

A DEGENERATE PCR SCREEN OF EUROSTA SOLIDAGINIS TO FIND GENETIC HOMOLOGUES TO GROEL AND CSPA

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Abstract

Eurosta solidaginis, the goldenrod gall fly, becomes freeze tolerant in early autumn. Many physiological changes occur to confer freeze tolerance, including the syntheses of cryoprotectant carbohydrates, changes in membrane lipid composition, increases in larval mass, and induction of ice nucleation (Morrissy and Baust 1976). Little is known about genes involved in the freeze tolerance capacity of *Eurosta solidaginis*. Two genes in *Escherichia coli*, *cspA* and *groEL*, are known to be involved in responses to temperature changes (Kandror and Goldberg, Ueguchi and Ito 1992). A degenerate polymerase chain reaction was carried out to determine if homologues to *cspA* and *groEL* exist in *Eurosta solidaginis*, using primers designed from a BLAST search of the gene sequences against *Drosophila melanogaster*. A fragment was amplified with the *cspA* primers, indicating the existence of a potential homologue. Further studies will be conducted to determine the sequence of this fragment.

Introduction

Eurosta solidaginis, commonly known as the goldenrod gall fly, is widely distributed throughout the United States and Canada (Uhler 1951). The life cycle of *E. solidaginis* begins when an egg is laid on the stem of the goldenrod plant. The eggs hatch in June, and the larvae subsequently bore into the stem of the goldenrod and develop. The larvae cause the plant tissue to form a gall, where in they proceed through 3 larval instar phases. The first two instars occur during June and early July, and the third develops in late July and early August. Beginning in early autumn the late third instars are unique in that they are freeze tolerant and remain so through winter (Bennett and Lee 1997). The larvae develop into pupae in late May and the fly emerges from the goldenrod plant in early June. It lives for 8 to 9 days, during which time it mates, and lays eggs that will subsequently form galls (Uhler 1951).

Changes associated with freeze tolerance in *E. solidaginis* include the synthesis of glycerol (Baust 1986), and the later synthesis of sorbitol and trehalose in response to low temperatures (Baust and Lee 1982). These carbohydrates are part of the multicomponent cryoprotective system (Baust 1986). Physiological changes include changes in membrane lipid composition (Bennett, et al. 1997) and increases in larval mass (Morrissy and Baust 1976 and Lee et al 1995). Calcium phosphate spherules, located in the malpighian tubules, may induce ice nucleation in the larvae, an adaptation that causes the larvae to freeze at relatively high subzero temperatures (Joanisse and Storey 1996). Freezing is accompanied by decreased activities of antioxidant enzymes, indicating that increased oxidative stress does not occur during freeze-thaw cycles (Joanisse and Storey 1996).

In addition to *E. solidaginis*, other freeze tolerant organisms include some species of plants, frogs, turtles, lizards, insects, and bacteria (Storey and Storey 1988). Studies of freeze tolerant species have led to the discovery of genes that may be involved in the freeze-thaw process, but little is known of genes in *Eurosta* that may aid in freeze tolerance. Two genes, *groEL* and *cspA*, that have been identified with temperature response in *Escherichia coli* are the focus of this study.

The *groEL* gene codes for the heat shock protein GroEL, and is located in the cellular cytosol (Langer et al. 1992). GroEL belongs to Hsp60 family and is composed of 14 identical 57-kDa subunits forming two symmetrical rings, each having a central cavity (Braig et al. 1994). Heat shock proteins are part of a family of proteins known as the chaperonins (Ellis and Van der Vies 1991). Chaperonins prevent protein aggregation, help catalyze refolding, and promote the selective degradation of heat damaged polypeptides (Hendrick et al 1993). They function by protecting polypeptides from irreversible denaturation induced by such stresses as high temperatures, ethanol, oxygen radicals, and heavy metals (Hendrick, et al 1993). GroEL interacts with another heat shock protein, GroES, (Hartl et al. 1992) to promote the proper assembly of a variety of proteins (Georgopoulos et al. 1973). GroEL binds substrate protein within its cavity and folds the polypeptides through ATP dependent cycles of protein release and rebinding (Martin et al. 1991).

The exact role of GroEL in temperature sensitive cellular processes is unclear. An earlier study showed that at least one of the gene products from the GroEL/GroES operon was necessary for growth at both 42°C and 17°C. Thus, the GroEL and GroES proteins are likely involved in fundamental cellular processes at stress temperatures (Fayet et al 1989). Another study suggested that GroEL overproduction suppresses the cold sensitivity of *sec* mutations (Danese et al 1995). The products of *sec* genes are required for protein translocation across the cytoplasmic membrane of *E. coli* (Bieker et al 1990). The most recent study contradicted previous findings by showing that a trigger factor (TF) was induced at cold temperatures, while the overproduction of GroEL/GroES reduced cell viability at low temperatures (Kandror and Goldberg 1997).

A second gene in *E. coli* associated with adjustment to temperature is the cold shock *cspA* gene. This gene is dramatically induced during the cold shock response and comprises more than 10 percent of the total protein synthesis in cells experiencing cold shock. CspA is 70 amino acids long and has a molecular mass of 7.4 kDa (Goldstein et al. 1990). More than 50 homologues of CspA have been identified in bacteria (Yamanaka et al 1998). A family of 17 cold shock proteins in *E. coli* alone have been discovered through gel electrophoresis (Jones et al. 1995), but some of the family members may be functionally redundant. The amino acid sequence of CspA is 43 percent identical to the cold shock domain of the eukaryotic Y-box protein family. These proteins bind to DNA and affect gene transcription, DNA replication, and DNA repair (Bouvet et al. 1995).

During a shift to low temperatures in *E. coli*, CspA is induced rapidly (up to 200-fold) and promotes transcription of other cold shock regions (Kandror and Goldberg 1997). High levels of CspA have been proposed to facilitate translation at low temperatures. The CspA mRNA is extremely unstable at 37°C, but it is dramatically stabilized upon cold shock, indicating that CspA expression at low temperatures may be regulated post transcriptionally (Fang 1997). CspA is necessary for transcription of mRNA at low temperatures because of its ability to destabilize secondary structures in mRNA (Jiang et al. 1997). The most recent study found that CspA functions as a transcription anti-terminator at rho-independent terminators in

vitro and in vivo and suggested that this occurs by preventing the formation of secondary structures in the nascent RNA (Bae et al. 2000).

Some homology between the *Drosophila melanogaster* genome and the sequences of groEL and cspA suggested that similar homology could exist between the two genes and *Eurosta solidaginis*, another member of Dipteren order of insects. If found in *Eurosta solidaginis*, the cspA and genes could be associated with its freeze tolerance.

Materials and Methods

Larvae of *Eurosta solidaginis* were collected from Colgate University property in Hamilton, NY, on November 8, 2000.

The sequences of GroEL and CspA were entered into a BLAST search in the National Institutes of Health database (www.ncbi.nlm.nih.gov:80/blast/blast.cgi) to search for homology in the *Drosophila melanogaster* genome. Primers for a degenerate polymerase chain reaction were designed using the BLAST results that showed areas of homology between the two sequences and the *Drosophila* genome. Primers for the degenerate polymerase chain reaction screen are shown in table 1.

Table 1: Primers used for degenerate PCR screen

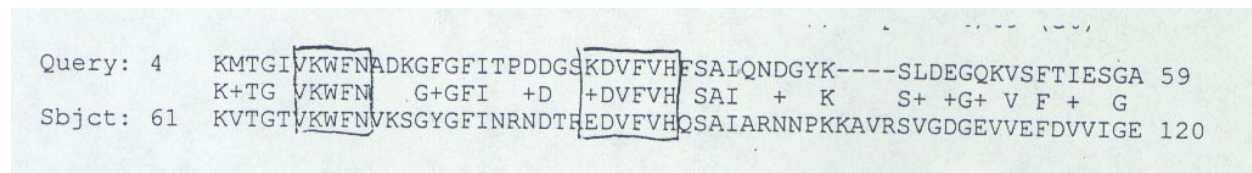
Gene	Forward Primer	Reverse Primer
GroEL	5'GTSCTGGCCGAYGCCCT3	5'CAGKCCRTCCTTBGTRAT3'
CspA	5'G TSAAGTGGTTCAA YGC3'	5'YTCCTRCASAAGCASGTA3'

The degenerate PCR screen was carried out with dilutions of the whole *Eurosta solidaginis* larvae to test for the presence of GroEL and CspA homologues. Temperature cycles for PCR were as follows: denaturation at 95°C for one minute, primer annealing at 50°C for one minute, and primer extension at 72°C for two minutes. These cycles were carried out 30 times. The PCR products were run on a 1.8% agarose gel to determine whether amplification occurred.

Results

The BLAST search between cspA and *Drosophila melanogaster* showed two regions of homology five and six amino acids long, flanking a fragment twenty-five amino acids long. The results are shown in figure 1.

Figure 1: BLAST results cspA vs. *Drosophila melanogaster*



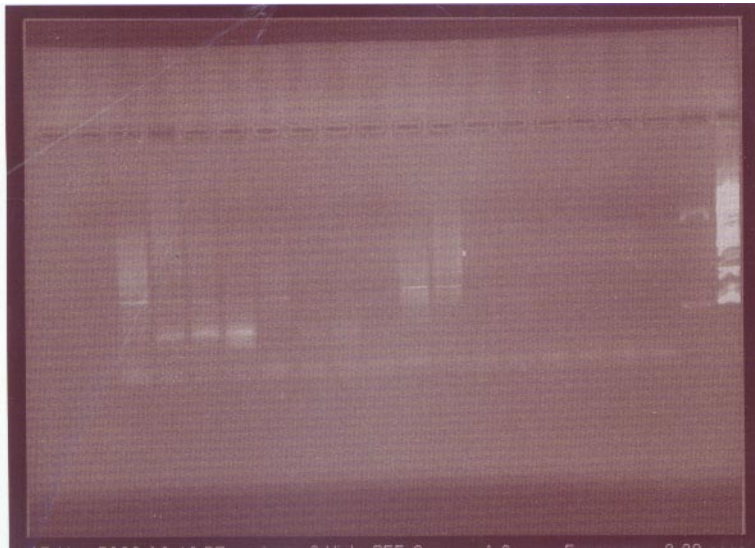
The BLAST search between cspA and *Drosophila melanogaster* showed regions of homology, both six amino acids long, flanking a region thirty-three amino acids long. The results are shown in Figure 2.

Figure 2: BLAST results groEL vs. *Drosophila melanogaster*

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Query: 3 AKDVKFGNDARVKMLRGVNLADAVKVTLGPKGRNVVLDKSFAGPTITKDGVSVAREIEL 62
          AKDV+FG + R ML+GV+VLADAV VT+GPKGRNV+++S+G+P ITKDGV+VA+ IEL
Sbjct: 23 AKDVRFGPEVRAMMLQGVDVLADAVAVTMGPKGRNVII EQSWGSPK ITKDGVTVAKSIEL 82
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There was no amplification with the groEL primers in the degenerate PCR screen of *Eurosta solidaginis*. However, an approximately seventy-five base pair fragment was amplified using the cspA primers in the 1:100, 1:1000, and 1:10,000 dilutions of the whole larvae. These results are shown in Figure 3.

Figure 3: 1.8% Agarose Gel of degenerate PCR products
Lane 18: basepair ladder, Lane 17: positive control, Lanes 2-9 cspA products, Lanes 10-17, groEL products. Amplification occurred in lanes 4 (1:100 dilution), 5 (1:1000 dilution), and 6 (1:10,000 dilution).



Discussion

In the degenerate PCR screen, it was determined that 50°C was the optimum annealing temperature, since no amplification occurred with the cspA primers at 60°C. The most successful dilution of the whole larvae for use in the PCR reaction was 1:10,000 because less amplification occurred at 1:100 and 1:1000 dilutions. The other bands that did not migrate as far as the PCR amplifications were likely transfer RNA fragments, as they were observed in the groEL lanes.

No amplification occurred with the groEL primers. This could have occurred because the annealing temperature was not optimal. In some reactions primer dimers were amplified, so the primer design might not have been ideal (results not shown). Additionally, A groEL gene homologue may not exist in *Eurosta solidaginis*. It was postulated that a GroEL homologue could exist in *Eurosta solidaginis* because previous studies showed that it was involved in maintaining fundamental cell processes at high and low temperatures (Ueguchi and Ito 1992). Although these previous studies indicated a role of groEL in response to cold temperatures,

recent studies have shown that GroEL may decrease the viability of *E. coli* cells exposed to cold temperatures (Kandror and Goldberg 1997). Since *Eurosta solidaginis* is a freeze tolerant organism, it would not be advantageous to have a heat shock gene that reduces viability at cold temperatures.

The amplification that occurred with the *cspA* primers was the expected fragment size of about seventy-five basepairs. The entire size of the gene, 225 basepairs, was not expected because we designed primers for a smaller fragment. This result indicates that a *cspA* homologue may exist in *Eurosta solidaginis*. The sequence of this fragment needs to be deduced to confirm the existence of a homologue. If confirmed, this *cspA* homologue may be an integral part of mRNA transcription in *Eurosta solidaginis* as it is in *E. coli*. It could be induced dramatically in the freeze response of *E. solidaginis*.

Future studies should attempt to confirm the *cspA* sequence in the amplified fragment from *E. solidaginis* and attempt to discern whether the gene is activated after October 2 to promote freeze tolerance. If the gene is activated exclusively during the period during which *E. solidaginis* is freeze tolerant, it likely has a vital role in the survival of *E. solidaginis* during the winter months.

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