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RESEARCH ARTICLE

INTERACTOME ANALYSIS OF PROTEIN KINASES, GERMINATION-RELATED AND HORIZONTALLY TRANSFERRED GENES OF *NOSEMA BOMBYCIS* USING STRING.

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Abstract

Nosema bombycis infects *Bombyx mori* upon spore germination utilizing a characteristic mechanism to invade host cell. The uptake of microsporidian spore is either by spore-host interaction or by endocytosis. The molecular mechanism leading to such interactions is not well elucidated. The expansions of *N. bombycis* genome have acquired many horizontal genes and interact with protein kinases, involved in defense mechanism and cell cycle events. Present study aimed towards understanding these interactions as spore germination being vital process in pebrine infects silkworms through spore endocytosis. Using STRING - the molecular functions of all these proteins and its functional partners in the interactome were analyzed and annotated. Further, the protein-protein interactions network was analyzed to study the functional interaction partners that could decipher the mechanism triggering uptake of spore through endocytosis. A total of 50 proteins including protein kinases, horizontal transfer and germination related genes were studied emphasizing CDC28 activation of VPS34 which inturn mediated the activation of CDC10 leading to spore wall formation. In conclusion, results highlighted the most possible mechanism triggering endocytosis of microsporidia and functional contribution of protein kinases and genes involved in horizontal gene transfer to the *N. bombycis* germination and survival strategy.

Introduction:-

Microsporidia have attracted much attention as they infect a variety of species ranging from protists to mammals[1]. Almost half of the reported genera of microsporidia use insects as primary hosts, and microsporidian infections usually have chronic and sublethal effects on their hosts. *Nosema bombycis*, the first named microsporidia species, is the causative agent of devastating pebrine disease in silkworm, *Bombyx mori*. *N. bombycis* infects silkworms both vertically (from mother to progenitor eggs) and horizontally (transovarially), damaging gut, malphigian tubules, silk glands and fat bodies causing larval inactivation and retarding the larval development and finally leading to silkworm death[2]. With the appropriate external stimuli trigger or direct contact with host cell, *N. bombycis* spore rapidly extrudes polar tube from the anterior end in a process called germination. Germination is the most important

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and foremost step in the infection of silkworm by *N. bombycis*[3]. The invasion into the host cell is by the mechanism of polar tube extrusion[4].

Comparative genomics of *N. bombycis* shows that the genome is astonishingly expanded as compared to the distantly related *N. ceranae* and this large genome size is due to the proliferation of host-derived transposable elements, horizontally transferred genes (HGT) from prokaryotes, and the production of segmental and tandem duplicates[5]. Based on the review of literature, 55 genes were identified to be involved in horizontal gene transfer, among these, 21 HGT genes had unknown function and 34 had predicted gene functions.

Further, protein kinases that form a large group of enzymes which transfer phosphate group from ATP to a number of proteins are involved in the mechanism of signal transduction leading to the *N. bombycis* infection[6]. These protein kinases are generally known to be involved in cell cycle events, cell proliferation, development, metabolism, signal transduction and stimulus to external signals. Genomic kinomes of *N. bombycis* revealed that there are 41 protein-kinases belonging to serine-threonine class[7]. Although there are studies that focused on actions of proteins derived from HGT and also germination related transcriptomics[8], there are no clear studies that depict the involvement of protein kinases in association to germination. The computational studies of physical and functional interactions prediction generates more robust interactome as the interactions integrate to provide completely annotated information. In view of this, understanding the interactome helps in understanding the biological significance of spore invasion, thereby leading to infection. The present study analyzes the functional interactions of acquired horizontal proteins and *N. bombycis* genomic protein kinases with highly expressed proteins during germination.

Methodology:-

The present investigation involves analysis of interaction of *N. bombycis* protein kinases, germination related proteins, horizontal transfer genes and infection mechanism during germination using the STRING software. . Among the fifty proteins, thirteen show specific expression during germination *of N. bombycis* spore, eighteen proteins acquired through horizontal gene transfer and nineteen protein kinases of *N. bombycis.* STRING database[9] provides critical assessment and integration of protein–protein interactions, including direct (physical) as well as indirect (functional) associations. All the proteins were queried in the query multiple search box with the interactions restricted to those available for *Encephalitozoon Cuniculi* as *E. cuniculi* is the closest microsporidian to *N. bombycis* as represented by Frankenhuyzen et al.,[10] and the sequence information is available. The prediction analysis methods including activation, inhibition, binding, co-expression and gene fusion were utilized. The confidence score >0.9 were selected to obtain interaction network representing >90% confidence in the prediction to establish an interactome network representing the *N. bombycis* infection in silkworm leading to pebrine disease.

Results and Discussion:-

A total of fifty proteins were analyzed to predict the protein interactions involved in germination of *N. bombycis* in silkworm (Table 1). Among these, forty proteins showed interactions atleast with one protein, whereas ten proteins did not interact. Fig. 1a represents overall protein-protein interaction network obtained in the present investigation. CDC28 was found to be the most interacting protein followed by Pho85 (ECU08_0230), which are protein kinases. CDC28 showed 19 interactions in the network forming the node 1 and 17 interactions were with other protein kinases and remaining two interactions with proteins expressed during germination. On the other hand, Pho85 showed 18 interactions, which is represented as node 2 (Table 4) and 16 such protein interactions were other protein kinases and two interactions with proteins expressed during germination. These two major nodes indicate protein kinases form crucial back bone for the interaction of horizontal proteins and *N. bombycis* protein kinases and could be implicated in eliciting there by causing the infection upon germination of *N. bombycis* spores. The functional annotation of all the proteins queried for protein-protein interactions utilized in this study is listed in table 1.

Proteins in the proposed network are highly interactive among themselves than for a random set of proteins of similar size that would be expected from the drawn genome. These strong interactions suggest that these proteins are biologically connected to elicit a response upon a specific signal, as a group. However, there was no significant pathway enrichment observed in cellular component and KEGG pathways. *N. bombycis* HGT, germination and protein kinase gene set includes genes encoding basic cellular functions such as transcription, translation, DNA replication and repair, cell cycle control, protein folding/turnover, intracellular trafficking and key enzymes for glycolysis, pentose phosphate pathway, trehalose metabolism and chitin biosynthesis (Table 1).

The mode of infection is either through polar tube intrusion or endocytosis and their protein interactors[3, 4, 11]. In the present study, the potential candidate interactor *i. e*., VPS34 (vacoular protein sorting 34) represented the major node of 18 protein interactors (Fig. 2) and is known to be involved in sporulation. VPS34 is known to initiate the formation of a forespore membrane at each spindle pole body and extends to form the spore envelope, which further requires binding of CDC10 through the Ptdlns(3) [12]. VPS34 converts phospho-inositol to phosphatidylinositol 3-phosphate, the key factor for sporulation and phosphorylates phosphatidylinositol to generate Ptdlns(3)P (Fig. 3). Phosphatidylinositol 3-kinase is also vital for cytoplasm to vacuole transport (Cvt) and autophagy as a part of the autophagy- specific VPS34 PI3-kinase complex I. These proteins are involved in endosome-to-golgi retrograde transport as part of the VPS34 PI3-kinase complex II.

Microsporidia invades host cells in two different ways, the first way of invasion being ejection of spore polar tube and piercing into the host cell in its close proximity. The second mode, host endocytosis of the infective spore but the spore escapes the abjection by endocytic vacuole of the host by discharging its polar tube. However, spore endocytosis mechanism remains unclear and the potential molecular players involved are yet to be elucidated. CDC10 protein or septins are GTPases involved in cytokinesis and spore wall formation[2, 13].

We hypothesize that major interaction of CDC28, binding and thereby activating CDC10 is crucial by which the microsporidia gets the signal for its invasive entry into the host (Fig. 1b). Endocytosis invasion is known to happen in *Encephalitozoon* species. The GO biological placement predicts that CDC10 is localised to the membrane of *N. bombycis*. Septin 7 of *O. colligata* is also known to be localised in its exospore[12]. Alternatively, a surface septin could also facilitate infection by simply helping to keep the parasite in close proximity to the host cell surface. Further, the present interactome also reveals the binding, catalysis and activation of CDC28 by VPS 34 mediates the activation of CDC10. The activation of VPS34 indeed might be by binding of sugars, anions or small molecules (Fig. 3).

N. bombycis intracellular parasitic lifestyle is designed to have highly reduced metabolism instead of energy investing pathways to synthesize basic biological building blocks (e.g. amino acids, sugars, nucleotides, lipids) and cofactors (e.g. ATP, NAD+, NADP+). The presence of one such enzyme like Mannose-1-phosphate guanyl transferase 2 is the key for carbohydrate metabolism and mannosylation of structural and functional proteins in microsporidians. In Mannosylation of polar tube (PTPs) and spore wall proteins plays very important role in the parasitic lifestyle of *E. cuniculi*, possibly as virulence factors reported for several fungal pathogens[14]. However, the present interactome reveals that PTP3 does not interact with Mannose-1-phosphate guanyl transferase 2, indicating its involvement in carbohydrate metabolism rather than mannosylation (Fig. 2). This is best evident (Table 2) with the maximum observed gene count correlated with the functions like phosphorylation and cell cycle processes as far as the biological function was considered. Further, another enzyme, dUTPase is involved in conversion of dUTP to dUMP and pyrophosphate thereby adding on to the ATP stealing mechanism from the host cell. Like many parasites *T. hominis* has lost the ATP-expensive pathways for the *de novo* biosynthesis of inosine 59-phosphate and for uridine mono-phosphate, the starting points for the biosynthesis of purines and pyrimidines for DNA and RNA biosynthesis[15]. Probably, *N. bombycis* might have acquired dUTPase through HGT from bacterium in the predicted manner.

The absence of mitochondria in *N. bombycis* indicates that they require energy for survival in the host. However, it possess several energy synthesizing pathway enzymes which could compensate for the energy synthesis, which further is achieved by stealing ATP from the host cell. Our analysis also includes such energy synthesizing pathway enzymes *viz.*, glucose-1-phosphate isomerase and transketolase based on transcriptome data of Ma et al.,[1]. Glucose-1-phosphate isomerases thus inter converts glucose-6-phosphate to fructose-6-phosphate in glycolysis pathway. This Glucose-6-phosphate can also be utilized by pentose phosphate pathway to synthesize ribulose-6-phosphate and NADPH, which are the key energy molecules that contribute towards the survival of spore in the host cell.

Several studies show the presence of microsporidia hexokinases involvement in glycolytic pathway; but the absence of hexokinase is evident through transcriptome data[8], furthered by distinctive absence of hexokinase activity in *Nosema gryllii*, in which activity of several glycolytic enzymes was detected in isolated pathogen cells[16] . Hexokinase catalyzes the first step in glycolysis and the pentose phosphate pathways. Therefore, microsporidia hexokinase activity within host cells could increase host synthesis of building blocks such as nucleotides, amino acids, and lipids, necessary for the rapid growth of parasites. Hexokinases are known to be present in microsporidians like *T. hominis, V. culicis and V. corneae*[14]. But the absence of hexokinase in our study is

compensated by the presence of Glucose-6-phosphate isomerase and transketolase. One of the interesting feature is *N. bombycis* has acquired phosphoglycerate mutase, which is another important enzyme during glycolysis and pentose phosphate pathways through horizontal gene transfer. In this analysis, there is strong triangular interaction between three enzymes (phosphoglycerate mutase, transketolase and glucose-6-phosphate isomerase) involved in energy synthesis. .

The genes acquired through HGT include enzymes involved in nucleotide synthesis (dUTPase, cytidylate kinase, uridine kinase, thymidine kinase). Further, the pathway analysis based on molecular functions revealed more number of genes participating in nucleotide binding, transferase and kinase activities (Table 3) thus implying its significance leading to elicit the infection upon endocytosis of spore by the host cell. CTP synthetase is the only nucleotide synthesis gene retained in the highly reduced genomes of the microsporidia[14]. The thioredoxin reductase acquired from (bacteria) interacts with thioltransferase, also acquired from bacteria suggesting that these defense proteins are involved in maintaining spore homeostasis and viability. These defense proteins might not interact with any germination expressed proteins. Fine example of role of defense proteins in microsporidia is presence of glutathione reductases and peroxidases, thioredoxin reductases and a superoxide dismutase in *T. hominis* [17]. Thymidine kinase, the pyrimidine salvage pathway enzyme interacts with phosphoglycerate mutase. Thymidine kinase acquistion as HGT is also found in the apicomplexan *Cryptosporidium*, from a bacterium[18].

There is an indication that calcium/calmodulin binding at the spore surface may commence a signaling cascade that causes spore activation[19]. Further, it is of interest to note that *E. cuniculi* genome encodes five calmodulin-dependent kinases in its minimal set of 32 protein kinases which could potentially participate in such process[7]. The clear concept of activation of germination through signaling pathways however has not been elucidated but, indications of calcium/calmodulin binding at the spore surface may commence signaling cascade cause spore activation. We hypothesize that calmodulin dependent protein kinase (ECU03_0630) interacting with ECU03_1290, ECU08_1620, CDC28, ECU08_0230, MRK1, ECU02_0550, ECU01_1320 and CDC5 (Fig. 1c) could be the stimulant for the host cell to initiate endocytosis (Fig. 3), which further requires experimental validation. The present investigation predicts and emphasizes the possible molecular mechanism of spore uptake through endocytosis and also unravels the protein interactors involved in ATP stealing mechanism and defense mechanism of *N. bombycis.*

Table 1:- List of proteins queried in STRING with its functional annotations and the String protein code

Query sequence name	String Protein code	Annotation
Thymidine kinase	TK	Key function in synthesis of DNA
Sugar permease	ECU11_1870	Transporter of β-galactosides
Deoxyuridine 5'triphosphate nucleotidohydrolase	ECU05_0280	Nucleotide metabolism
Thioredoxin reductase	ECU01_0680	Catalyze reduction of thioredoxin
Mevalonate kinase	ECU10_1510	Catalyze the rate-limiting step for the production of isopentenyl pyrophosphate
Extracellular serine proteinase	SPL2	involved in the degradation of proteins
Translation initiation factor E2B gamma subunit	ECU05_1360	mRNA-binding protein involved in translation elongation
2,3-bisphosphoglycerate phosphoglycerate mutase	ECU10_1060	synthesis of 2,3-bisphosphoglycerate
Cytidylate kinase	ECU03_1270	Pyrimidine metabolism
Molybdenum cofactor synthesis protein 3	ECU03_1290	Uncharacterized
Thioltransferase	ECU09_1375	Antioxidant defense system
Microtubule-associated protein 1A	ECU02_0130	Cell cycle protein
Nucleoporin NUP170	ECU06_0470	Nuclear pore complex proteins
Transketolase 1	ECU06_0120	Catalyzes d-xylulose to erythose-4-phosphate
Glutamate NMDA receptor-associated protein 1	ECU07_0290	Ion channel protein
glucose-6-phosphate isomerase	ECU05_0650	Interconverts glucose-6-phosphate and fructose-6-phosphate
protein phosphatase PP2-A regulatory subunit A	ECU09_1490	Uncharacterized

mannose-1-phosphate guanylyltransferase	ECU11_0690	Fructose and mannose metabolism
Nuclear transcription factor Y subunit	ECU10_0260	Uncharacterized
Alanyl-tRNA synthetase	ECU02_1490	Catalyses the attachment of an amino acid to its cognate transfer RNA molecule
Protein peanut	CDC10	Predicted septin
60S ribosomal protein L6	ECU08_1790	Ribosomal protein
MADS domain containing protein	ECU07_1730	Uncharacterized
PTP3	PTP3	Sporoblast-to-spore polar tube biogenesis.
SPL2	SPL2	Degradation of proteins
CK2	CKA1	Casein kinases
PHO85	ECU08_0230	Regulates their perception of and response to stress from the environment
CRK7	CTK1	hyperphosphorylates the C-terminal heptapeptide repeat domain (CTD)
CDC2	CDC28	Required for entry into S-phase and mitosis
GSK	MRK1	Cell cycle regulation
PLK	CDC5	Protein kinase involved in mitotic exit. 'septum-promoting factor'
AUR	IPL1	Chromosome segregation during the later part of each cell cycle.
NEK	ECU11_1500	Cell cycle regulation
WEE	ECU08_1620	Negative regulators of mitosis
CDC7	CDC7-1	Initiation of DNA synthesis
HASPIN	ECU03_0890	Mitotic spindle positioning and mitotic arrest regulation
TTK	MPS1	Involved in the regulation of the onset of mitosis.
PEK	ECU02_0550	Cell cycle regulation
GEK	ECU01_1320	Protein kinase C
NUAK	ECU08_1480	SNF1-related protein kinase
CHK1	CHK1	Serine/threonine-protein kinase
KIN1	KIN1	Serine/threonine protein kinase involved in regulation of exocytosis
MKC	ECU03_0630	Calmodulin-dependent protein kinase
CK1-A	ECU03_0910	Casein kinase 1 homolog (involved in DNA repair)
ATM	ECU03_1100	Phosphatidylinositol-4-kinase catalytic subunit
RIO2	ECU09_1260	Cell cycle regulation

Table 2:- List of genes involved in different aspects of biological process along with the Gene Ontology ID obtained based on the interactome of protein kinases, horizontally transferred genes and germination genes.

Pathway ID	Pathway description	observed gene count
GO.0008150	biological process	21
GO.0009987	cellular process	20
GO.0044238	primary metabolic process	19
GO.0071704	organic substance metabolic process	19
GO.0008152	metabolic process	19
GO.0016310	phosphorylation	18
GO.0006793	phosphorus metabolic process	18
GO.0006796	phosphate-containing compound metabolic process	18
GO.0044237	cellular metabolic process	18
GO.0044763	single-organism cellular process	16
GO.0044699	single-organism process	16
GO.0043170	macromolecule metabolic process	16
GO.0019538	protein metabolic process	15
GO.0044260	cellular macromolecule metabolic process	15

GO.0006468	protein phosphorylation	14
GO.0006464	cellular protein modification process	14
GO.0043412	macromolecule modification	14
GO.0044267	cellular protein metabolic process	14
GO.0007049	cell cycle	9
GO.0000278	mitotic cell cycle	7
GO.0022402	cell cycle process	7
GO.1903047	mitotic cell cycle process	7
GO.0051301	cell division	7
GO.0000280	nuclear division	6
GO.0007067	mitotic nuclear division	6
GO.0048285	organelle fission	6
GO.1902589	single-organism organelle organization	6
GO.0006996	organelle organization	6
GO.0016043	cellular component organization	6
GO.0071840	cellular component organization or biogenesis	6
GO.0050794	regulation of cellular process	5
GO.0051726	regulation of cell cycle	4
GO.0007059	chromosome segregation	2

Table 3:- List of genes involved in different aspects of molecular processes obtained based on interactome of protein kinases, horizontally transferred genes and germination genes indicating nucleotide binding activites.

pathway ID	pathway description	observed gene count
GO.0003674	molecular_function	20
GO.0003824	catalytic activity	19
GO.0001882	nucleoside binding	18
GO.0001883	purine nucleoside binding	18
GO.0017076	purine nucleotide binding	18
GO.0032549	ribonucleoside binding	18
GO.0032550	purine ribonucleoside binding	18
GO.0032553	ribonucleotide binding	18
GO.0032555	purine ribonucleotide binding	18
GO.0035639	purine ribonucleoside triphosphate binding	18
GO.0097367	carbohydrate derivative binding	18
GO.0043168	anion binding	18
GO.0000166	nucleotide binding	18
GO.1901265	nucleoside phosphate binding	18
GO.0036094	small molecule binding	18
GO.0043167	ion binding	18
GO.0097159	organic cyclic compound binding	18
GO.1901363	heterocyclic compound binding	18
GO.0005488	binding	18
GO.0016301	kinase activity	17
GO.0016772	transferring phosphorus-containing groups	17
GO.0016740	transferase activity	17
GO.0005524	ATP binding	17
GO.0030554	adenyl nucleotide binding	17
GO.0032559	adenyl ribonucleotide binding	17
GO.0016773	phosphotransferase activity, alcohol group as acceptor	15
GO.0004672	protein kinase activity	14
GO.0004674	protein serine/threonine kinase activity	14
GO.0004693	cyclin-dependent protein serine/threonine kinase activity	3
GO.0019205	nucleobase-containing compound kinase activity	2

Table 4:- The genes represented in the two major nodes of the interactome

Node 1	Node 2
CDC10, CDC28, CDC5, CDC7-2, CHK1, CKA1, CTK1, ECU01_0680, ECU01_1320, ECU03_0630, ECU03_0890, ECU03_0910, ECU03_1100, ECU03_1290, ECU05_0650, ECU05_1360, ECU07_0290, ECU08_0230, ECU08_1480, ECU08_1620, ECU08_1790, ECU09_1260, ECU09_1375, ECU09_1490, ECU10_0260, ECU10_1060, ECU11_1500, IPL1, KIN1, MRK1, TK	CDC10, CDC28, CDC5, CDC7-2, CHK1, CKA1, CTK1, ECU02_0550, ECU02_1490, ECU03_0630, ECU03_0910, ECU03_1290, ECU05_0280, ECU05_0650, ECU06_0120, ECU07_0290, ECU08_0230, ECU08_1480, ECU08_1620, ECU08_1790, ECU09_1260, ECU09_1375, ECU10_1060, ECU11_0690, ECU11_1500, IPL1, KIN1, MPS1, MRK1, VPS34

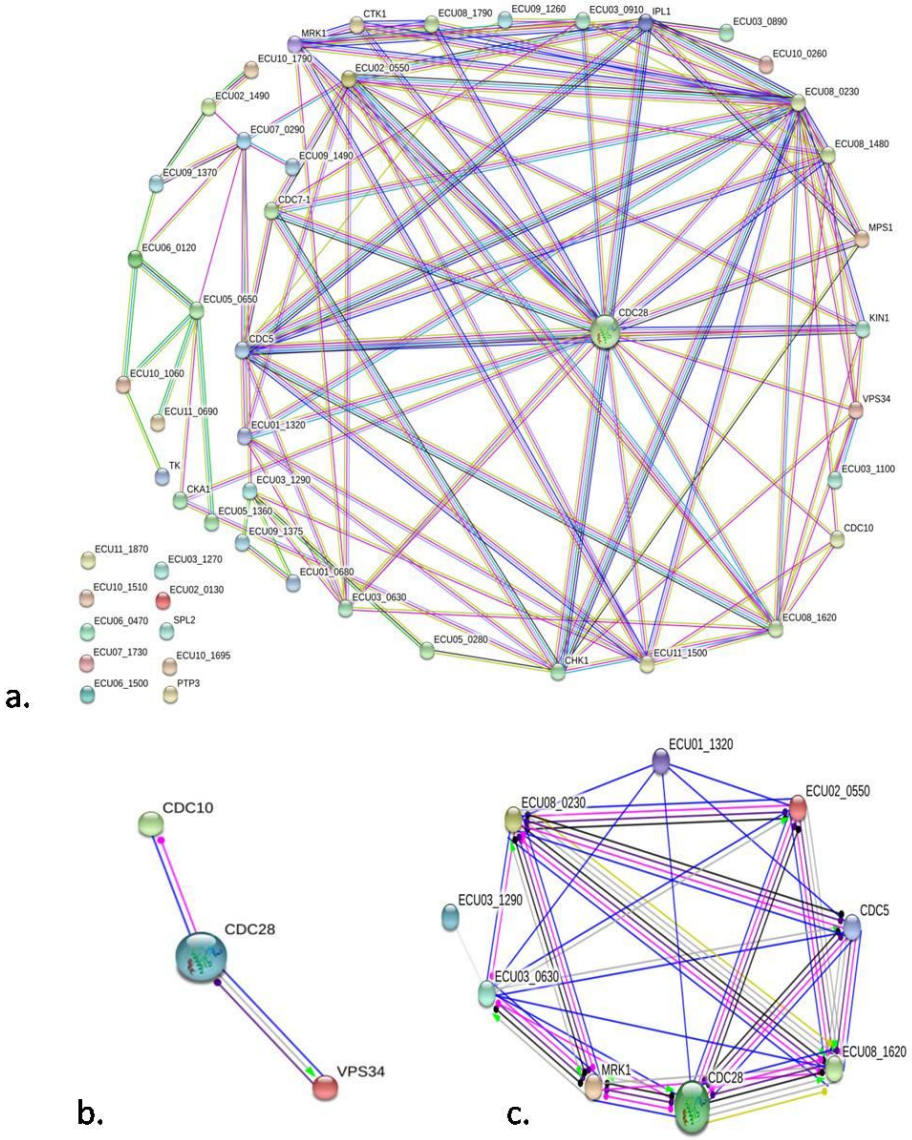

Figure 1:- The interactome of protein kinases, horizontally transferred genes, germination related genes of *Nosema bombycis* using STRING. a) Overall interaction b) Specific interaction of CDC28, VPS34 and CDC10; c) Interactome representing proteins interacting with calmodulin dependent protein kinases, ECU03_0630. (Blue line indicates known interactions from curated database, magenta-known experimentally determined interaction; green–predicted interactions of gene neighborhood; red - predicted interactions of gene fusions; dark blue - predicted interactions of gene co-occurrence; light green – text mining; black –co-expression and purple –protein homology).

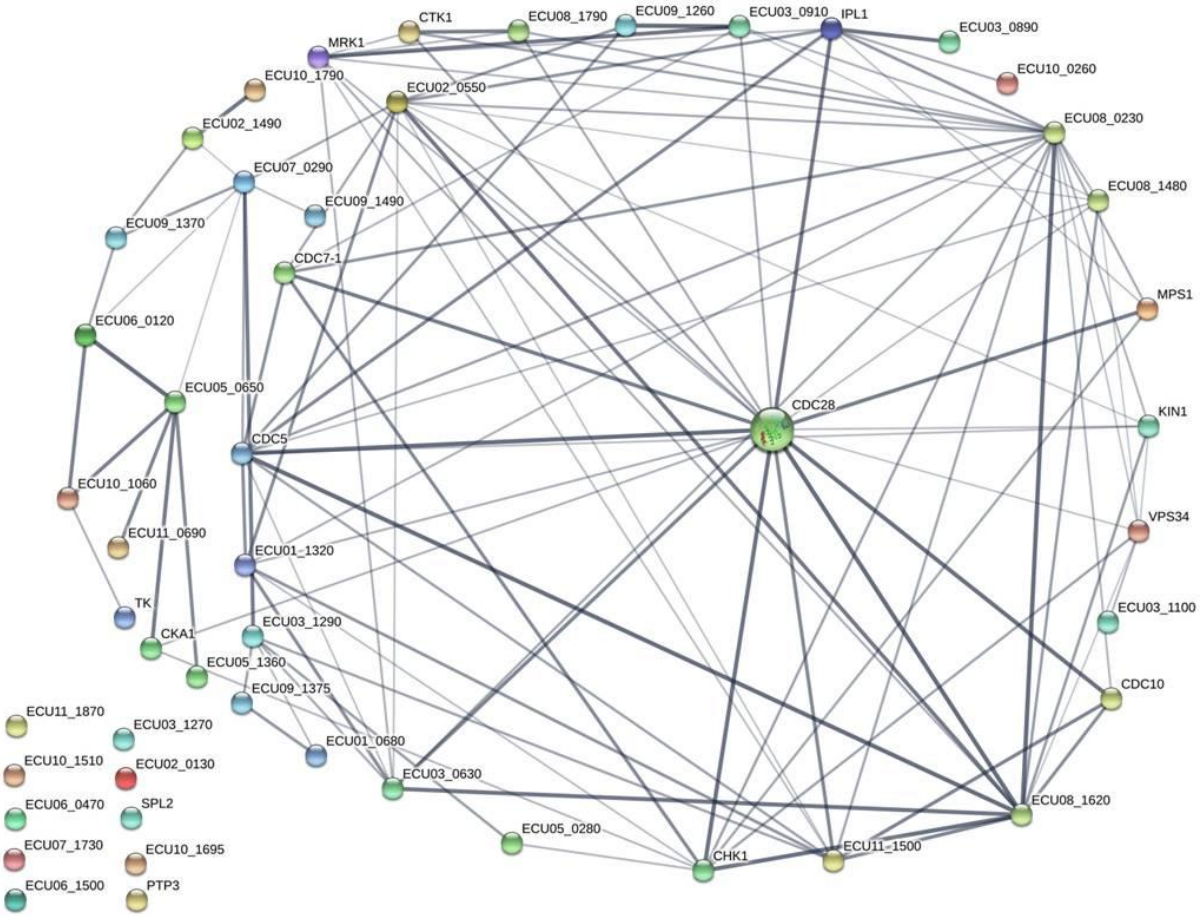

Figure 2:- Protein interactome of protein kinases, horizontally transferred genes, germination related genes of *N. bombycis* represented by the different intensity of the interactions based on data support. The circles outside the network indicated non-interacting partners.

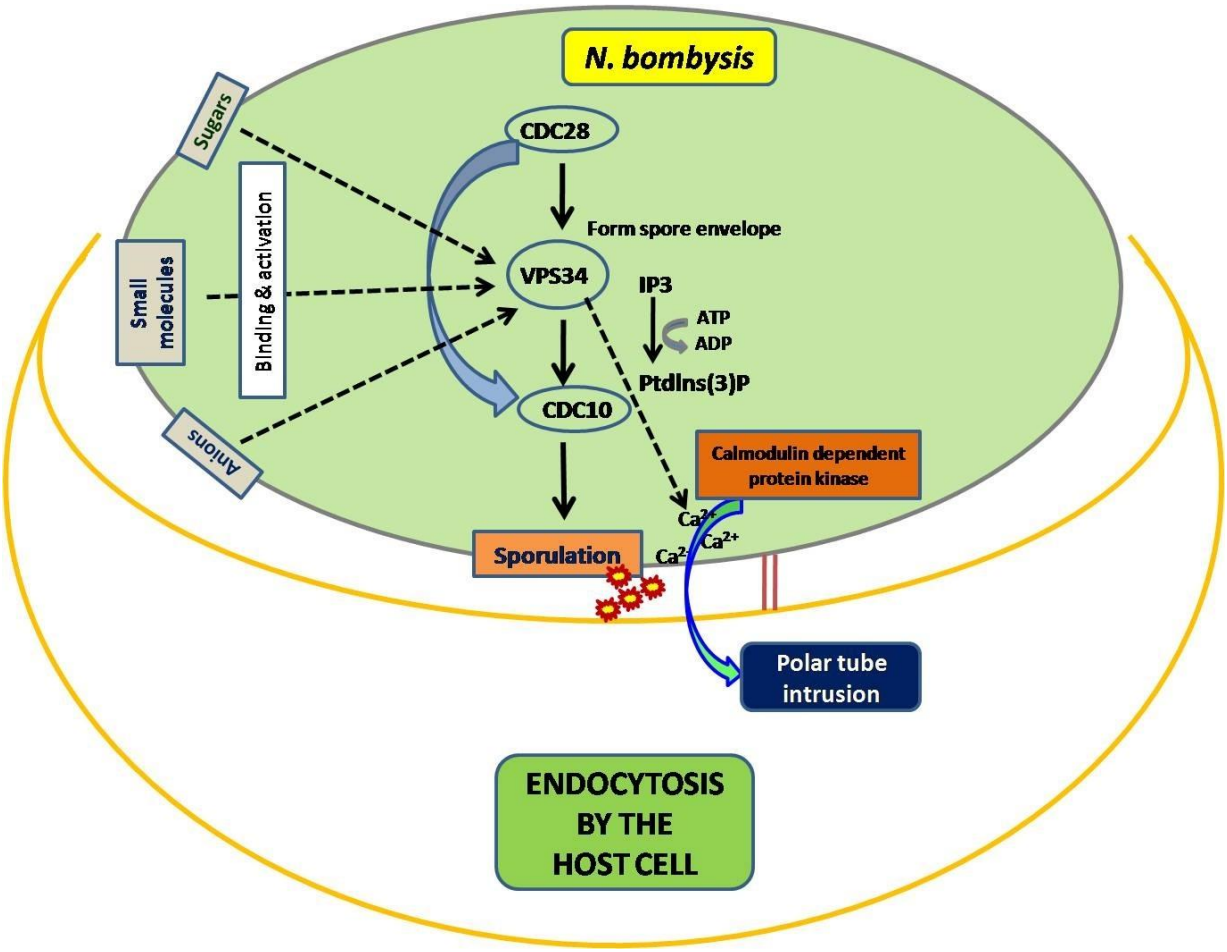

Figure 3:- Pathway representing the mode of interaction leading to endocytosis of *N. bombycis* based on protein-protein interacting network.

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