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New Genetic Tools for Improving SIT in *Ceratitis capitata*: Embryonic Lethality and Sperm Marking

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ABSTRACT: Environment friendly sterile insect technique (SIT) is being applied effectively as a component of area-wide integrated pest management (AW-IPM) for *Ceratitis capitata* since 1970s. Nevertheless improved biological strategies are needed to increase the efficacy of AW-IPM. Transgenic approaches should increase and widen the applicability of such programmes to different pest species. In this respect two major strategies are followed: First an approach to cause sterility was designed without interfering with spermatogenesis to maintain males and their sperm as competitive as possible. We followed a strategy, which is based on the expression of a lethal factor under the control of a promoter that is active at early blastoderm stages. The system employs the ectopic expression of a hyperactive proapoptotic gene that causes embryo-specific lethality when driven by the tetracycline-controlled transactivator tTA under the regulation of a cellularization gene enhancer/promoter. The system has been tested successfully in *Drosophila melanogaster* (Horn & Wimmer 2003). We tried the direct transfer of the *Drosophila* system to *Ceratitis capitata* by injecting the respective constructs that carry *Drosophila*-derived promoters. Unfortunately, the cellularization specific promoters from *Drosophila* seem not functional in *Ceratitis*. Therefore, the corresponding enhancers/promoters from *Ceratitis* were isolated and subsequently the tTA was brought independently under the control of each enhancer/promoter region. These constructs were injected in *Ceratitis* for further evaluation. Second, we have engineered a medfly strain carrying a sperm marking system. This strain carries two fluorescent markers. One (turboGFP) marker is under the control of the spermatogenesis specific *β2-tubulin* promoter from *Ceratitis* and is therefore sperm specifically expressed. The second (DsRed) is under the control of the polyubiquitin promoter of *Drosophila*. Released males from this strain could be distinguished from wildtype males in the monitoring process. In addition, monitoring of the mating success of released sterile and fluorescently sperm-marked males by trapping females and examine their spermathecae would be possible. This effective and easily recognizable sperm marking will make novel studies possible on medfly reproductive biology and using sperm marked strains could optimize releasing strategies in SIT-based AW-IPM.

Key Words: cellularization, conditional embryonic lethality, insect transgenesis, sperm marker, *beta2-tubulin*.

INTRODUCTION

The Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), is one of the most devastating and economically important insects among 250 pest species belonging to the Tephritidae family (Khoo et al. 1991). A native of Sub-Saharan Africa, medfly has spread into the Mediterranean basin to parts of Central and South America, Hawaii and Australia in less than 200 years. Moreover, in the latter half of the last century, it was sporadically detected in different areas of the United States like California, Florida and Texas. The success of *C. capitata* infestation is partially dependant on its highly polyphagous nature (*C. capitata* attacks more than 250 different fruits, vegetables and nuts) and on the ability to adapt its multivoltine cycle to different temperate climates to overwinter as larvae in different crops.

Biological approaches to insect pest management offer alternatives to insecticidal control. The Sterile Insect Technique (SIT) is regarded as an ecologically safe method for area-wide control. SIT reduces the pest population by mass release of sterilized pest organisms (Knipling 1955). The SIT process involves mass rearing and release of individual flies rendered sterile through gamma or X-ray irradiation exposure. SIT has been proven in many areas to be effective against medfly. An example: Southern Mexico (MOSCAMED program), where the eradication of *C. capitata* was achieved over a region of 15.000 km² in 1982 (Hendrichs et al. 1983) and a barrier was created through Guatemala (Villasenor et al. 2000). For many pest

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species including medfly, SIT proved to be most effective by releasing only sterile males (Robinson 2002). 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 (Bushland 1971; Cayol et al. 1999). Recently a transgene-based embryonic sterility system was successfully established in *Drosophila melanogaster* (Meigen) (Horn and Wimmer 2003) and this system may provide an alternative to the use of radiation in area-wide integrated pest management programs involving the SIT.

Another problem in SIT programs is the monitoring: up to now in some SIT programs, mass reared pupae are sterilized and dusted with fluorescent powders which enable sterilized flies to be distinguished from wild flies when recaptured in traps in the release area. This monitoring system implies some disadvantages: the fluorescent dyes are expensive, dangerous for human health and error prone, because they can be transferred from sterilized marked flies to wild type ones (Hagler and Jackson 2001). Beside dyes, there have been genetic based approaches to improve the monitoring in various species. A dominant mutation has been isolated for *C. capitata*, which affects the third stripe on the abdomen (Niyazi et al. 2005). Field-cage studies have shown comparable sexual compatibility and mating competitiveness, including data on genetic sexing strains (GSS). Another system describes a transgenic sexing strain in *Anopheles stephensi* (Catteruccia et al. 2005). In this system the spermatogenesis specific *β2-tubulin* (*β2t*) promoter from *Anopheles gambiae* drives enhanced green fluorescent protein (EGFP). This system has been proven under small-scale conditions as a good sexing strain in *Anopheles stephensi.*

Our aim is the development of a sperm marking system for *C. capitata*, which is based on the use of the *C. capitata* spermatogenesis-specific *β2t* promoter driving a fluorescent marker (Scolari et al. 2008). After thorough strain evaluation and a test phase for fitness, accuracy and stability of the sexing procedure as well as the stability of these strains, they could be used for different purposes. A possible application might be the use as a transgenic sexing strain in combination with the ability for an easy monitoring in an operational SIT program. The system will also help in providing more detailed information on reproductive biology of *C. capitata.* The aim of the studies reported here was to establish and evaluate such embryonic lethality and sperm marking systems in *C. capitata*. Functional large-scale SIT activities, like those established for *C. capitata*, are ideal for comparing the effectiveness and usefulness of novel transgenic SIT approaches.

MATERIAL AND METHODS

In-situ hybridization. The RNA probes were made with a DIG-RNA-labeling Kit (Roche, Mannheim) and hybridizations were performed as described in Davis et al. (2001).

Plasmid. p*B*[*s1*-tTA; *PUb*-DsRed1] was constructed as previously described in Schetelig et al. (2007).

RESULTS

1) Evaluation of a transgenic approach to sterilize flies with an embryonic lethal transgene combination in *C. capitata*.

An approach to cause sterility was designed without interfering with spermatogenesis to maintain males and their sperm as competitive as possible. We followed a strategy based on the expression of a lethal factor under the control of a promoter that is active at early blastoderm stages. When the male is homozy-

gous for the combination of the necessary gene constructs, each fertilization event will lead to embryonic lethality (Horn and Wimmer 2003). The advantage of this system lies in the proposed high competitiveness of such males, since their reproductive organs will not be affected and matings actually lead to sperm transfer. However, it is very important that the promoter is active only in early stages of development. Then the lethal phase can be overcome while developing under permissive conditions in the rearing facilities, whereas after release non-permissive conditions will not affect the males themselves but only their progeny (Wimmer 2005). The system employs the ectopic expression of a hyperactive proapoptotic gene that causes embryo-specific lethality when driven by the tetracycline-controlled transactivator tTA under the regulation of a cellularization gene enhancer/promoter. The system has been tested successfully in *Drosophila melanogaster* (Horn and Wimmer 2003). We want to transfer this binary expression system to *C. capitata* to evaluate it in comparison with the effective conventional SIT using radiation-induced sterility.

We first tried the direct transfer of the Drosophila-derived system to *C. capitata*. Therefore we injected the driver construct p*B*[*s1*-tTA; *PUb*-DsRed1], which contains the tTA gene under the control of the *Drosophila melanogaster serendipity* α *(sry*α*)* promoter region (Schetelig et al. 2007), together with the p*hsp*-p*Bac* (Handler and Harrell 1999) into the germline of *C. capitata*. We got four independent transgenic lines. These lines where tested for tTA expression by whole mount *in-situ* hybridizations with a RNA probe to tTA. None of the four transgenic lines expressed the *tTA*. Representative three of the four lines are shown in Figure 1.

Thus, the cellularization specific *sry*α promoter from *Drosophila* seems not to be functional in *C. capitata*. In order to get functional promoters for use in our system, we searched for endogenous promoters of *C. capitata*. To obtain *C. capitata* genes and their promoters which lead to specific expression at the blastoderm stage, we first carried out PCR-based cDNA subtractions of different embryonic stages and identified several cellularization-specific genes (Schetelig et al. 2007). After that we isolated the corresponding enhancers/promoters by inverse PCR (iPCR) and subsequently brought the tTA independently under the control of each enhancer/promoter region. We injected the construct carrying the cellularization specific promoter of *sub1_68* from *C. capitata* into the germline of *C. capitata* and got transgenics with cellularization specific expression of tTA (Figure 2). However, the expression as detected by whole mount *in-situ* hybridizations was relatively weak.

2) Development of a transgenic sperm marking system for *Ceratitis capitata*.

To develop a sperm marking system for the fruit fly *C. capitata*, we isolated the spermatogenesis specific gene *β2t* from *C. capitata* by degenerate primer PCR. By rapid amplification of cDNA ends (RACE), we amplified the complete *β2t* and isolated the upstream region by iPCR. The observed upstream region of *β2t* was fused to the fluorescent marker turboGFP (tGFP). *β2t*-tGFP was then inserted in a *piggyBac* (p*B*) transposon vector carrying a red (DsRed) fluorescent marker driven by a polyubiquitin promoter (*PUb*): *β2t*-tGFP in p*B*[*PUb*-DsRed1]. The advantage of this transposon vector is that it carries a characterized fluorescent marker driven by the ubiquitiously expressed polyubiquitin promoter from *Drosophila melanogaster* (Handler and Harrell 2001). The construct p*B*[*β2*-tGFP; *PUb*-DsRed1] was injected with a transposase source into the posterior end of *C. capitata* embryos to cause germline transformation. Flies from this transformation have a red body and males have green fluorescent testes (Figure 3). For sperm use and remating analyses different fluorescent

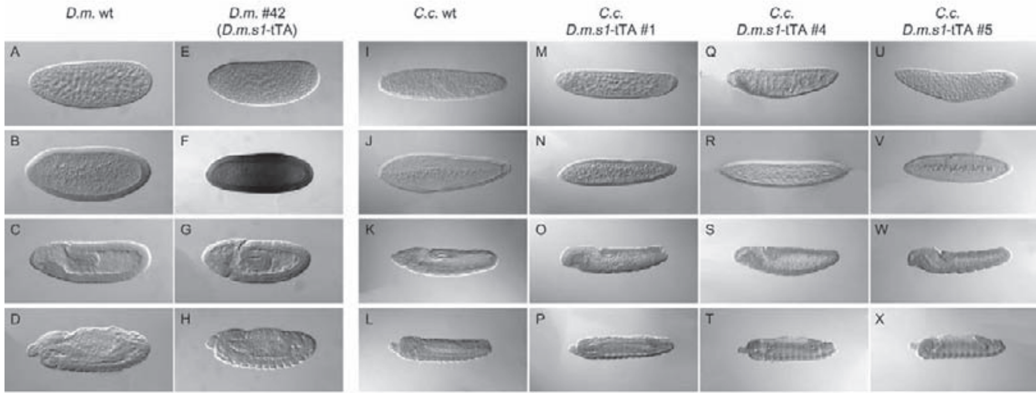

Fig. 1. Cellularization specific promoter of *Drosophila* is apparently nonfunctional in *C. capitata*. Shown are whole mount *in-situ* hybridizations using a tTA RNA probe. The embryogenesis of each strain (columns) is pictured with four embryonic stages: first row = blastoderm; second row = cellularization; third row = germ band elongation; fourth row = germ band retraction.
D.m. wildtype (A-D): no expression of tTA at all stages. *D.m.* #42 (Horn and Wimmer 2003) (E-H): tTA is cellularization specifically expressed in this strain, which carries the *D.m.s1*-tTA.
C.c. wildtype (I-L): no expression of tTA at all stages. *C.c. D.m.s1*-tTA #1 (M-P), *C.c. D.m.s1*-tTA #4 (Q-T) and *C.c. D.m.s1*-tTA #5 (U-X): no expression of tTA at all stages.

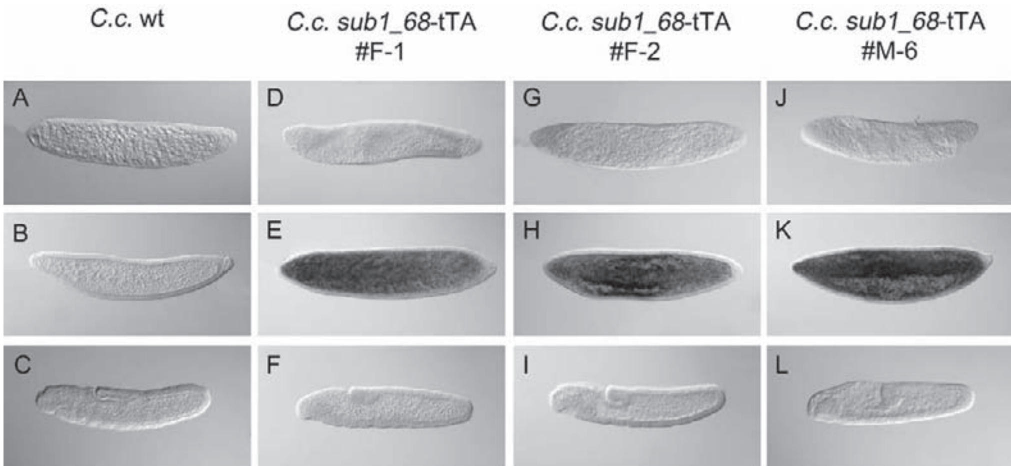

Fig. 2. Endogenous promoter of a cellularization specific gene of *C. capitata* causes weak tTA expression. Shown are whole mount *in-situ* hybridizations using a tTA RNA probe. The embryogenesis of each strain (columns) is pictured with three embryonic stages: first row = blastoderm; second = cellularization; third = germ band elongation.
C.c. wildtype control (A-C): no expression of tTA at all stages.
C.c. sub1_68-tTA #F-1 (D-F), *C.c. sub1_68*-tTA #F-2 (G-I) and *C.c. sub1_68*-tTA #M-6 (J-L): cellularization specific expression of tTA driven by the *C.c. sub1_68* promoter.

marked sperms would be helpful. Therefore we are developing also a system carrying a green body and a red testes marker.

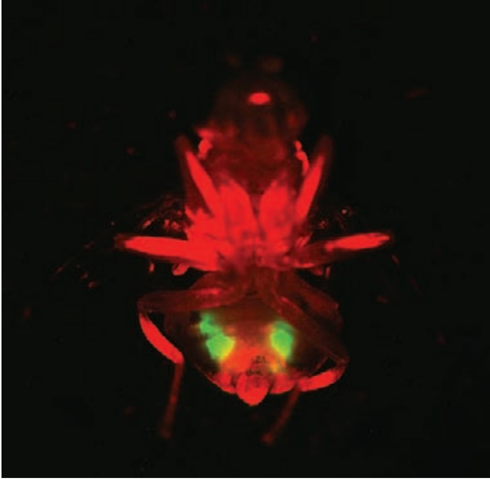

Fig. 3. Transgenic marked *C. capitata* male carrying two different types of molecular markers. The male has a red fluorescent body marker (*PUb*-DsRed) and a testes-specific green fluorescent marker (*β2t*-tGFP).

DISCUSSION

1) Evaluation of a transgenic approach to sterilize flies with an embryonic lethal transgene combination in *Ceratitis capitata*.

A direct transfer of the driver construct carrying the cellularization specific *D. melanogaster sryα* promoter to *C. capitata* showed that this promoter is not functional in *C. capitata*. Thus, we can assume that the complex interaction between enhancers and promoters of stage-specifically expressed genes (Blackwood and Kadonaga 1998) is different between *D. melanogaster* and *C. capitata*. A *Drosophila melanogaster* promoter might not act as an adequate alternative to an endogenous *C. capitata* promoter to enable strong expression rates.

For a highly specific embryonic lethality system we need promoters mediating strong expression specific to early embryonic stages. Therefore we isolated several cellularization genes from *C. capitata* (Schetelig et al. 2007) and their promoters. We could show that the *Ceratitis* cellularization specific promoter from *sub1_68* fused to tTA expressed tTA specifically at cellularization stages (Figure 2). So this promoter might be sufficient for using it for our embryonic lethality system. But the staining time for the color reaction of 24h for the depicted whole mount *in-situ* hybridizations was not in an estimated average time window for *in-situ* hybridizations on *C. capitata* with RNA probes (average of staining time to the color reaction is completed in *C. capitata* embryos is between 0.5 and 3h). So we suppose that the specific, albeit low expression of this construct will probably not be sufficient for generating a successful and safe lethality system. To better promote the tTA expression we wish to isolate varying long versions of the *sub1_68* upstream region which might include more regulating elements. We will also isolate and test upstream regions from other *C. capitata* cellularization specific genes.

2) Development of a transgenic sperm marking system for *Ceratitis capitata*.

After thorough strain evaluation and test phase for fitness, accuracy and stability, the developed sperm marked strains are a first step for improving the following objectives (Scolari et al. 2008):

- Reproductive biology in *Ceratitis capitata*. Thus far little is known about the mechanisms of remating behavior and the consequential sperm use. With the sperm marking system it should be possible to widen our the knowledge of the reproductive biology of the medfly. The obtained results will also be important to further improve strategies of SIT programs.
- Monitoring of an area wide SIT program. Because *β2t* is expressed in the testes, released males from these strains could be distinguished from wildtype males in the monitoring process during SIT. Together with the information on trapped females in the field and dissection of their sperm storage organs, this would allow for a bet-

ter evaluation of the efficiency of SIT programs.

- In principle these strains can also be used as sexing lines that could be sorted automatically for males and females during larval development (presuming gonadal fluorescence is detectable). However, since *Ceratitis capitata* has already an effective sexing strain, this might be more important for the development of SIT in other Tephritids.

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