Development of an Embryonic Lethality System in Mediterranean Fruit Fly *Ceratitis capitata*

M. F. SCHETELIG[1], C. HORN[2], A. M. HANDLER[3] and E. A. WIMMER[1]

[1]*Department of Developmental Biology, Johann-Friedrich Blumenbach Institute of Zoology and Anthropology, Georg-August-University Göttingen, Justus-von-Liebig-Weg-11, 37077 Göttingen, Germany*
[2]*Structural and Computational Biology Unit, EMBL Heidelberg, Meyerhofstraße 1, 69117 Heidelberg, Germany*
[3]*USDA/ARS, Center for Medical, Agricultural, and Veterinary Entomology, Gainesville, FL 32608, Florida, USA*

ABSTRACT The Mediterranean fruit fly *Ceratitis capitata* (Wiedemann) is one of the world´s most destructive insect pests, costing farmers billions of dollars annually. Improved biological strategies are needed to increase the efficacy of area-wide integrated pest management (AW-IPM) programmes. Transgenic methodology could enhance and widen the applicability of the sterile insect technique (SIT) as a component of AW-IPM programmes and a transgenic approach to sterilize insects with an embryonic lethal transgene combination instead of conventional radiation was successfully tested in *Drosophila melanogaster* Meigen. This system is currently being transferred to *C. capitata*, in order to test its feasibility in this species and compare its effectiveness to radiation sterilization. Therefore two strategies are being followed: (1) direct transfer of the constructs used in *D. melanogaster* and assessment of their functionality in *C. capitata*, and (2) isolation of genes active during early embryonic development of *C. capitata* for use in an embryonic lethality system with endogenous components. If proven functional and effective in *C. capitata*, such a system might be transferable to other insect pests.

KEY WORDS *Ceratitis capitata*, cellularization, cDNA-subtraction, conditional embryonic lethality, sterile insect technique (SIT), insect transgenesis

1. Introduction

The Mediterranean fruit fly *Ceratitis capitata* (Wiedemann) is one of the worlds' most important pests of fruits and vegetables, attacking more than 260 different fruits, vegetables and nuts. The direct damage caused by feeding larvae and the quarantine status of this insect have major impacts on many agricultural economies. Biological approaches to insect pest management offer alternatives to insecticidal control. The sterile insect technique (SIT) is a powerful component of area-wide integrated pest management (AW-IPM) programmes to suppress or eliminate populations of economically important pest species by the mass-release of radiation-sterilized insects (Knipling 1955). However, the use of radiation for sterilizing insects does have some adverse effects on their competitiveness which in turn reduces the efficiency of the technique (Cayol et al. 1999, Calkins and Parker 2005).

Recently a transgene-based embryonic sterility system was successfully established in *Drosophila melanogaster* Meigen (Horn

M.J.B. Vreysen, A.S. Robinson and J. Hendrichs (eds.), Area-Wide Control of Insect Pests, 85–93.

and Wimmer 2003) and this system may provide an alternative to the use of radiation in AW-IPM programmes integrating the SIT. The aim of the studies reported here was to establish and evaluate such a system in *C. capitata*.

2. Transgene-Based Sterility System in *Drosophila melanogaster*

A novel transgenic approach was developed to induce sterility without interfering with gametogenesis or with other larval and adult stages of the insect life cycle. Sterility is based on the transmission of a transgene combination that causes dominant embryo-specific lethality in subsequent progeny. This dominant lethality is suppressible by additives in the larval diet, thereby enabling rearing of such strains. This should allow the generation of competitive sterile insects that can transfer competitive sperm (Horn and Wimmer 2003), carrying the transgene, to wild females. The embryos produced by the females will carry the dominant transgene and, in the absence of the additives, the embryos will die. For the effector gene causing organismal lethality, a hyperactive allele of the pro-apoptotic gene *head involution defective* (*hid*) was chosen, which induces cell death when expressed ectopically (Grether et al. 1995). To avoid down regulation of HID by developmental signalling pathways, the phosphoacceptor-site mutant allele hid^{Ala5} (Bergmann et al. 1998) was used. To limit the effect of the transgenes to the embryonic stage, enhancer-promoters of genes that are expressed at high levels but are specific to the cellularization stage were used. In *D. melanogaster* the genes *serendipity α (sry α)* and *nullo* encode structural components of the microfilament network that are specifically required for blastoderm cellularization (Ibnsouda et al. 1993, Postner and Wieschaus 1994). To establish conditional embryonic lethality, a suppressible binary expression system based on the tetracycline controlled transactivator tTA (Gossen and Bujard 1992) was employed. By adding tetracycline to the larval diet the transgene activity can be suppressed. In *D. melanogaster*, hid^{Ala5} specifically causes embryonic lethality when driven by tTA under the control of the enhancer-promoter from a cellularization gene, and can be suppressed by tetracycline provided maternally to the egg (Horn and Wimmer 2003). Due to the inhibition of the tTA-DNA binding by tetracycline, the tTA protein functions as a switch to discriminate restrictive from permissive conditions. Under restrictive conditions (without tetracycline) 99.9% of *D. melanogaster* embryos that inherited one copy of the transgene combination were killed. Under permissive conditions (with tetracycline), lethality was suppressed which allowed the continuous generation of large numbers of transgenic insects (Fig. 1). Strains homozygous for the transgene combination can be propagated on tetracycline-containing food. Males from these *D. melanogaster* strains are competitive in laboratory mating assays and transmit the transgene combination, which causes dominant embryonic lethality in offspring. Thus the transgene-based suppressible embryo-specific lethality system may enable competitive sterile insects to be produced without irradiation and is therefore of interest for improving conventional SIT and widening its applicability.

3. Transfer of the Transgene Embryonic Lethality System to *Ceratitis capitata*

3.1. Direct Transfer of the Drosophila*-Used Transgenes to* Ceratitis capitata

For fast and easy transfer of the embryo-specific lethality system from the model organism to the pest species, direct use of the *D. melanogaster* transformation constructs in *C. capitata* was pursued (Horn and Wimmer 2003). This involved taking the driver construct pBac{3xP3-EYFP;>>s1-tTA>>} (Horn and Wimmer 2003) and digesting it with *Bgl*II. The fragment, which contains the *tTA* gene under control of the -276:+45 *sry α* promoter region, was inserted into the *Bgl*II site of the transformation vector pB[PUb-

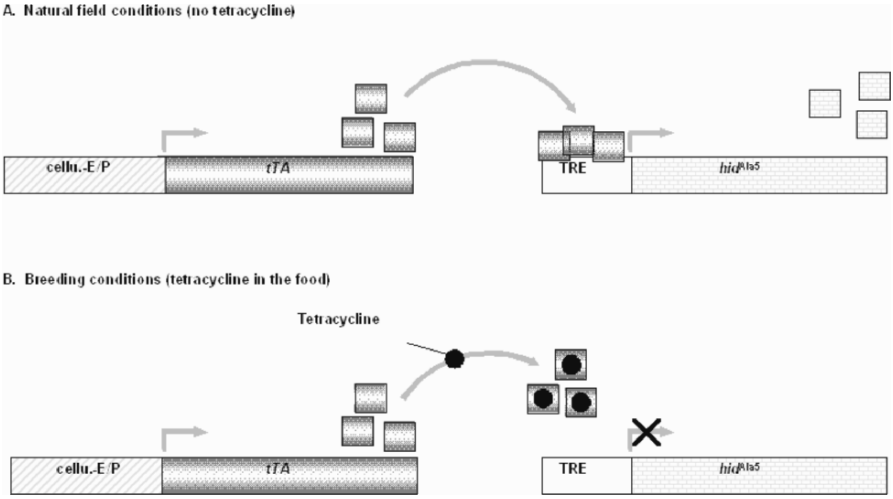

Figure 1. *Binary expression system for conditional embryonic lethality. Enhancer-promoters of the cellularization genes (cellu.-E/P)* sry α *or* nullo *were selected, which mediate gene expression exclusively during early embryogenesis. The tetracycline controlled transactivator (tTA) is based on a bacterial-viral fusion protein and mediates gene expression by binding to a* tTA-response element (TRE)*. The main advantage of this system is that targeted gene expression can be controlled by the food supplement tetracycline. (a) Under natural field conditions there is no tetracycline and the tTA proteins bind to the* TRE *leading to the expression of* hid^{Ala5}*, which causes lethality. Induction of lethality is limited to the early cellularization stage of embryogenesis because the genes* sry α *and* nullo*, are exclusively expressed at the cellularization stage. (b) Under laboratory rearing conditions the larval diet contains tetracycline, which binds to tTA. Tetracycline-bound tTA cannot bind to the* TRE *thereby suppressing* hid^{Ala5} *expression and allowing all progeny to survive.*

DsRed1] (Handler and Harrell 2001). For germ-line transformation this construct was injected together with a helper plasmid into the posterior of early *C. capitata* embryos, resulting in four transgenic lines. These are currently being analysed for transgene-mediated *tTA* expression.

The results will determine: (1) whether the complex interaction between enhancers and promoters of stage-specifically expressed genes (Blackwood and Kadonaga 1998) is the same or different between *D. melanogaster* and *C. capitata,* and (2) whether a *D. melanogaster* promoter can act as an adequate alternative to an endogenous *C. capitata* promoter to enable high expression rates to be obtained.

3.2. Embryonic Cellularization Genes from Ceratitis capitata

3.2.1. Searching for sry α *and* nullo *by Degenerate Polymerase Chain Reaction (PCR)*

According to fossil records, the phylogenetic distance between *Drosophila* spp. and *Ceratitis* spp. can be estimated to be around 100 million years (Naumann 1994). Since the cellularization genes *sry α* and *nullo* are fast evolving even within the Drosophilidae (Ibnsouda et al. 1993, Hunter et al. 2002), it might be challenging for the *D. melanogaster* constructs to function in a more distantly related species such as *C. capitata*. Therefore, we started to isolate cellularization genes *sry α* and *nullo* in *C. capitata*.

To obtain specific DNA sequences of *C. capitata* homologues of the genes *nullo* and *sry α*, mRNA from 0-48 hour embryo collections was isolated and translated into double-stranded cDNA for later PCRs. Degenerate primers were designed on the basis of amino acid sequence comparisons between known drosophilid *nullo* and *sry α* proteins (Ibnsouda et al. 1998, Hunter et al. 2002) under the following conditions: primer length between 18-29 base pairs and a maximum of 64 permutations. Using the cDNA collection and the degenerate primers, gradient PCRs were carried out (annealing temperature: gradient from 39°C to 50°C) with all suitable primer combinations. If possible, nested PCRs were performed after the primary gradient PCRs for a more selective amplification and a reduction of background. As a control, gradient PCRs were carried out with only one of the degenerate primers to check whether these already lead to non-specific amplifications. The DNA fragments of possibly interesting bands were cut out of an agarose gel, purified (QiaEX II Gel Extraction Kit, Qiagen, Hilden), ligated into the vector pCRII (TA Cloning Kit Dual Promoter (pCRII), Invitrogen) and transformed. The DNA clones were sequenced and analysed by "basic local alignment search tool" (BLAST) algorithms (Altschul et al. 1997) as well as *in situ* hybridizations to whole mount *C. capitata* embryos.

Unfortunately none of the BLAST hits matched the *nullo* or *sry α* genes from drosophilids. Also none of the *in situ* hybridizations with probes from sequences with no BLAST hits gave expression patterns comparable to *D. melanogaster nullo* or *sry α*. Thus the cellularization-specific genes could not be obtained by this degenerate PCR approach based on sequence similarities to drosophilid genes. One reason for this might be the fast evolution of developmental genes in drosophilids (Schmid and Tautz 1997).

3.2.2. Cellularization in Ceratitis capitata

Because *nullo* and *sry α* homologues from *C. capitata* could not be isolated by PCR with degenerate primers, blastoderm-specifically expressed genes were isolated in an independent experiment. For this purpose, we first determined the time window of cellularization in *C. capitata* and this knowledge was used to select differentially expressed genes by cDNA subtractions (3.2.3.).

To determine the time window of cellularization, embryos were fixed at one hour intervals after oviposition followed by immunofluorescence staining of cell membranes and nuclei. For comparison, the same staining was done on *D. melanogaster* whose embryonic development lasts 22 hours at 25°C and whose cellularization takes place between 2 hours 10 minutes and 2 hours 40 minutes after oviposition. In contrast to *D. melanogaster*, embryonic development in *C. capitata* takes 48 hours and cellularization takes place later and for a longer period from nine to 12 hours after oviposition (Fig. 2). In *C. capitata*, the typical elongation of the nuclei could not be observed during the slow phase of cellularization as described for *D. melanogaster* (Lecuit and Wieschaus 2000).

3.2.3. Enrichment of Cellularization-Specific Gene Transcripts by cDNA-Subtraction

In *D. melanogaster* cellularization genes are highly and exclusively expressed during the superficial cleavage of insect embryos (Postner and Wieschaus 1994, Lecuit and Wieschaus 2002). Thus isolating one or more of these genes and particularly their promoters in *C. capitata* would allow a *C. capitata*-specific embryonic lethality system to be generated. With knowledge of the cellularization time window (3.2.2.) a stage-specific screening was performed. Since strongly expressed genes, which exist during all stages of embryogenesis, would prevent a successful and effective cDNA screen for cellularization-specific genes, a cDNA subtraction approach was used (Diatchenko et al. 1996) for the selective isolation of genes, which are specifically expressed during *C. capitata* cellularization.

Using the cDNA transcripts isolated from the cellularization stage those cDNA transcripts, which are also present in other embry-

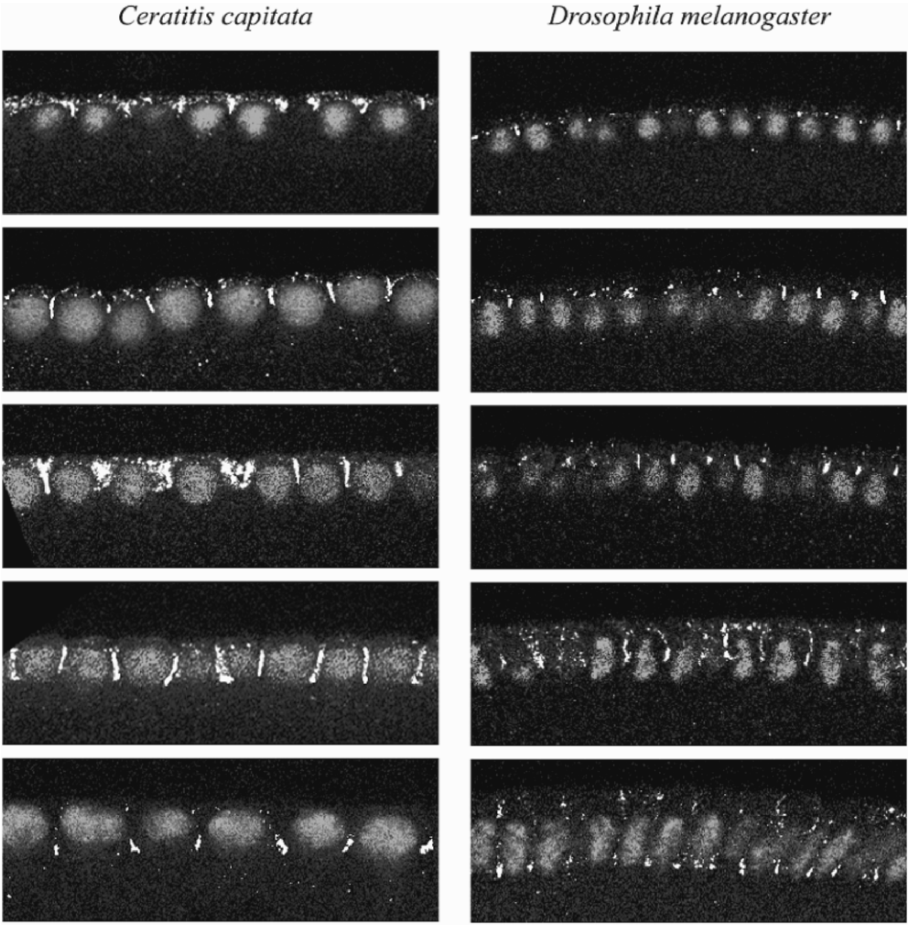

Figure 2. *Superficial cleavage during insect development. Comparison of the cellularization of (left side)* Ceratitis capitata *and (right side)* Drosophila melanogaster *embryos. The immunofluorescence staining with primary armadillo antibody and secondary Alexa488-marked antibody shows the invagination of the cell membrane (bright white stripes between the large gray nuclei). Nuclei are stained with propidiumiodide (large gray balls). In* C. capitata*, cellularization takes places between nine hours (upper, left panel) and 12 hours (lower, left panel) after oviposition. In* D. melanogaster*, cellularization takes places between two hours and ten minutes (upper, right panel) and two hours and 40 minutes (lower, right panel) after oviposition. Panels between represent intermediate stages of cellularization in chronological (vertical) order.*

onic developmental stages, were subtracted. Two subtractions were carried out: (1) a [0-6 hours + 15-21 hours] double-stranded cDNA collection from a double-stranded cDNA collection of cellularization stages (9-12 hours), and (2) a [0-6 hours + 15-48 hours] double-stranded cDNA collection from a double-stranded cDNA collection of a widened "cellularization" time window (7.30-12.30 hours).

The second subtraction was performed because only 4% of the isolated genes in the first subtraction were identified as cellulariza-

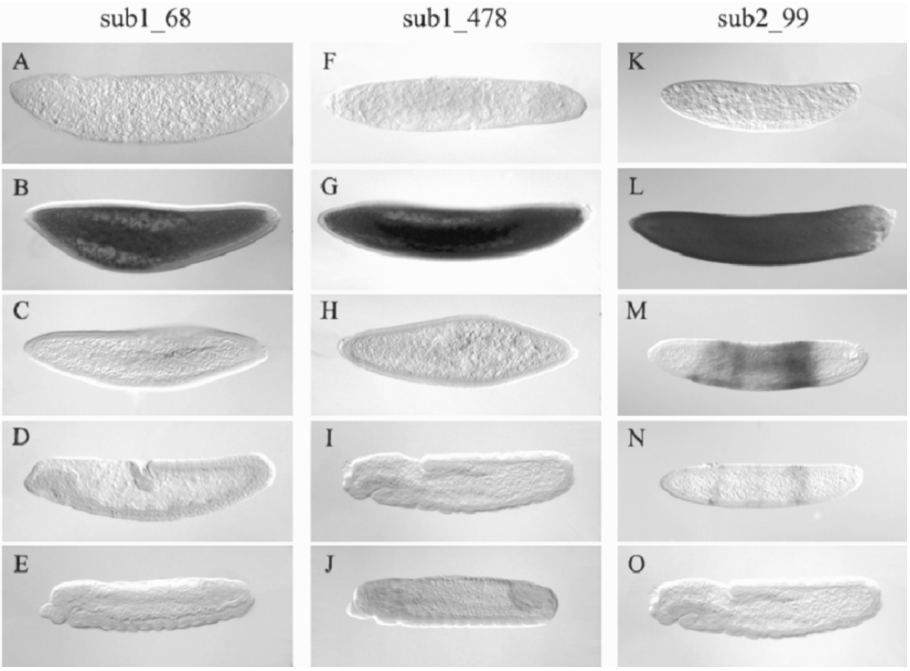

Figure 3. *Cellularization-specific gene expression patterns 1.* Ceratitis capitata *gene sequences* ***sub1_68*** *and* ***sub1_478.*** *(a) and (f) preblastodermal embryo without expression, (b) and (g) strong expression at the onset of cellularization, (c) and (h) very weak expression at the end of the cellularization, (d) and (i) no expression during gastrulation, (e) and (j) no expression during after germ band retraction.* ***Sub2_99*** *(k) preblastodermal embryo without expression, (l) strong expression at the onset of cellularization, (m) strong expression restricted to the centre of the embryo and lacking at the anterior and posterior pole during the slow phase of cellularization, (n) weak expression reduced to three stripes in the centre of the embryo at the fast phase of cellularization, and (o) no expression during and after germ band elongation.*

tion specific by *in situ* hybridizations and BLAST searches. In addition many housekeeping genes had been amplified. The second subtraction conditions were improved by using a widened cellularization time window to make sure that cellularization sequences, produced at earlier time points and also responsible for cellularization, could be identified. Furthermore, the subtracted cDNA pool was expanded to 48 hours to improve the exclusion of non-differentially expressed genes. This increased the efficiency to ~12%. PCR products from these subtractions were agarose gel purified, ligated and transformed into the vector pCRII (for details see 3.2.1). Transformants were pre-selected by restriction enzyme digest patterns and their plasmids then isolated and sequenced. The DNA clones obtained were analysed by *in situ* hybridizations to whole mount *C. capitata* embryos.

From 720 transformants (subtraction 1 (sub1): 550; subtraction 2 (sub2): 170), putative identical clones were identified by enzyme restrictions and 106 probably different clones sequenced (sub1: 45; sub2: 61). Six of the 106 clones were expressed exclusively during cellularization of *C. capitata* (sub1: two; sub2: four; Figs. 3 and 4). Additional three clones were highly expressed during cellularization, but their expression was not

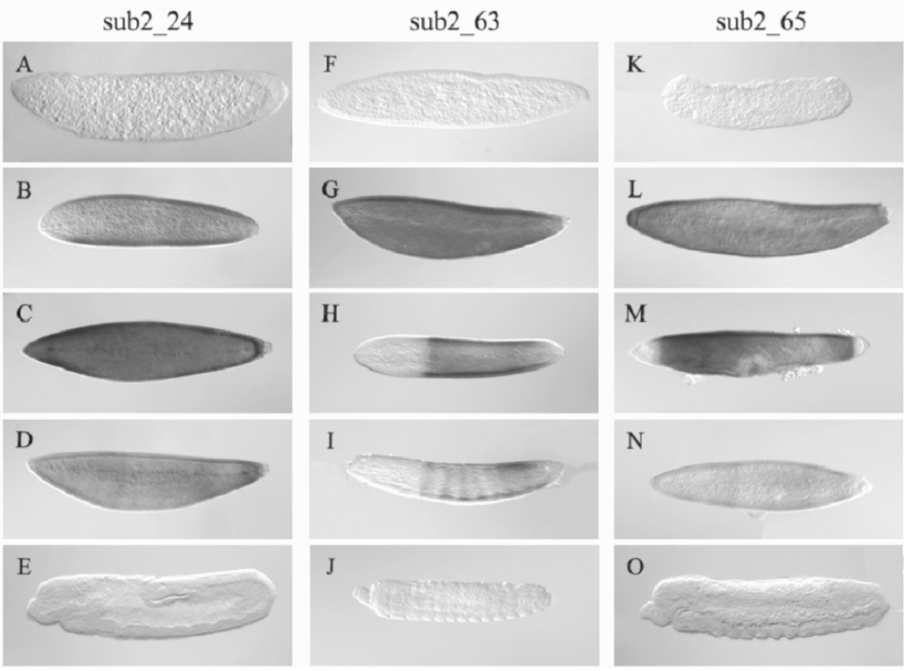

Figure 4. *Cellularization-specific gene expression patterns 2.* Ceratitis capitata *gene sequences* ***sub2_24****. (a) preblastodermal embryo without expression, (b) strong expression at the onset of cellularization, (c) very strong expression during the slow phase of cellularization, (d) reduced expression during the fast phase of cellularization, (e) no expression during germ band retraction.* ***sub2_63*** *(f) preblastodermal embryo without expression, (g) strong expression at the onset of cellularization, (h) strong expression exclusive of the posterior and anterior pole in the slow phase of cellularization, (i) weak expression ending up in stripes exclusive of the posterior and anterior pole in the fast phase of cellularization, (j) no expression after germ band retraction.* ***sub2_65 (k)*** *preblastodermal embryo without expression, (l) strong expression at the onset of cellularization in the whole embryo, (m) very strong expression exclusive of the anterior and posterior pole during the slow phase of cellularization, (n) weak expression reduced to broad stripes at the fast phase of cellularization, and (o) no expression after germ band retraction.*

restricted to this stage (data not shown).

4. Conclusions

A transgene-based embryonic lethality system established in *D. melanogaster* is being evaluated following injection of the driver construct of the binary expression system into *C. capitata* embryos. It will be interesting to determine whether the *sry α* promoter from *D. melanogaster* is also active in *C. capitata* as well as to what extent and in which stages it leads to expression.

To search for cellularization-specific genes in *C. capitata* by cDNA subtraction, the cellularization time window of *C. capitata* embryogenesis was first determined. Six cellularization-specifically expressed candidate genes were isolated by the cDNA subtraction screen. Current work involves searching for the promoter/enhancer regions of these genes by inverse PCR of genomic DNA for use in a

C. capitata specific transgene-based embryonic lethality system.

Once the promoters/enhancers are available, a transgenic sterility system for *C. capitata* will be constructed and its fitness and competitiveness compared to flies sterilized by radiation.

5. Methods

Secondary antibodies (Jackson Immunoresearch) were obtained commercially. The anti-Armadillo antibody (mAb N2 7A1) (Peifer et al. 1994) was obtained from the Developmental Studies Hybridoma Bank (University of Iowa). Antibody stainings were performed as described by MacDonald and Struhl (1986). For the cDNA subtraction the Clontech PCR-Select cDNA Subtraction Kit (BD Biosciences, Heidelberg) was used. The RNA probes for *in situ* hybridization were made with DIG-RNA-labelling Kit (Roche, Mannheim) and hybridizations were performed as described in Davis et al. (2001).

6. Acknowledgements

This work is supported by the Robert Bosch Foundation (EAW) within the programme "International Research into the Development of Sustainable Agriculture and Forestry" and the USDA National Research Initiative Competitive Grants Program (AMH).

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