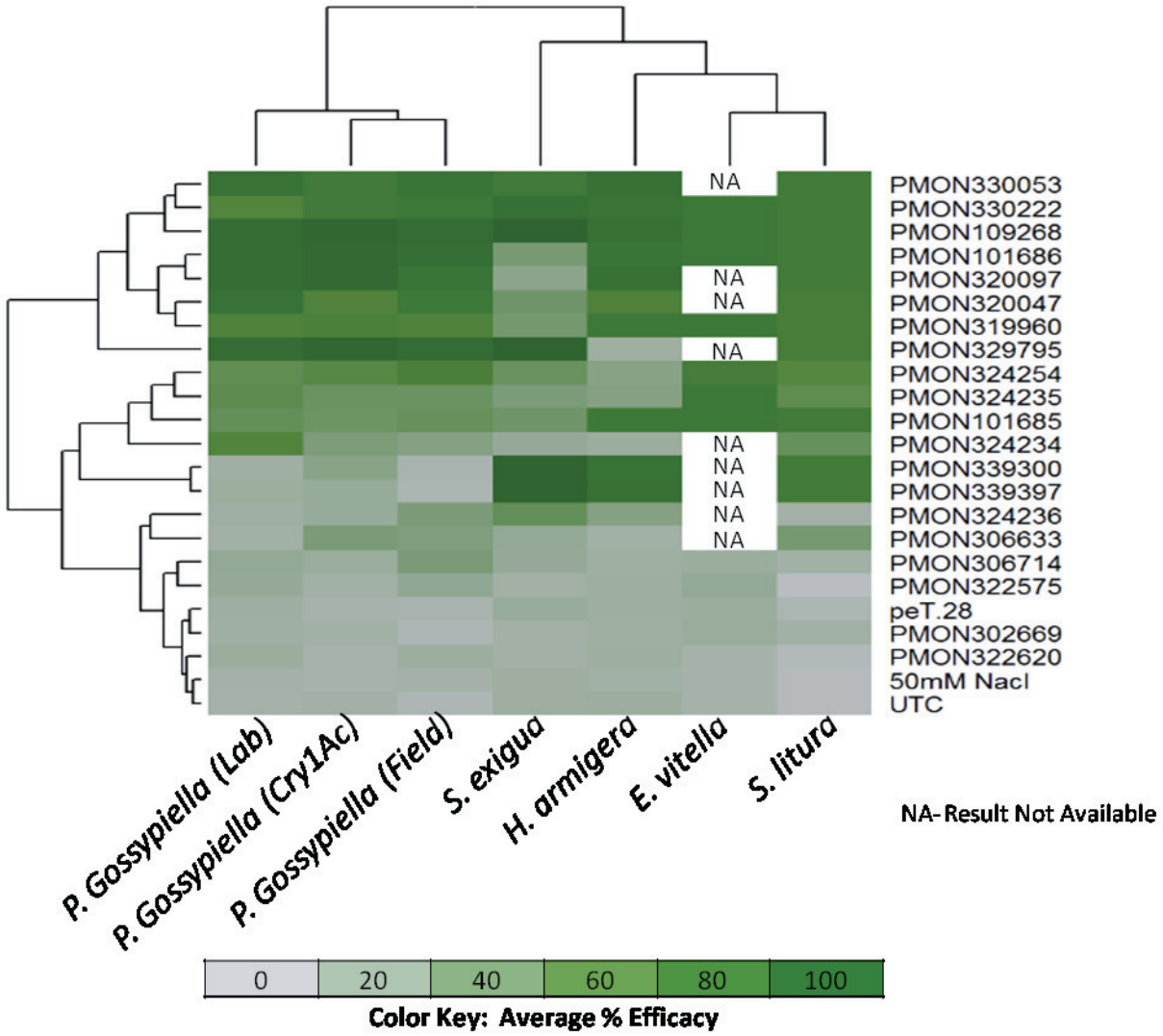
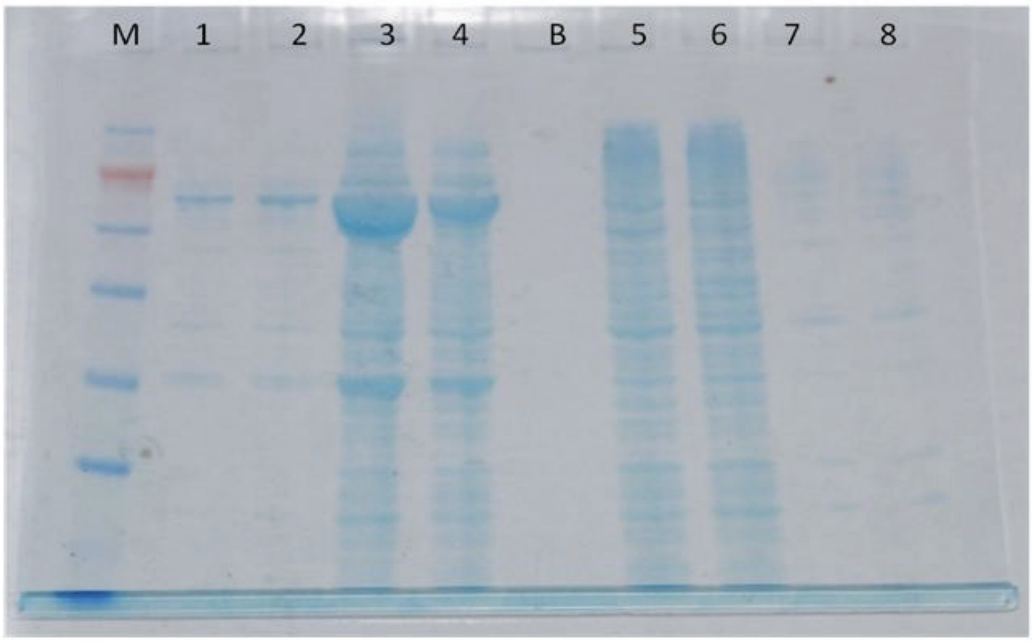


Figure 6: Gel analysis for Protein expression screening



M- SeeBlue® Plus2 pre-stained Standard(Invitrogen)
1- pMON109268 LB+IPTG 1 o/n **T**
2- pMON109268 LB+IPTG 2 o/n **T**
3- pMON109268 AIM 2 o/n **T**
4- pMON109268 AIM 1 o/n **T**
B- Blank
5- pET-28a(+) AIM 1o/n **T**
6- pET-28a(+) AIM 2 o/n **T**
7- pET-28a(+) LB+IPTG 1o/n **T**
8- pET-28a(+) LB+IPTG 2 o/n **T**

Note: T refers to total protein and o/n refers to overnight

4. Conclusion:

Out of the 17 constructs tested using *E. coli* direct bioassay method TIC868 (PMON329795) showed 100 percentage mortality and efficacy at 1X concentration of the crude protein against the tested insects except *H. armigera* (*E. vitella* not tested). Similarly TIC2160 variants (PMON330222, PMON330053, PMON320097, PMON320047) showed 75-95 percentage mortality. Where as TIC6757 (PMON339300 and PMON339397) showed 100 percentage mortality and efficacy for *H. armigera, S. exigua, S. litura* but very less (10-25%) percentage mortality and efficacy for *P. gossypiella*. However constructs PMON322620, PMON302669, PMON322575 and PMON306714 were not effective to any of the tested insect species at the 1x concentration tested (Figure 5).

Gel analysis revealed that there is no induction on null vector (plasmid with out desired gene). In addition AIM media gave good amount of total protein when compare to LB media

induced with IPTG. Time comparison study showed that there were high amount of protein induction upon 48 hrs induction (Figure 6).

5. References:

1. Procedure for Preparation of Semi-synthetic diet for Lepidopteran Insects-BPD-ENT-MET-3238.