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A transposon-mediated germline transformation system for the honeybee *Apis mellifera*

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Eidesstattliche Erklärung

Ich versichere an Eides statt, dass die Dissertation von mir selbständig und ohne unzulässige fremde Hilfe erstellt worden ist. Die Dissertation wurde in der vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

Düsseldorf, den 31. Oktober 2012

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I Einleitung

I.1 Die Honigbiene *Apis mellifera*

Die Honigbiene (*Apis mellifera*) hat als weltweit wichtigster Bestäuber der Blütenpflanzen großen Anteil an der Aufrechterhaltung des Ökosystems (Rader et al., 2009, Aebil et al., 2012). Daneben sind auch die Bienenprodukte Honig und Wachs für den Menschen von großem Interesse. Umso erschreckender ist das plötzliche Bienensterben, das seit etwa zehn Jahren auf der nördlichen Hemisphäre beobachtet wird (Cox-Foster et al., 2007, Neumann and Carreck, 2010). Hierbei wird vom sogenannten *colony collapse disorder* Syndrom (CCD) gesprochen, welches sich durch verwaiste Stöcke mit Brut und Futtervorräten auszeichnet, ohne dass tote Bienen vorgefunden werden (Cox-Foster et al., 2007). Trotz aller Bemühungen ist es bisher nicht möglich gewesen das Bienensterben auf einen bestimmten Faktor zurückzuführen. Höchstwahrscheinlich handelt es sich bei der Ursache des CCD um eine Kombination mehrerer Erreger (Yang and Cox-Foster, 2007). Man konnte zeigen, dass die Varroa-Milbe (*Varroa destructor*), welche die Bienen befällt, großen Einfluss auf den plötzlichen Tod ganzer Völker hat (Genersch et al., 2010, Guzman-Novoa et al., 2010). Die Varroa Milbe schwächt das Immunsystem der Biene und erleichtert somit die Infektion durch Viren. In einigen Fällen werden die Viren sogar direkt von der Milbe übertragen (Bowen-Walker et al., 1999, Yang and Cox-Foster, 2007).

Neben ihrer wichtigen Rolle im weltweiten Ökosystem wird die Biene durch ihre vielfältigen und faszinierenden Eigenschaften, in vielen Forschungsbereichen der Biologie als Modellorganismus angesehen. Zu diesen Eigenschaften zählen unter anderem eine komplementäre Geschlechtsbestimmung, kooperatives Verhalten, Kastendifferenzierung in Arbeiterinnen und Königin und eine eusoziale Lebensweise.

In eusozialen Insekten leben mehrere überlappende Generationen miteinander, wobei sich dieser Verband in fruchtbare und unfruchtbare Mitglieder teilt und die Nahrungsbeschaffung sowie Brutpflege kooperativ durchgeführt wird. Bei den unfruchtbaren Mitgliedern einer Honigbienenkolonie handelt es sich um die Arbeiterinnen, die den größten Anteil des Volks ausmachen. Die fruchtbaren

Mitglieder sind die Königin und die männlichen Drohnen, wobei die Drohnen nur in Frühjahr und Sommer leben. Die Honigbiene zeigt ein weites Spektrum an Verhaltensweisen, welche sich sowohl zwischen den Kasten als auch zwischen den Geschlechtern unterscheiden (Winston, 1987). Eine Jungkönigin macht bei Geschlechtsreife einige Orientierungsflüge, um sich danach auf dem sogenannten Hochzeitsflug mit mehreren Drohnen zu paaren und anschließend den Rest ihres Lebens in einer Kolonie Eier zu legen (Winston, 1987). Wird das Volk zu groß für die alte Behausung, werden neue Königinnen herangezogen. Kurz vor deren Schlupf schwärmt die alte Königin mit 50-90 % des Bienenstaates aus, um eine neue Kolonie zu gründen (Lampeitl, 1982). Da die Nachkommen einer Königin in erster Linie Arbeiterinnen und damit steril sind, findet durch das Aufziehen von neuen Königinnen und Schwärmen die eigentliche geschlechtliche Vermehrung statt (Tautz, 2008). Die neuen Königinnen eines Volks versuchen sich gegenseitig abzustechen bis in der Regel nur noch eine Königin übrig bleibt, die dann Zeit ihres Lebens von den Arbeiterinnen versorgt wird. Es kann jedoch auch zu einem oder mehreren sogenannten Nachschwärmen kommen, bei dem eine der frischgeschlüpften Jungköniginnen mit einem weiteren Teil des Volkes auszieht und eine weitere neue Bienenkolonie gründet (Winston, 1987).

Neben dem Versorgen der Königin gehen die Arbeiterinnen einer Vielfalt von weiteren Aufgaben nach, welche jede Arbeiterin abhängig von ihrem Alter übernimmt. Dazu gehören das Reinigen der Waben, die Wachsproduktion, der Wabenbau, die Larvenaufzucht und das Verteidigen den Stockes gegenüber Räubern und Fressfeinden (Winston, 1987). Zum Ende ihres Lebens hin übernehmen die Arbeiterinnen das Sammeln von Pollen und Nektar. Dabei orientieren sie sich am Stand der Sonne und an polarisiertem Licht und können die Informationen zu Standort und Entfernung einer Futterquelle, kodiert in ihrer Tanzsprache, an Artgenossen weitergeben (von Frisch, 1967). Im Vergleich zu den Arbeiterinnen zeigen Drohnen ein weniger umfangreiches Verhaltensrepertoire. Zunächst werden sie bis zur Geschlechtsreife von den Arbeiterinnen gefüttert und gepflegt. Dann fliegen sie aus, um sich an Drohnensammelpunkten mit ebenfalls ausfliegenden Jungköniginnen zu paaren (Lampeitl, 1982, Winston, 1987).

Drohnen schlüpfen in der Regel aus unbefruchteten Eiern, während weibliche Bienen aus befruchteten Eiern schlüpfen (Dzierzon, 1845). Diese Art der Geschlechtsbestimmung bezeichnet man als Haplodiploidie. Bei der Honigbiene ist

nicht etwa der Befruchtungszustand des Eis für die Geschlechtsfestlegung verantwortlich, sondern die allelische Komposition am geschlechtsbestimmenden Lokus (SDL). Daher wird die Geschlechtsbestimmung bei der Honigbiene als komplementäre Geschlechtsbestimmung bezeichnet (Beye et al., 2003a). Liegt das Gen *complementary sex determiner* (*csd*) am SDL in zwei unterschiedlichen Allelen vor, entwickelt sich aus dem Ei ein Weibchen. Die Csd-Proteine nehmen nur im heterozygoten Zustand ihre aktive Form ein und beeinflussen so weitere Gene der geschlechtsbestimmenden Kaskade auf weibchenspezifische Weise (Gempe et al., 2009b). Bei hemi- oder homozygotem Allelzustand entwickelt sich ein Männchen, wobei nur die haploiden Männchen fertil sind. Die diploiden Drohnen werden bereits im frühen Larvenstadium von den Arbeiterinnen erkannt und aufgefressen.

Ob sich aus einer weiblichen Larve eine Arbeiterin oder eine Königin entwickelt, ist nicht genetisch bedingt, sondern hängt von der Zusammensetzung des Futters ab, mit dem sie während der frühen larvalen Entwicklung gefüttert wird (Haydak, 1970). Enthält dieses Futter einen entsprechend hohen Anteil Royalaktin (ein Bestandteil des Gelee Royale) werden diverse Stoffwechselprozesse so geschaltet, dass es zu einer schnelleren Entwicklung, stärkerem Größenwachstum und zur Ausbildung von funktionsfähigen Ovarien kommt und sich somit eine Königin entwickelt (Kucharski et al., 2008, Kamakura, 2011b).

Die genetische Basis dieser entwicklungs- und verhaltensbiologischen Eigenschaften der Honigbiene ist weitestgehend unbekannt. Dank des sequenzierten Genoms (Weinstock et al., 2006) kann man viele Orthologe zu Genen in *Drosophila melanogaster* finden, doch ist deren funktionelle Analyse in der Honigbiene in erster Linie nur durch Mikroinjektion oder Fütterung von dsRNA möglich. In solchen RNA Interferenz (RNAi) Experimenten konnten die Transkripte einiger Gene abgebaut und so Informationen über die Genfunktion gewonnen werden (Gempe et al., 2009b, Kamakura, 2011b, Nissen et al., 2012). Diese Experimente erfolgten bisher vorrangig an Eiern und Larven. Über die erfolgreiche Manipulation von adulten Honigbienen mittels dsRNA gibt es bisher nur wenige Berichte (Mussig et al., 2010, Mustard et al., 2010). Durch Injektion kann die dsRNA nur zu bestimmten Zeitpunkten in der Entwicklung und lokal begrenzt in den Organismus eingebracht werden. Auch durch die Fütterung von dsRNA kann nicht in alle Entwicklungsprozesse eingegriffen werden, zum Beispiel nicht innerhalb der Embryonalentwicklung oder während des Puppenstadiums. Durch Injektion und Fütterung von dsRNA kann auch kein

konstanter Abbau von Gentranskripten über einen längeren Zeitraum erzielt werden. Hinzu kommt eine hohe Variation im jeweils ausgeprägten Phänotyp zwischen den einzelnen manipulierten Individuen. Zudem wurde gezeigt, dass die dsRNA Einfluss auf die Expression von Nicht-Zielgenen nehmen kann (Jarosch and Moritz, 2012).

Durch die Verwendung von transgenen Honigbienen könnte man diese Schwierigkeiten umgehen. Transgene Linien würden zudem Vorteile für die Untersuchung von Genfunktionen liefern: Bienenköniginnen legen bis zu 1500 Eier am Tag (Gary, 1992, Katzav-Gozansky et al., 2001) und würden so in kurzer Zeit und über längere Zeiträume eine große Menge an Nachkommen produzieren, die einen konstanten Phänotypen in Bezug auf das manipulierte Gen zeigen. In Kombination mit entsprechenden Promotoren wäre es möglich in jedem Gewebe und zu jeder Zeit in der Entwicklung Gene zu exprimieren oder ihre Transkripte abzubauen. Transgene Linien der Honigbiene würden die Möglichkeit bieten, nahezu jedes Gen auf seine Funktion hin zu untersuchen. Des Weiteren könnte man durch die transgene Expression von dsRNA gegen virale Transkripte die Vermehrung von Viren unterdrücken und somit Krankheiten der Honigbiene untersuchen und möglicherweise bekämpfen. Bisher wurde kein System zur Generierung transgener Honigbienen etabliert. Auch wurden bisher keine Honigbienenpromotoren zur möglichen Expression von Fremdgenen identifiziert. Die Schwierigkeiten bei der Etablierung einer Transformationsmethode liegen bei den charakteristischen Eigenschaften der Honigbiene: Durch die Kastendifferenzierung ist es nötig transgene Königinnen aufzuziehen, da nur diese Nachkommen produzieren. Auch die Haltung und Vermehrung von Bienen ist aufgrund ihrer Organisation in Kolonien aufwendig und unter Laborbedingungen schwierig.

I.2 Transposons

Um die Funktion von Genprodukten während der Entwicklung oder innerhalb von Stoffwechselprozessen zu verstehen, ist es erforderlich genetische Informationen stabil in das Genom eines Organismus zu integrieren. In den meisten Modellorganismen werden transgene Linien durch den Einsatz von Transposons erzeugt (Lin et al., 1994, OBrochta and Atkinson, 1996, Sinzelle et al., 2006). Diese sogenannten *springenden Gene* bilden eine große Gruppe von genetischen

Elementen, die eine Rekombinationsreaktion durchführen können und sich so von einem Ort des Genoms an einen anderen transferieren. Transposable Elemente machen große Teile des Genoms von Pro- und Eukaryoten aus und führen durch das Springen im Genom zu genetischer Variation und Mutationen. Im Genom der Honigbiene wurden außer einigen wenigen, stark degradierten *mariner*-Transposons keine Mitglieder der großen Transposonfamilien gefunden (Weinstock et al., 2006). Bei *mariner*-Transposons handelt es sich um Mitglieder der Klasse II-Transposons, die DNA-Transposons (Finnegan, 1989). DNA-Transposons werden als Ganzes aus dem Chromosom ausgeschnitten und an anderer Stelle wieder eingefügt. Im Gegensatz dazu werden transponierbare Elemente der Klasse I über reverse Transkription einer RNA transponiert. Da dieser Vorgang einer retroviralen Integration ähnelt werden sie entsprechend Retrotransposons genannt (Finnegan, 1989, Gogvadze and Buzdin, 2009). Im Genom der Honigbiene konnten keine aktiven Retrotransposons identifiziert werden (Beye et al., 2006, Weinstock et al., 2006).

DNA-Transposons bestehen natürlicherweise aus einem Gen und zwei flankierenden Bereichen, den sogenannten invertierten Wiederholungen (*inverted repeats*, IR) die acht bis 50 bp lang sein können. Das Gen kodiert für das Enzym Transposase, das die Transposition katalysiert. Das Transposasegen und die invertierten Wiederholungen sind aufeinander abgestimmt und spezifisch für jeden Transposontyp (Finnegan, 1989). Um Transposons für die Integration von Fremdgenen in das Genom eines Organismus zu nutzen, werden die natürlich vorkommenden transponierbaren Elemente abgeändert (OBrochta and Atkinson, 1996, Handler, 2002). Das Gen, welches für die Transposase kodiert wird aus dem Transposon entfernt um ein unkontrolliertes Transponieren im Organismus zu vermeiden. Stattdessen wird eine Expressionskassette, bestehend aus Promoter und Reportergen, zwischen die invertierten Wiederholungen inseriert. Das Konstrukt wird dann beispielsweise durch Injektion zusammen mit einer Transposasequelle in einen Embryo eingebracht. Letztere kann in Form von Transposase-mRNA oder einem Helferplasmid, auf dem ein Promotor und das Gen für die Transposase liegen, zur Verfügung gestellt werden (Berghammer et al., 1999, Pavlopoulos and Averof, 2005). Im Organismus wird daraufhin das Transposase-Protein synthetisiert, welches das Transposon ins Genom transponiert.

I.2.1 Transposon-vermittelte Transformation von Insekten

Die genetische Transformation eines Insekts mit Hilfe eines transposablen Elements wurde 1982 zum ersten Mal bei der Taufliege *D. melanogaster* gezeigt. Hierbei wurde das P-Element verwendet, welches in einigen *Drosophila*-Stämmen natürlich vorkommt (Rubin and Spradling, 1982). Auch wenn es bei *D. melanogaster* standardmäßig benutzt wird um fremde DNA dauerhaft in das Fliegengenom zu integrieren, ist der Ansatz auf diese Spezies und einige eng verwandte Arten aus der Familie der *Drosophilidae* begrenzt (O'Brochta and Handler, 1988). Die Versuche, Spezies aus anderen Insektenordnungen mit dem P-Element zu transformieren schlugen fehl. Doch fand man im Laufe der letzten dreißig Jahre weitere transposable Elemente, wie *hobo*, *mariner*, *minos, piggyBac* und *hermes*, wobei es insbesondere mit dem *piggyBac*-Transposon möglich war, Arten verschiedenster Insektenordnungen zu transformieren (OBrochta and Atkinson, 1996).

I.2.2 Das *piggyBac*-Transposon

Das *piggyBac*-Transposon ist ein vielversprechender Kandidat um die Honigbiene genetisch zu transformieren. Mit ihm konnten schon die Transformationen von Spezies aus vier Insektenordnungen erzielt werden. Dazu gehörten unter anderem *Ceratitis capitata* und *Musca domestica* (Diptera; (Handler et al., 1998, Hediger et al., 2001), *Bombyx mori* (Lepidoptera(Tamura et al., 2000), *Athalia rosae* (Hymenoptera; (Sumitani et al., 2003) und *Tribolium castaneum* (Coleoptera; (Lorenzen et al., 2003). Das *piggyBac*-Transposon gehört zur Gruppe der TTAA-spezifischen transposablen Elemente innerhalb der DNA-Transposons, das heißt es integriert spezifisch in eine TTAA-Sequenz und dupliziert diese im Verlauf der Transposition (Fraser et al., 1995). Das *piggyBac*-Transposon wurde ursprünglich in der Aschgrauen Höckereule *Trichoplusia ni* entdeckt (Fraser et al., 1983) und besteht aus 2472 bp. Diese beinhalten die je 13 bp langen invertierten terminalen Wiederholungen (*inverted terminal repeats*, ITR) und die 19 bp langen invertierten subterminalen Wiederholungen (*inverted repeats,* IR; Abb. I-1). Der für

die Transposase kodierende Bereich innerhalb der invertierten Wiederholungen ist 1,8 kb lang (Cary et al., 1989).

Abbildung I-1: Schematische Darstellung des natürlich vorkommenden *piggyBac*-Transposons. Dargestellt sind der ORF der *piggyBac*-Transposase, die duplizierte TTAA-Intergrationssequenz, die invertierten subterminalen Wiederholungen (IR), sowie die invertierten terminalen Wiederholungen (ITR). Abbildung nach Handler (2002).

Zur Transformation von Insekten wurde das Gen für die *piggyBac*-Transposase aus dem Transposon entfernt und durch eine Marker-Expressionskassette ersetzt, die es ermöglicht Transformanten zu identifizieren. In Spezies, in denen weißäugige Mutantenlinien zur Verfügung standen, wurde mit den Genen *white* oder *vermillion* als Marker gearbeitet, deren Genprodukte die Pigmentierung der Augen bewirken (Handler and McCombs, 2000, Lorenzen et al., 2002). In transformierten Zellen werden diese Gene exprimiert, was zu einer mosaikartigen oder vollständigen Pigmentierung der Komplexaugen führt. An dieser Pigmentierung ist eine erfolgreiche Transformation zu erkennen. Die Gene der Fluoreszenzproteine EGFP oder DsRed wurden in Spezies als Reporter genutzt, in denen keine weißäugigen Mutantenlinien zur Verfügung standen (Horn and Wimmer, 2000, Horn et al., 2002). Als Promotor, der diese Markergene exprimieren sollte, wurde unter anderem der Promotor des Polyubiquitin-Gens von *D. melanogaster* benutzt (Davis et al., 1995). In vielen Spezies etablierte sich der künstlichen Promotor 3xP3 bzw. 6xP3 (Berghammer et al., 1999, Posnien et al., 2011). Hierbei handelt es sich um drei bzw. sechs hintereinanderliegende Pax6-Bindestellen (P3) in Kombination mit einem Core-Promotor (Vgl. I.3). Der Promotor wird durch Anlagerung des Proteins Pax6 aktiviert. Pax6 besitzt eine Masterregulatorfunktion in der Augenentwicklung und ist in Struktur und Funktion innerhalb der Metazoa konserviert (Horn and Wimmer, 2000, Horn et al., 2002). Ein P3-basierter Promotor vermittelt also die Expression eines Reportergens in allen Zellen, die an der Augenentwicklung beteiligt sind. Da der Promotor 3xP3 in allen bisher untersuchten

Insekten die Expression eines Reportergens (*dsRed* oder *egfp*) in Gehirn oder Augen initiierte wird seine Funktion als universell angesehen (Berghammer et al., 1999, Handler, 2002). Daher ist ein P3-basierter Promotor ein vielversprechender Kandidat um die Expression von Reportergenen während der Augenentwicklung der Honigbiene zu initiieren und so die Identifizierung von Transformanten zu ermöglichen.

I.3 Promotoren zur gezielten Expression von Transgenen

Promotoren sind wichtige Werkzeuge in der Gentechnik, da durch sie die Expression von Fremdgenen vermittelt wird. Sie werden beispielsweise durch Transposons in das Genom eines Organismus eingebracht und exprimieren dort gewebe- und/oder zeitabhängig Reportergene.

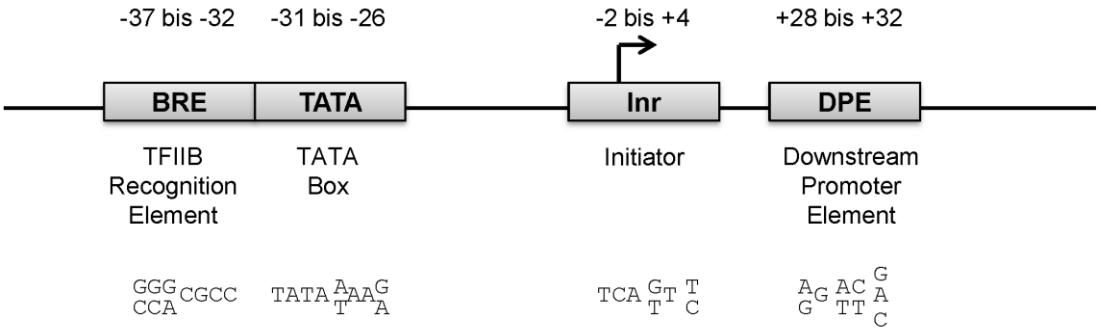

Abbildung I-2: Schematische Darstellung der Elemente des Core-Promotors. Die dargestellten Elemente können zur basalen Transkription eines nachgeschalteten Gens beitragen. Dabei können, müssen aber nicht alle Elemente vorhanden sein. Die unterhalb der Promotorelemente dargestellten Konsensus-Sequenzen beziehen sich auf die Sequenzen der Spezies *D. melanogaster*. Die oberhalb der Promotorelemente dargestellten Zahlen markieren die Position ausgehend vom Transkriptionsstart (+1). Abbildung nach Smale and Kadonaga (2003).

Ein Promotor ist in erster Linie eine Erkennungssequenz für die RNA-Polymerase II. Innerhalb dieser 20-200 bp langen Sequenz liegen die Sequenzelemente TATA-Box, Initiator (Inr), TFIIB Recognition Element (BRE) und Downstream Promoter Element (DPE) (Abb. I-2). Diese Elemente sorgen teils gemeinsam, teils unabhängig voneinander dafür, dass die Proteine der Transkriptionsmaschinerie an die DNA binden und das meist strangabwärts gelegene

Gen transkribiert wird (Smale and Kadonaga, 2003). Dieser Bereich, der einzig zur Bindung des Transkriptionskomplexes und für die Initiation der Transkription sorgt, wird Core-Promotor genannt (Abb. I-2).

Neben den Elementen des Core-Promoters gibt es noch weitere regulatorische Elemente, die zum Beispiel zur zeitlichen und räumlichen Spezifizierung der Transkription beitragen. So ist es möglich, dass manche Gene nur in einer bestimmten Entwicklungsperiode oder einem speziellen Gewebetyp exprimiert werden. Auch diese spezifizierenden Sequenzelemente liegen meist strangaufwärts des entsprechenden Gens (Yao and White, 1994, Novina and Roy, 1996, Hoffmann et al., 1997). Promotoren von Hitzeschockproteinen werden bevorzugt in der Gentechnik eingesetzt. Durch sie ist es möglich, die Expression von Fremdgenen zu jedem beliebigen Zeitpunkt mittels Änderung der Umgebungstemperatur an- oder auszuschalten (Monsma et al., 1988, Grotewiel et al., 1998, Lam and Thummel, 2000, Cheng et al., 2001). Die Genexpression unter der Kontrolle von *hsp*-Promotoren kann auch mit Hilfe eines Lasers gezielt in einzelnen Geweben aktiviert werden (Uhlirova et al., 2002). Hitzeschockproteine sind Chaperone, die den Proteinen der Zelle helfen, ihre Konformation auch unter nicht optimalen Bedingungen, wie Hitze, Kälte oder einem falschen pH-Wert, einzuhalten (Hartl, 1996). In der Promotorregion von Hitzeschockgenen befinden sich konservierte Sequenzmotive, die sogenannten Hitzeschockelemente (HSE) (Lindquist, 1986).

4P Typ	n**TTC**nn**GAA**nn**TTC**nn**GAA**n
3P Typ	n**TTC**nn**GAA**nn**TTC**n
Gap Typ	n**TTC**nn**GAA**nnnnnnn**GAA**n
Step Typ	n**TTC**nnnnnnn**TTC**nnnnnnn**TTC**

Abbildung I-3: Verschiedene Klassen von Hitzeschockelementen (HSE). Dargestellt sind Nukleotidsequenzen verschiedener HSE-Klassen; die konservierten GAA- und revers komplementären TTC-Sequenzen sind in Großbuchstaben dargestellt. Klassifizierung und Abbildung nach Sakurai and Enoki (2010).

Durch die Konserviertheit der Hitzeschockelemente innerhalb der Eukaryoten sind sie leicht zu identifizieren (Lindquist, 1986). Sie bestehen aus mehreren invertierten Wiederholungen der Sequenz nGAAn die direkt hintereinander liegen. Man kann sie entsprechend ihres Aufbaus in wenigstens vier Klassen einteilen (Abb.

I-3) (Sakurai and Enoki, 2010). Im Fall von zu großer Hitze binden Hitzeschockfaktoren (HSF), die immer in der Zelle vorliegen, an die Hitzeschockelemente und initiieren dann die Expression der Hitzeschockproteine (Abb. I-4) (Zhong et al., 1998, Ahn and Thiele, 2003). Da die bindenden Hitzeschockfaktoren immer trimerisch vorliegen, bestehen die Hitzeschockelemente aus mindestens drei Einheiten (Pirkkala et al., 2001). Existierende Hitzeschockelemente innerhalb einer potentiellen Promotorsequenz geben somit einen Hinweis auf eine temperatursensible Expression der dahinterliegenden Gene.

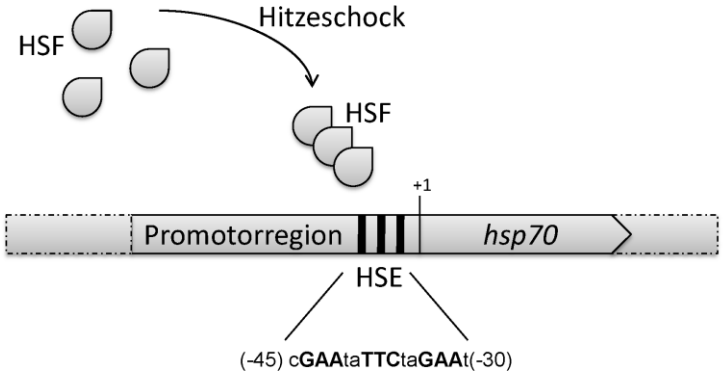

Abbildung I-4: Schematische Darstellung einer Hitzeschockreaktion am Beispiel des *hsp70*-Gens von *D. melanogaster*. Die frei in der Zelle vorliegenden Hitzeschockfaktoren (HSF) erfahren bei zu hohen Temperaturen eine Konformationsänderung und bilden Trimere. Diese können an die in der Promotorregion lokalisierten Hitzeschockelemente (HSE) binden, wodurch die Transkription des *hsp70*-Gens und damit die Synthese der entsprechenden Hitzeschockproteine (HSP) vermittelt wird. +1, -30 und -45 markieren die Nukleotidpositionen ausgehend vom Transkriptionsstart.

Auch in der Honigbiene konnten Hitzeschockproteine aufgrund ihrer Sequenzähnlichkeit innerhalb der Eukaryoten identifiziert werden (Schlesinger, 1990, Severson et al., 1990). Chacon-Almeida et al. (2000) gelang es die Proteine Am-Hsp70 und Am-Hsp83 mit einem Antikörper gegen Hsp70 aus Rindern nachzuweisen, was die strukturelle Konserviertheit der Proteine verdeutlicht. Es konnte gezeigt werden, dass die Proteinmenge von Am-Hsp70 und Am-Hsp83 nach einem vierstündigen Hitzeschock bei 42 °C erhöht wurde (Chacon-Almeida et al., 2000). Durch die Proteinuntersuchungen können bisher nur indirekt Rückschlüsse auf Promotoreigenschaften der Hitzeschockproteine in der Honigbiene gezogen werden. Ebensowenig wurden für die Honigbiene andere speziesspezifische Promotoren charakterisiert, mit denen eine Expression von Fremdgenen möglich wäre. Der einzige bisher in der Honigbiene funktionell getestete Promotor ist der

CMV Promotor des humanen Cytomegalovirus. Mit seiner Hilfe erfolgte die Expression des Fluoreszenzproteins EGFP in neuronalem Honigbienengewebe (Kunieda and Kubo, 2004).

In der Taufliege *D. melanogaster* sind bereits Promotoren mit vielfältigen Expressionseigenschaften charakterisiert worden. Zum Beispiel wird der Promotor des Gens *actin5c* (*act5c*) in Experimenten eingesetzt, in denen Gene konstitutiv überexprimiert werden sollen (Chavous et al., 2001). Actin5c ist ein Teil des Cytoskeletts und wird daher in allen Zellen eines Organismus exprimiert. Der gewebespezifische Promotor des *elav*-Gens wird in *D. melanogaster* für die Expression von Transgenen in neuronalen Geweben eingesetzt (Campos et al., 1987, Yao and White, 1994, Wu et al., 2011). Der Promoter des *hsp70*-Gens aus *D. melanogaster* wird in einer Vielzahl von Experimenten benutzt um Fremdgene zu definierten Zeitpunkten zu exprimieren. Er ist ein klassischer Hitzeschockpromotor, der die Expression eines nachgeschalteten Transgens nach einem mehrstündigen Hitzeschock vermittelt (Grotewiel et al., 1998, Lam and Thummel, 2000). Er wird nicht nur in *D. melanogaster*, sondern auch in anderen Arten der Gattung *Drosophila* und in Spezies anderer Insektenordnungen, eingesetzt (Uhlirova et al., 2002, Ramos et al., 2006). Es wurde gezeigt, dass der *hsp70*-Promotor von *D. melanogaster* auch Gene in Primaten-Zelllinien exprimieren kann (Pelham, 1982). Sein Aufbau scheint somit strukturell konserviert zu sein und auch durch die Hitzeschockfaktoren anderer Spezies erkannt zu werden. Nach seinem Vorbild wurden die Promotoren der entsprechenden orthologen *hsp70*-Gene auch aus anderen Spezies isoliert und zur Expression von Transgenen eingesetzt (Bienz and Pelham, 1986, Halloran et al., 2000). Aufgrund der strukturellen Konserviertheit vieler Gene zwischen der Taufliege, der Honigbiene und anderen Insektenspezies kann angenommen werden, dass die Promotoren der Honigbienengene ähnliche Charakteristika aufweisen, wie die Promotoren ihrer orthologen Gene in *D. melanogaster*.

I.4 Zielsetzung

Die Honigbiene *Apis mellifera* gilt als Modellorganismus für Entwicklungs- und Lernprozesse, sowie für soziales Verhalten. Die diesen Eigenschaften zu Grunde liegenden Genfunktionen sind weitestgehend unbekannt. Um Gene zu manipulieren und somit ihre Funktion zu identifizieren, müssen sie gezielt exprimiert oder ihre Transkription unterbunden werden. Dies kann durch genetische Transformation erreicht werden.

Ziel der vorliegenden Arbeit ist die Etablierung eines transposon-vermittelten Systems zur Transformation der Honigbiene. Dazu soll ein *piggyBac*-basiertes Transposon verwendet werden, welches das Markersystem 6xP3 kombiniert mit dem Reportergen *rubia*, beinhaltet. Mit *piggyBac*-basierten Transposons wurden bereits Spezies aus vier Insektenordnungen transformiert. Das Markersystem 3xP3 bzw. 6xP3 in Kombination mit einem Fluoreszenzgen hat sich innerhalb der Insekten etabliert, da mit ihm ein einfaches Identifizieren von Transformanten möglich ist. Im Zuge der Entwicklung eines Systems zur Generierung transgener Honigbienen soll eine Methode zur Aufzucht von transgenen Honigbienenköniginnen etabliert werden, welche die im Genom integrierten Transgene an ihre Nachkommen weitergeben.

Speziesspezifische Promotoren sind essentiell um Transgene gezielt zu transkribieren. Für die Honigbiene sind bisher keine speziesspezifischen Promotoren bekannt. Ziel dieser Arbeit ist daher die Identifizierung und Charakterisierung von konstitutiven, gewebespezifischen und temperaturinduzierbaren Promotoren für die Honigbiene. Diese sollen zunächst in Insektenzellen (*Sf*21-Zellen) auf ihre Fähigkeit hin untersucht werden, die Transkription von Reportergenen zu veranlassen. Weiter soll untersucht werden ob die Genexpression unter der Kontrolle potentieller Hitzeschockpromotoren, von Temperaturänderungen beeinflusst werden kann. Zudem soll ein potentiell neuronenspezifischer Promotor mittels eines Elektroporationsexperiments direkt in lebenden Zellen des Honigbienengehirns getestet werden.

In Kombination mit dem entwickelten *piggyBac*-Transposon soll die Funktion eines Promotors auch in der Honigbiene untersucht werden. Dazu wird nach dem Markersystem 6xP3-*rubia* eine weitere Expressionskassette in das Transposon integriert, die aus dem Honigbienenpromotor *Am-actin5c* und dem Reportergen *egfp*

besteht. Mit diesem erweiterten Konstrukt sollen transgene Königinnen generiert werden, in deren Nachkommen die Funktion des Promotors getestet werden soll.

I.5 Thesen

Folgende Thesen können im Rahmen dieser Arbeit aufgestellt werden:

- *Sf*21-Zellen weisen ähnliche Transkriptionseigenschaften wie Honigbienenzellen auf. Die *Sf*21-Zellen eignen sich daher zum Test von Honigbienenpromotoren und deren Genexpressionseigenschaften.
- Die Promotoren der Honigbienengene *Am-actin5c*, *Am-hsp70* und *Am-hsp83* initiieren die Expression eines Reportergens in *Sf*21-Zellen.
- Die Expression von Reportergenen unter der Kontrolle des Promotors des Hitzeschockgens *Am-hsp83* lässt sich durch Temperaturveränderungen beeinflussen.
- Der Promotor des Honigbienengens *elp2l* initiiert die Expression von Reportergenen im Gehirn der Honigbiene.
- Mit dem *piggyBac*-basierten Transposon ist es möglich, Fremdgene in das Honigbienengenom zu integrieren.
- Honigbienenköniginnen, die als Embryo durch das *piggyBac*-basierte System transformiert wurden, haben die Transgene stabil in ihr Genom integriert und geben sie an ihre Nachkommen weiter.
- Der künstliche Promotor 6xP3 ist in Kombination mit dem Reportergen *rubia*, welches für ein rotes Fluoreszenzprotein kodiert, geeignet um transgene Honigbienen im Puppenstadium zu identifizieren.
- Der Promotor *Am-actin5c* vermittelt die Expression des Reportergens *egfp* in transgenen Honigbienen.

II Manuscripts

Manuscript I

Honeybee promoter sequences for targeted gene expression

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Key words:

Apis mellifera, honeybee promoters, gene expression, insect cell culture, electroporation

Abstract

The honeybee, *Apis mellifera*, displays a rich behavioural repertoire, social organisation, caste differentiation and has an interesting mode of sex determination, but we still know little about the underlying genetic programs. We lack stable transgenic tools in honeybees that would allow for genetic control of gene activity in stable transgenic lines. As an initial step towards a transgenic method, we identified promoter sequences in the honeybee that can drive constitutive, tissue specific and cold shock induced gene expression. We identified the promoter sequences of *Am-actin5c*, *elp2l, Am-hsp83* and *Am-hsp70* and showed that, except for the *elp2l* sequence, the identified sequences were able to drive reporter gene expression in *Sf*21 cells. We further demonstrated that the putative neuron specific *elp2l* promoter sequence can direct gene expression in the honeybee brain via electroporation experiments. The identification of these promoter sequences is an important initial step in studying the function of genes with transgenic experiments in the honeybee, an organism with a rich set of interesting phenotypes.

Introduction

Honeybees (*A. mellifera*) are economically important pollinators of wild flowers and crop plants, and they express remarkable features, which establish the honeybee as an interesting model for understanding basic biological phenomena. However, we lack tools to manipulate genes in stable transgenic lines, which would facilitate the dissection of the genetic control of honeybee behaviour and development (Menzel et al., 2006). Examples of interesting honeybee features include a rich behavioural repertoire, social organisation, caste differentiation and divergent mode of sex determination. Sex determination in honeybees is controlled by the genotype of a single gene called *complementary sex determiner* (*csd*; Beye et al., 2003), which differs from the well studied sex chromosome systems (Goodfellow and Lovellbadge, 1993; Cline and Meyer, 1996). Females are heterozygous for the *csd* gene, whereas males are homozygous or hemizygous (Hasselmann et al., 2008; Gempe et al., 2009), but the basis of sexual differentiation is only partially understood (Gempe and Beye, 2011).

A honeybee colony consists of thousands of essentially sterile female workers and one single fertile female, the queen (Winston, 1987), that lays up to 1500 eggs per day (Gary, 1992; Katzav-Gozansky et al., 2001). This pronounced caste differentiation relies on differential nutrition with royal jelly (Haydak, 1970; Kamakura, 2011) and epigenetic control of gene activity (Kucharski et al., 2008). Honeybee workers perform a rich and diverse repertoire of behavioural tasks that are employed to maintain the colony (von Frisch, 1967; Winston, 1987; Seeley, 1995). The behavioural activities of workers are performed in highly coordinated ways and involve task specialisation and division of labour (Robinson, 1992; Page and Erber, 2002). Workers also show impressive cognitive abilities; for instance, they can learn abstract concepts, such as ´same´ and ´different´ (Giurfa et al., 2001). Furthermore, workers can communicate the location of food sources to other colony members of the hive via waggle dances (von Frisch, 1967).

In this study, we identified for the first time honeybee promoter sequences that are suitable for driving transgenic transcription. Targeted gene expression in the honeybee is an important tool for basic and applied research in the honeybee. In a previous study, Kunieda and Kubo (2004) were able to drive the expression of the EGFP reporter gene in the honeybee brain by the human cytomegalovirus (CMV)

promoter. They introduced the respective plasmid into honeybee nervous cells by electroporation. Transgenic studies in the fruit fly *Drosophila melanogaster*, the medfly *Ceratitis capitata* and the red flour beetle *Tribolium castaneum* showed that endogenous promoters are essential tools for controlling gene expression in the respective species (Kalosaka et al., 2006; Schinko et al., 2010). We followed this approach and tried to identify honeybee promoter sequences that would promote transcription in neuronal tissue, in the entire organism and only after temperature dependent induction. We used the genetic information of orthologs in *D. melanogaster*, whose promoters are widely used in transgenic experiments, to identify putative candidate promoters. In *D. melanogaster*, the *actin5c* (*act5c*) gene is ubiquitously expressed throughout development (Fyrberg et al., 1983), and its promoter sequence has been used to drive the overexpression of transgenes (Chung and Keller, 1990; Chavous et al., 2001). The expression of the elav (embryonic lethal abnormal vision) gene in *D. melanogaster* is restricted to neuronal tissues (Campos et al., 1987; Yao and White, 1994), and its promoter sequence has been widely used to genetically direct expression to the fly brain (Robinson et al., 2002; Wu et al., 2011). Gene transcription of heat shock proteins (hsp) can increase by 10 to 1000-fold after stress conditions, such as heat shock (Lindquist, 1984; Lindquist, 1986). The *Dm hsp70* promoter sequence has been widely used to induce knockdown of genes or express cDNA at specific development stages by shifts in the environmental temperature (Grotewiel et al., 1998; Monsma et al., 1988; Lam and Thummel, 2000; Cheng et al., 2001). Promoters of *hsp* genes have also been successfully used in other species, including the lepidopteran insects *Bicyclus anynana* and *Bombyx mori* (Monsma et al., 1988; Uhlirova et al., 2002; Ramos et al., 2006; Schinko et al., 2010).

In this study, we cloned the honeybee orthologs of these genes, which are candidates for these tissue-specific promoters, general and inducible promoter sequences, and we studied whether they can induce expression of the reporter gene rubia in *Sf*21 insect cells or in the honeybee brain. Our attempts to study the expression of our reporter gene constructs directly in honeybees failed regardless if we transferred the plasmids via injection (Beye et al., 2002) or via sperm (Robinson et al., 2000) to the embryos (some information of this non-functional pilot study is given in the supplementary information SI1). Here, we report on the first honeybee candidate promoter sequences that may be suitable for directing the expression of

genes in nervous tissue, the entire organism and at all developmental stages after a temperature shift.

Experimental Procedures

Cloning of putative promoter sequences

We amplified the genomic region upstream of the corresponding translation start site of the genes *Am-actin5c*, *Am-hsp70* and *Am-hsp83* by PCR from honeybee genomic DNA and the *Dm-hsp70* gene from *D. melanogaster* (Pelham, 1982) genomic DNA. The putative transcription start sites were revealing the 5´end of the transcript via RACE experiments (FirstChoice^{TM} RLM-RACE Kit) and RT-PCRs. All the oligonucleotides that were used in this study are listed in the supplementary information (SI6). The sequence information for the amplified genes was obtained from NCBI. We cloned these amplicons into the pIZ/V5 His plasmid (Invitrogen, Darmstadt, Germany) using the *Sac*I/*Xba*I or the *Hind*III/*Xba*I restriction sites and thereby replaced the OpIE2 promoter sequence just upstream of the pUC ori site. To produce the *elp2l* putative promoter sequence with the 5'UTR, we first amplified the 2000 bp upstream of the transcriptional start site from genomic DNA by PCR. We cloned this fragment into the pGEM-T plasmid (Promega, Mannheim, Germany) using the *Hind*III and *Xba*I restriction sites. This produced the plasmid pGEM-T_fne_prom, in which a new restriction site (*BsaI*) was introduced by an oligonucleotide in the PCR. In the second step, we amplified the 5´UTR region of the *elp2l* gene from honeybee cDNA. We cloned this fragment into the plasmid pGEM-T_fne_prom downstream of the transcription start by using the *BsaI/XbaI* restriction sites. Next, we cloned this entire *elp2l*-derived sequence into the pIZ/V5 His plasmid by using the *Hind*III/*Xba*I restriction sites. We amplified the CMV promoter sequence from the pEPI-EGFP plasmid that was kindly provided by Prof. Hans J. Lipps, University of Witten (Sinclair, 1987, Kunieda and Kubo, 2004, Stehle et al., 2007). The IE^{hr5} promoter sequence was amplified by PCR from the plasmid LA928, which was kindly provided by Dr. Luke Alphey, University of Oxford (Granados and Federici, 1986, Gong et al., 2005). The amplified CMV and IE^{hr5} promoter sequences were cloned into the pIZ/V5 His plasmid using the *Hind*III/*Xba*I restriction sites. We used the pIZ/V5 OpIE2 plasmid from Invitrogen (Darmstadt, Germany; (Theilmann and Stewart, 1992)). We inserted the *rubia* reporter gene expressing a red fluorescence protein downstream of the 5'UTRs by using the *Xba*I and *Sac*II restriction sites. To construct the pIZ/V5 *elp2l* EGFP plasmid, we replaced *rubia* with the *egfp* gene using the *Xba*I/*Sac*II restriction sites. The *egfp* gene was

amplified by PCR. We used the peGFP-C1 plasmid from Clonetech (Saint-Germain-en-Laye, France) and the pBluebac plasmid from Invitrogen (Darmstadt, Germany).

Cell culture

*Sf*21 cells (Biochrom, Berlin, Germany) were kept adherent at 27 °C in 6-well plates and maintained according to the manufacturer´s instructions. We transfected 2.5 µg of plasmid DNA into 10^6 cells using Roti insectofect reagent (Roth, Karlsruhe, Germany).

Expression analysis in *Sf*21 cells

We imaged each of the 10 wells representing independent transfection experiments for each construct. These images were taken under constant excitation and fluorescence detection settings to semi-quantify the relative expression of Rubia proteins for each of the putative promoter sequences. We measured the light intensities from the three most luminous cells of each picture (N=10). We dragged a line across each of the three most luminous cells in each picture, which produced an intensity profile in the LSM 510 Meta software. We estimated the average across the cells and pictures that resulted in relative intensity values (ranging from 0 to 250) for each promoter. Red fluorescence was detected by a confocal laser microscope (Zeiss LSM 510 Meta) at a wavelength of 561 nm 48 h after the transfection with the plasmids.

Expression analysis under temperature shift conditions

*Sf*21 cells were transfected with the plasmids containing either the *Am-hsp70*, *Am-hsp83* or *Dm-hsp70* promoter sequences and were kept at 27 °C for 48 hours prior to the temperature shift experiments. We either heated (34 °C) or cooled (16 °C) the *Sf*21 cells for 1.5 h. Immediately afterwards, we took four non-overlapping pictures of the cells in each well. To estimate the relative increase in fluorescence after the temperature shifts, we counted the number of cells above a fluorescence threshold. We took four non-overlapping pictures of the non-shifted transfected cells for each transfection and set the threshold above the brightest fluorescent cell in each picture. Based on these four thresholds, we generated a mean threshold for the respective transfection experiment. We counted the number of fluorescent cells

above this mean threshold for cells that were treated with higher or lower temperatures and those that were not. We calculated the relative proportion of fluorescent cells above the threshold to all of the cells that were present in the picture that we detected by white light settings. We compared the relative number of fluorescent cells under the shifted and non-shifted temperature conditions by the Mann-Whitney-U test, which we conducted in IBM SPSS Statistics (20.0.0).

Electroporation

Adult worker honeybees were caught, anesthetised on ice and immobilised by putting them in a small plastic tube. A total of 260 nl of the peGFP-C1, pBluebac and pIZ/V5 *elp2l* EGFP plasmids were injected into the median ocelle of a worker honeybee by using the method of Mussig et al. (2010), at a concentration of 2 µg/µl. Teflon insulated platinum wire electrodes (diameter 0.125 mm) were used. The insulation was removed at the tip of the electrodes and they were flattened by maintaining them between two metal plates and hitting them with a hammer. The electrodes were introduced in the complex eye, through a cut performed in the dorsal part of the complex eye. The electroporation of the brain was performed with 5 pulses for 25 ms at 100 V using a CUYEDIT 21 electroporator (Nepagene, Ichikawa, Japan). The mortality rate was of 41.4% (58/140 animals). The brains of the surviving bees were dissected three days after electroporation, fixed, dehydrated and cut into slices of 100 µm. They were stained with a mouse-derived antibody against GFP (mAB 3E6, Invitrogen, Darmstadt, Germany) and with a CY5-conjugated secondary antibody (goat anti-mouse, Jackson immunoresearch, Suffolk, UK) and imaged with the Leica TCS-SP2 confocal microscope using a 10x dry objective (HC PL APO CS 10x0.4, Leica, Bensheim, Germany). The Helium Neon (HeNe) 633 nm laser line was used to detect the Cy-5 signal (emission 670 nm). All brains were imaged at different laser powers: Figure 5A: laser power 50%, photomultiplier tube (PMT) 580; Figure 5B: laser power 70%, PMT 580; Figure 5C: laser power 94%, PMT 651. Thus the negative control brain was scanned at the highest intensity. Since we were interested only in detecting GFP signal and not in quantifying them, we did not normalize the positive brains to controls. In all pictures we used a pixel resolution of 1024 x 1024 in *xy* axis and an 8 bit intensity resolution. The voxel size was of 0.73 µm x 0.73 µm in figure 5A and of 1.46 µm x 1.46 µm in figure 5B and 5C.

Results

Honeybee derived promoter sequences direct gene expression in *Sf*21 cells

We amplified putative promoter sequences of *Am actin5c*, *elp2l*, *Am hsp70* and *Am hsp83* gene from the honeybee genome by PCR (Fig. 1). We cloned the fragments into the pIZ/V5-His plasmid (Invitrogen) upstream of the translational start codon of the reporter gene, *rubia*. We amplified a minimum of 1000 bp upstream of the translation start site of the corresponding gene which included the entire 5´ untranslated region (5´UTR). We identified 5´ ends of transcripts via RT-PCR experiments to assign putative transcriptional start points. Our putative promoter sequence of the *actin5c* gene of *Apis mellifera* (GB12453) encompasses a 1420 bp fragment upstream of the translation start site, including the entire 5´ untranslated region (UTR; Fig. 1). The *Am actin5c* gene is the ortholog of the *actin5c* gene of *D. melanogaster.* The fruit fly gene is expressed throughout development (Chung and Keller, 1990), and its promoter sequence has been employed to constitutively express transgenes (Chavous et al., 2001). The putative promoter sequence of the elp2l gene (GB18785) encompasses 2000 bp upstream of the translation start with 207 bp of that being the 5´UTR. The elp2l gene is annotated on NCBI as a homolog of the elav gene of *D. melanogaster* (further information on homology is shown in SI2). The *elav* promoter sequence has been widely used in transgenic fruit flies to drive the neuronal expression of genes (Samson and Chalvet, 2003; Wu et al., 2011). The putative promoter sequence of the *Am hsp70* gene (GB14852), including the 5´UTR, encompasses 1001 bp upstream of the translation start codon. The expression of the *Am hsp70* gene increased after heat shock in foraging honeybee workers (Elekonich, 2009), suggesting that the promoter sequence can drive expression after a heat shock. The putative promoter sequence of the *Am hsp83* gene (GB14494) encompasses 1000 bp sequence upstream of the translational start codon, including a 490 bp 5´UTR. We suggest the promoter of *Am hsp83* gene is a good candidate for driving temperature shift inducible transcription as the transcription of the ortholog gene *Se hsp83* in the lepidopteran insect *Spodoptera exigua* was inducible by cold shock (Xu et al., 2011). We listed the sequences of the putative promoters in the supplementary information (SI3).

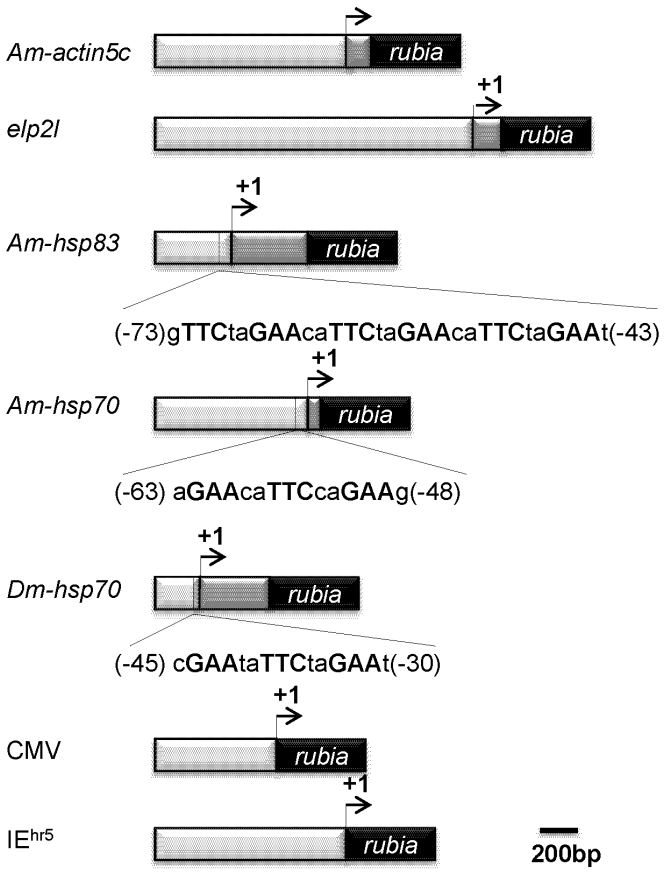

Figure 1: Schematic presentation of the expression cassettes that were cloned into the pIZ/V5-His plasmid. Light grey boxes indicate the genomic region upstream of the transcription start; dark grey boxes show the 5´UTR region. Black boxes indicate the region encoding for the red fluorescence protein Rubia. The scale indicates the sizes of the boxes (bp). The position of the HSE (heat shock element) sequence motifs and their position upstream of their respective transcription start sites are indicated below the boxes; +1 denotes the transcription start site.

We checked for conserved heat shock elements (HSEs) in the sequences of our putative *Am hsp70* and *Am hsp83* promoter sequences. These elements consist of a highly conserved nGAAn sequence or the corresponding palindromic sequence nTTCn that are present in different copy numbers. These elements are required to bind the heat shock factors that promote transcription of the heat shock protein genes (Pirkkala et al., 2001; Sakurai and Enoki, 2010). We found six HSEs 43 to 73 bp upstream of the transcription start site in the *Am hsp83* sequence (Fig. 1). In the *Am hsp70* sequence, we identified three HSEs 48 to 63 bp upstream of the transcription start site (Fig. 1). The presence of these reiterated motifs in the putative *Am hsp83* and *Am hsp70* promoter sequences suggests a possible role for these sequences in driving expression after temperature shock. We compared the gene

expression function of the honeybee sequences in subsequent experiments to the already characterised *hsp70* promoter of *D. melanogaster* (Pelham, 1982), which has been widely used as inducible promoter in transgenic flies (Monsma et al., 1988); to the CMV (human cytomegalovirus) promoter (Sinclair, 1987; Kunieda and Kubo, 2004); and to the baculo virus derived OplE2 and IEhr5 promoters (Granados and Federici, 1986; Theilmann and Stewart, 1992; Cartier et al., 1994).

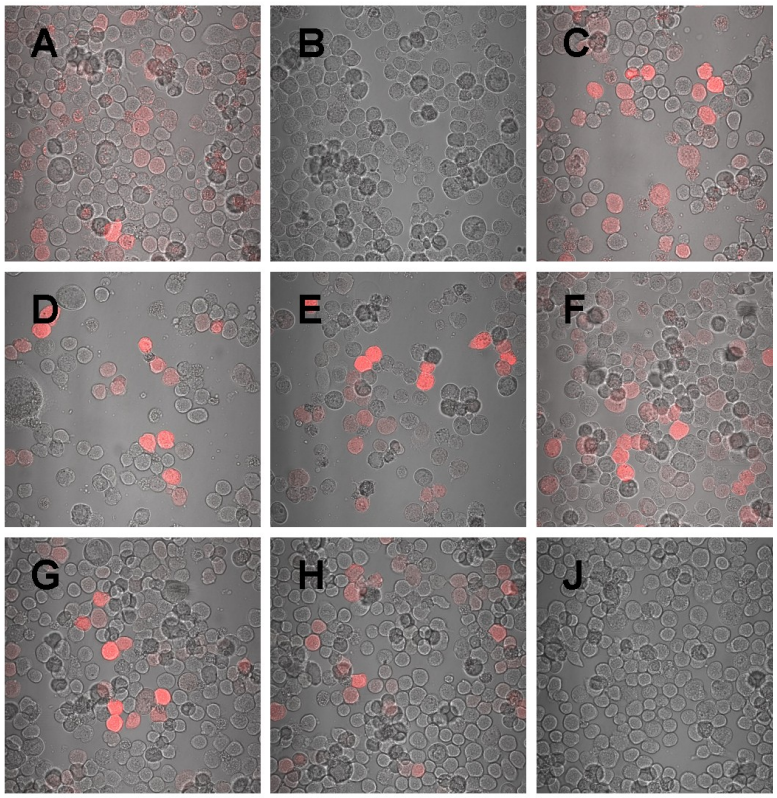

Figure 2: Expression of Rubia protein driven by the putative promoter sequences in *Sf*21 cells. The images are presented as overlays of the red fluorescence detection picture and the bright light picture, which were taken by a confocal microscope. A-H: 10^6 cells were transfected with 2.5 µg of the pIZ/V5 plasmid driving Rubia protein expression with the indicated putative promoter sequences. **A:** *Am-actin5c*; **B:** *elp2l*; **C:** *Am-hsp70*; **D:** *Am-hsp83*; **E:** *Dm-hsp70*; **F:** OplE2; **G:** CMV; **H:** IE^{hr5}; **J:** control, untransfected *Sf*21 cells.

We transfected 2.5 µg of the respective plasmids into 106 Sf21 cells using Roti Insectofect reagent. We studied the *Sf*21 cells 48 h after transfection to determine whether they showed red fluorescence signals compared to non transfected control cells. In *Sf*21 cells that were transfected with plasmids containing the *Am actin5c, Am hsp70* or *Am hsp83* putative promoter sequences, we observed red fluorescence compared to untransfected cells (Fig. 2A, C, D, J), suggesting that the cloned honeybee sequences can drive expression of the Rubia protein in *Sf*21 cells. We also observed red fluorescence in cells that were transfected with vectors containing the *Dm hsp70*, the OplE2, the IEhr5 and the CMV promoter sequences (Fig. 2 E-H). Cells transfected with the plasmid containing the putative *elp2l* promoter sequence displayed no fluorescence (Fig. 2B), suggesting that the Rubia protein was not expressed. This result shows that the elp2l promoter, which is supposed to drive

expression in the neuronal substrate in honeybees, does not drive expression in *Sf*21 cells, which were derived from ovarian tissues. This negative result, however, indicates that the core promoter sequence alone is not sufficient to drive expression of the reporter gene in our assay.

Table 1: The relative strength of the honeybee promoter sequences in *Sf*21 cells as measured by fluorescence intensities of Rubia proteins.

promoter	expression strength
Am-actin5c	++
elp2l	-
Am-hsp70	+
Am-hsp83	+++
Dm-hsp70	+++++
OplE2	+++++
CMV	++
IE^{hr5}	++++
untransfected	-

Red fluorescence intensities were measured from the picture data. The three brightest cells per picture for each promoter sequence were counted. We averaged the fluorescence intensity values from 10 independent transfection experiments. We ordered the values of the fluorescence intensities from "+++++" (OplE2) to "-" (untransfected).

We compared the relative strength of the promoter activity by semi quantifying the fluorescence intensity of single cells. Because the fluorescence intensities of transfected cells are strongly affected by the stage of cell's cell cycle, we choose the three brightest cells for each picture. These cells should represent cell samples that are presumably at similar cell cycle stages and this procedure allows us to approximate fluorescence intensities and therefore relative expression levels for each promoter. We analyzed 10 independent transfection experiments for each promoter. For each transfection we imaged a set of cells (we detected per picture a mean cell number of 134 (±44 SEM) cells and 17(±7 SEM) transfected cells; SI4 shows the number of detected cells for each promoter construct) and measured the average

light intensity values for each of the three brightest cells in the picture. We used the same excitation and detection settings at the microscope and the relative light intensities could range from 0 to 250.

We found according to the arbitrary fluorescence intensities the following order of expression (high to low): OplE2, *Dm hsp70*, IEhr5, *Am hsp83*, *Am actin5c*, CMV and *Am hsp70* (Tab. 1). This result indicates that the honeybee derived promoter sequences induce lower levels of expression than the promoters that are derived from the baculovirus (OplE2, IEhr5)). These viral promoters are strong promoters and are highly adapted to the lepidopteran *Sf*21 cells (Theilmann and Stewart, 1992; Cartier et al., 1994).

The *Am hsp83* promoter derived expression is enhanced by temperature shift

We studied whether the expression level of the rubia reporter gene driven by the *Am hsp70*, *Am hsp83* and *Dm hsp70* promoters can be enhanced by shifting the constant incubating temperature of 27 °C to higher (34 °C) or lower (16 °C) temperatures for 1.5 hours. From one transfection experiment we split the transfected cells and studied the effect of temperature shifts and non-shifts on expression in independent cell samples. We did not study the same cell samples before and after the temperature shifts because the microscopical observations and microbial infection of the cell culture (due to these observations) could both stress the cells and could possibly induce expression that is independent of our treatment (the temperature shifts). We first determined whether the fluorescence intensities of the untransfected *Sf*21 cells were affected by temperature shifts, but we did not observe any effect (data not shown).

To estimate the relative increase in fluorescence after the temperature shifts, we obtained a fluorescence threshold under non-shifted condition (constant temperature of 27 °C). We took four pictures of different cells (mean number of detected cells per picture 145 (±58 SEM)) for each transfection in which the temperature was non-shifted and set the threshold above the brightest fluorescent cell for each picture. From these four thresholds, we built a mean threshold for the respective transfection. We shifted the temperature (to 16 and 34°C for 1.5 h) of the independent cell samples (but derived from the same transfection) and determined the number of cells above that threshold (Fig. 3; 4) that we obtained from the non-shifted cell samples. If more fluorescent cells over the threshold were detected after a

temperature shift this was taken as evidence of an increase in expression due to the temperature shift. To determine these numbers, we took four pictures of different cells for each transfection. Each of these pictures (shifted and non-shifted cells) comprises a mean number of 145 (±58 SEM) cells and 10 (±6 SEM) transfected cells (SI5 shows the detailed numbers of cells for each promoter construct). All over we studied for each promoter construct cells from nine different transfection experiments.

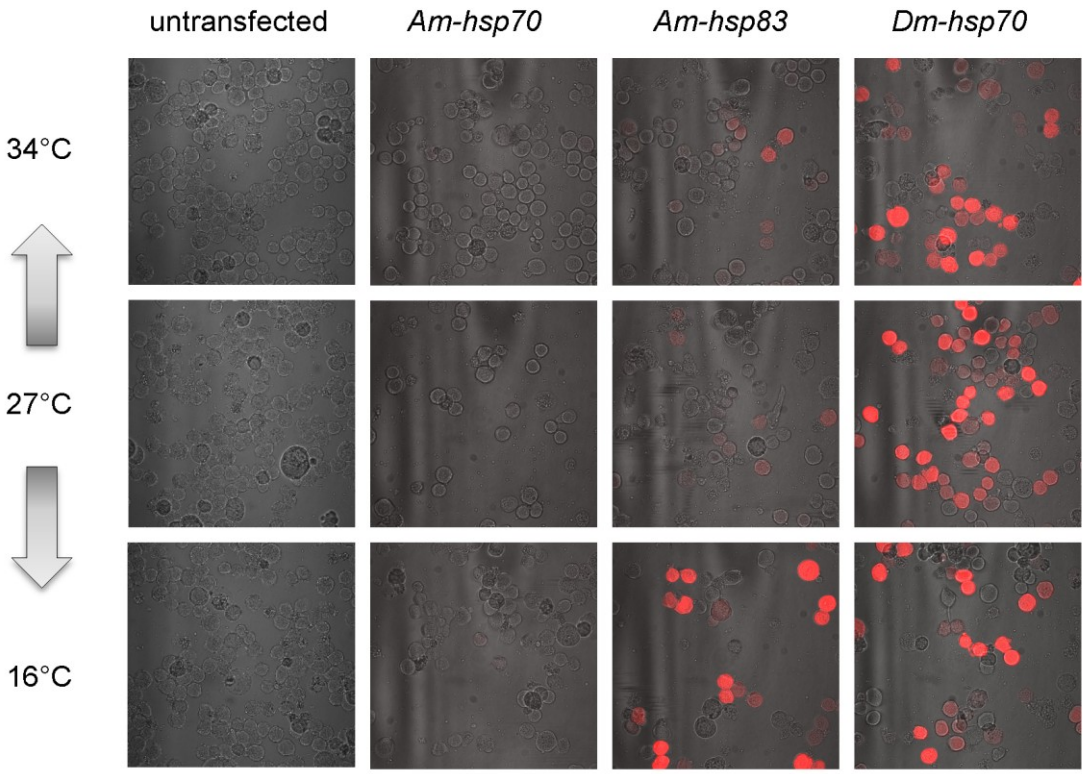

Figure 3: The effects of temperature shifts on *hsp* promoter-derived expression of Rubia protein in *Sf*21 cells. Temperature shifts were performed for 1.5 hours 48 h after transfection. Heat shock was performed at 34 °C; cold shock was performed at 16 °C.

For the *Am hsp83* promoter sequence, the number of fluorescent cells above the threshold increased almost three-fold after 16 °C treatment (Mann Whitney U test; $p < 0.001$; Fig. 3; 4A) compared to the number of cells above the threshold in the constant temperature condition. Temperature shifts to 34 °C did not increase the number of fluorescent cells ($p > 0.05$; Fig. 3, 4A). We conclude that shifting the temperature to 16 °C increases the Am hsp83 promoter-driven expression of Rubia protein in *Sf*21 cells. Shifting the cells containing the *Am hsp70* or the *Dm-hsp70* promoter sequence to 16 °C or to 34 °C did not increase the number of fluorescent cells above the threshold compared to the cells held at a constant temperature (Mann

Whitney U test, p > 0.05; Fig. 3, 4B, C). The latter result suggests that temperature shifts were not able to enhance protein expression of genes regulated by the *Am hsp70* or *Dm hsp70* promoter sequences in *Sf*21 cells.

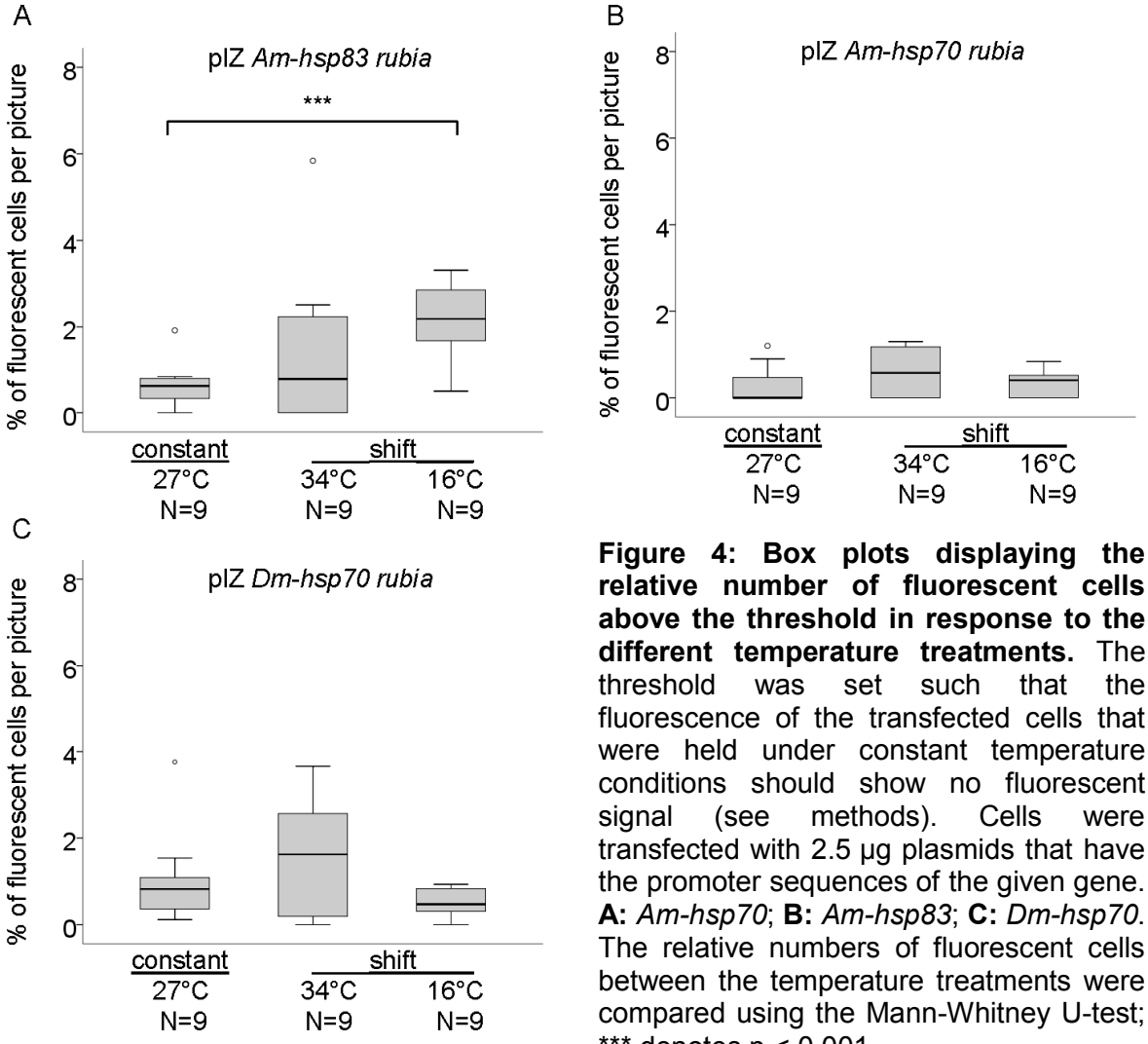

Figure 4: Box plots displaying the relative number of fluorescent cells above the threshold in response to the different temperature treatments. The threshold was set such that the fluorescence of the transfected cells that were held under constant temperature conditions should show no fluorescent signal (see methods). Cells were transfected with 2.5 µg plasmids that have the promoter sequences of the given gene. **A:** *Am-hsp70*; **B:** *Am-hsp83*; **C:** *Dm-hsp70*. The relative numbers of fluorescent cells between the temperature treatments were compared using the Mann-Whitney U-test; *** denotes p < 0.001.

The *elp2l* promoter sequence can direct gene expression in the honeybee brain

Our study showed that the *elp2l* promoter sequence is not able to drive expression of the Rubia protein in *Sf*21 cells, which are ovarian derived (Fig. 2E). A first survey of transcription in different honeybee tissues via RT-PCR proposes a confined expression of the *elp2l* honeybee gene in neuronal tissue (Schulte, personal communication). To further test, whether the putative *elp2l* promoter sequence can direct expression in honeybee nervous tissue, we introduced the pIZ/V5 *elp2l* EGFP

plasmid into honeybee brains via electroporation. This pIZ/V5 *elp2l* EGFP plasmid consists of the *elp2l* promoter sequence, which was cloned in front of the EGFP reporter gene. The electroporation method for honeybees was introduced by Kunieda and Kubo (2004) to introduce DNA into the honeybee brain. We injected 520 ng of the respective plasmid into each brain via the middle ocellus and electroporated them afterwards. In addition to the pIZ/V5 *elp2l* EGFP plasmid, we also electroporated the peGFP-C1 plasmid that contains a CMV promoter, which has been previously shown to drive EGFP protein expression in the honeybee brain (Kunieda and Kubo, 2004). We also electroporated with the pBluebac plasmid, which comprises the polyhedrin gene promoter sequence from baculovirus and comprised no reporter gene. We employed this plasmid as negative control to avoid non-specific effects from the electroporation procedure. Three days after the electroporation (58.6 % of the bees survived), we immunostained the brains and detected the EGFP protein via mouse anti-EGFP antibody and a secondary goat anti-mouse antibody, which is conjugated with the fluorophore Cy5. Electroporation with the pIZ/V5 *elp2l* EGFP plasmid produced Cy5 fluorescence signals in distinct parts of the brain, indicating that the EGFP protein is expressed (N = 4; Fig. 5A). The expression is found in peripheral

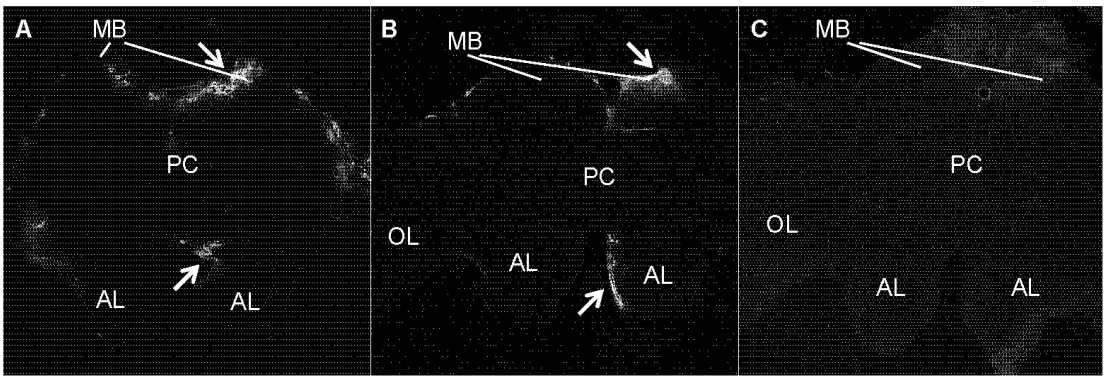

Figure 5: Immunostaining of EGFP protein expression after electroporation of plasmid DNA into the honeybee brain. Examples of the immunostaining of EGFP protein with mouse-derived anti-EGFP antibody that was detected with a Cy5-conjugated goat anti-mouse antibody. **A:** peGFP-C1; **B:** pIZ/V5 *elp2l* EGFP; **C:** pBluebac; AL: antennal lobe; PC: protocerebrum; MB: mushroom bodies; OP: optical lobes; arrows indicate regions of strong Cy5 fluorescence signal denoting EGFP expression.

parts of the protocerebrum, the antennal lobes and the mushroom bodies. When we electroporated brains with peGFP-C1, which served as a positive control (Kunieda and Kubo, 2004), we detected a similar Cy5 fluorescence pattern in the brain as with our pIZ/V5 *elp2l* EGFP plasmid (N = 3; Fig. 5B). This result suggests that the CMV

and the *elp2l* promoter produce similar expression patterns only in the peripheral parts of the brain, most likely due to the lack of entire spreading of the DNA during the electroporation procedure. In brains which we electroporated with the pBluebac plasmid (N = 5; Fig. 5C), we detected no fluorescence, indicating that our method produced no artificial Cy5-fluorescence signal. Taken together, these results show that our honeybee derived *elp2l* promoter sequence can direct expression of genes in the honeybee brain.

Discussion

In this study, we cloned several general, tissue specific and inducible candidate promoter sequences from the honeybee (*A. mellifera*) and showed that the general (*Am-actin5c*) and inducible promoters (*Am-hsp70* and *Am-hsp83*) can drive expression in lepidopteran-derived *Sf*21 cells. We also showed that the *Am-hsp83* is a cold shock-inducible promoter in *Sf*21 cells. Because we lack transgenic methods in honeybees, we took advantage of the *Sf*21 cell system. If the candidate promoters from the honeybee genome can induce expression in the heterologous system of *Sf*21 cells, we took it as a first hint that they may also drive expression in honeybees. We also identified a candidate of tissue specific promoter that can drive expression in nervous cells of the honeybee brain but not in *Sf*21 cells. Hence, we identified candidate honeybee promoters that can drive ubiquitous, nervous tissue specific and inducible expression of transgenes in the honeybee.

We showed that the *Am-actin5c* promoter sequence can drive gene expression in *Sf*21 cells, suggesting that this sequence harbours the relevant sequence information required to promote transcription in this cell line. In *Sf*21 cells, the *Am-actin5c*-derived expression is weak compared to viral promoters. In *D. melanogaster,* the endogenous promoter sequence of the *actin5c* gene has been widely used to drive constitutive overexpression of transgenes (Bond and Davidson, 1986, Thummel et al., 1988). We thus also suggest that the *Am-actin5c* promoter sequence can be used in an equivalent way to promote constitutive expression of transgenes in the honeybee.

We showed that the putative *elp2l* promoter cloned fragment could not drive gene expression in *Sf*21 cells. This result suggests that the general core promoter sequence that is located directly upstream of the transcription start site and contains the putative TATA-Box and the putative initiator sequence is not sufficient to drive gene expression in the *Sf*21 cells. This negative result shows that the core promoter sequence alone is not sufficient to direct expression of the reporter gene in *Sf*21 cells. We electroporated the *elp2l* promoter sequence into the brain of honeybees and showed that this sequence can drive gene expression in the nervous tissue. We suggest that activation of the *elp2l* promoter requires some species or tissue specific factors (the *Sf*21 cells are derived from ovarian cells) to promote transcription. The promoter sequence of the *D. melanogaster elav* gene, which is a homolog of the

elp2l gene, has been widely used to express transgenes in neuronal tissues (Yao and White, 1994, McGuire et al., 2004). For instance, Wu et al. (2011) rescued *D. melanogaster* mutants of neuronal receptors PlexB by expressing the *plexB* gene under the control of the *elav* promoter in all neurons. The *elav* gene of *D. melanogaster* has two paralogous copies, the *fne* (*found in neurons*) and the *rbp9* (*riboprotein 9*) genes (Samson and Chalvet, 2003), whereas the *elp2l* gene of the honeybee is a single copy gene, that expresses in neuronal tissues, but not in muscle tissues (Schulte, personal communication). All of the fruit fly paralogous genes are expressed solely in neuronal tissues. The Elav protein is present in all neurons throughout development (Robinow and White, 1991), whereas proteins from the *fne* gene were found in neurons of the central nervous system and in the peripheral nervous system during embryogenesis (Samson and Chalvet, 2003) of the fruit fly. *rbp9* is exclusively expressed in neurons of the CNS in *D. melanogaster* (Kim and Baker, 1993). From our experimental evidence, we suggest that the *elp2l* promoter can direct expression of transgenes in the honeybee brain.

We also identified *Am-hsp83* as a cold shock inducible promoter in *Sf*21 cells. A heat shock at 34 °C did not increase the level of expression in *Sf*21 cells. A previous study that measured the level of *Am*-Hsp83 protein expression in the honeybee showed that the *Am-hsp83* promoter is also inducible by a heat shock (Chacon-Almeida et al., 2000). Chacon-Almeida et al. (2000) showed that honeybee workers treated for 4 h with 42 °C showed a higher level of *Am*-Hsp83 protein expression than control worker bees, which were maintained at 34 °C. We suggest that in our study, the temperature for a heat shock was not high enough or that the *Sf*21 cell system cannot mimic the cellular heat shock conditions of honeybees. Consistent with our results, the *Se-hsp83* gene in *Spodoptera exigua*, a lepidopteran insect, also increased the level of transcription after cold shock (Xu et al., 2011). We thus suggest that we have identified a promoter sequence of the honeybee that can induce higher levels of expression after a temperature shift, *Am-hsp83*.

The *Am-hsp70* promoter was not inducible after temperature shifts from 27 °C to higher (34 °C) or to lower (16 °C) temperatures. However, Elekonich (2009) showed that the amount of *Am*-Hsp70 protein in the heads of worker honeybees increases after shifting the temperature from 33 °C to 42 °C or 46 °C for 4 h, suggesting that the *Am-hsp70* gene is a heat inducible gene, which is consistent with our identification of three heat shock elements in the cloned fragment. A similar motif

consisting of three HSEs is present as well in the promoter sequence of the *D. melanogaster hsp70* gene, a motif which is required for temperature shift induced expression in that species (Pelham, 1982). The heat shock elements indicate in general a temperature-associated expression pattern of the downstream gene (Lindquist, 1986, Sakurai and Enoki, 2010). Therefore, we speculate that we have cloned the *Am-hsp70* promoter sequence but that the temperature shift we applied was not sufficient or that the *Am-hsp70* promoter requires cellular conditions of the honeybee that we cannot mimic in *Sf*21 cells. However it cannot be excluded that we missed regulatory regions of the promoter that are important to control the heat shock induced expression. The *Am-hsp83* promoter sequence contains a HSE motif consisting of six HSEs, whereas the *Am-hsp70* promoter sequence only contains three HSEs. This finding supports our result, that the activity of *Am-hsp83* promoter can get increased during a temperature shift whereas the activity of *Am-hso70* promoter cannot get increased under the same conditions.

Transgenic studies in the fruit fly *D. melanogaster*, the medfly *C. capitata* and the beetle *T. castaneum* showed that endogenous promoters are essential tools for controlling gene expression in the respective species (Kalosaka et al., 2006, Schinko et al., 2010). Overexpression and ectopic expression of genes and knockdown studies that were mediated by endogenous promoters have been very informative for gaining insights into the underlying genetic programs in *D. melanogaster* (McGuire et al., 2004). The honeybee promoter sequences that we identified here are thus valuable genetic tools for controlling the expression of transgenes in the honeybee. A transgenic method has yet to be established in honeybees, but stable transformants mediated by transposons have been successfully applied in different insect species (OBrochta and Atkinson, 1996). *PiggyBac* and *Minos* are some of the more widely used transposons. Both transposons are absent in the honeybee genome and are good candidates to produce transgenic honeybees. The *piggyBac*-mediated transposition of transgenes has been used in at least 13 insect species that belong to three insect orders (Lepidoptera, Diptera and Coleoptera; reviewed in Handler (2002)). *Minos*-derived transpositions have been used in a wide range of species, for instance, in the mosquito *Anopheles stephensi* (Catteruccia et al., 2000), in the coleopteran *T. castaneum* (Pavlopoulos et al., 2004) or in the medfly *C. capitata* (Zwiebel et al., 1995) and even outside the class of insects in the crustacean

Parhyale hawaiensis (Loukeris et al., 1995, Catteruccia et al., 2000, Pavlopoulos et al., 2004).

The control of transgene expression by endogenous promoters will greatly facilitate the genetic analysis of the rich behavioural repertoire, the social organisation, caste differentiation and sex determination of the honeybee. The *Am-actin5c* promoter is a candidate for driving ubiquitous expression, while the e*lp2l* promoter can promote expression in the brain. The *elp2l* promoter may thus enable us to study genes that specify the neuronal substrate that is orchestrating the rich behavioural repertoire of the honeybee. The *Am-hsp83* promoter will allow us to direct the expression or gene knockdowns by temperature shifts, which will enable us to study the function of genes at defined developmental stages.

Taken together, the identification of the first honeybee promoter sequences is an initial and important step in studying the function of genes by transgenic tools in the brain or at different developmental stages in the honeybee, an organism that displays a rich set of interesting and unexplored phenotypes.

Acknowledgments

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Supplementary information

SI1: Sperm mediated or microinjected plasmid transfer into honeybee embryos: pilot experiments

Microinjection: We injected 10 and 50 pg of the plasmids pIZ/V5 His *Am-actin5c rubia*, pIZ/V5 His *elp2l rubia* or pIZ/V5 His CMV *rubia* per egg into honeybee embryos but we could not observe any reporter gene expression. Injecting high concentrations of plasmid DNA had a lethal effect on the embryos.

Sperm mediated plasmid transfer: We introduced plasmid DNA into honeybee eggs via sperm mediated gene transfer (SMGT) (Francolini et al., 1993). We mixed 5µl honeybee sperm with different concentrations of plasmid DNA (50, 500, 5000 or 15000 ng) pIZ/V5 His *Am-actin5c rubia* or pIZ/V5 His CMV *rubia.* We instrumentally inseminated 24 (3 with 50ng, 4 with 500 ng 5 with 5000ng and 12 with 15000 ng) honeybee virgin queens with this mixture following the procedure described in Robinson et al. (2000). From these 24 queens 19 produced offspring. We collected 50 L1-larvae from each queen, isolated DNA. We performed PCRs designed to amplify fragments of the pIZ/V5 plasmids, that were inseminated mixed with the sperm. We were not able to amplify the corresponding fragments of pIZ/V5 plasmids in any of the offspring, suggesting that the copy number of the sperm mediated transfer of plasmids was too low to be detected in the PCRs.

SI2: Blastp search of *D. melanogaster* Elav protein amino acid sequence against the honeybee protein database at NCBI

Score	Expect	Methods	Identities	Positives	Gaps
427 bits	2e-146	Compositional matrix adjust.	224/389(58%)	267/389(68%)	68/389(17%)

```
Query  136  GSQNGSNGSTETRTNLIVNYLPQTMTEDEIRSLFSSVGEIESVKLIRDKSQVYIDPLNPQ  195
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Sbjct  15   GSTLGQASQEESKTNLIVNYLPQSMTQDEIRSLFSSIGEVESCKLIRDKL----------  64

Query  196  APSKGQSLGYGFVNYVRPQDAEQAVNVLNGLRLQNKTIKVSFARPSSDAIKGANLYVSGL  255
                GQSLGYGFVNY RP+DAE+A+N LNGLRLQNKTIKVS+ARPSS+AIKGANLYVSGL
Sbjct  65   ---TGQSLGYGFVNYHRPEDAEKAINTLNGLRLQNKTIKVSYARPSSEAIKGANLYVSGL  121

Query  256  PKTMTQQELEAIFAPFGAIITSRILQN---------AGNDTQ------------------  288
            PK MTQQ+LE +F+P+G IITSRIL +           G D
Sbjct  122  PKNMTQQDLENLFSPYGRIITSRILCDNITVRQFVTGGGDYLPEKMMMDPLNNLNRLPTG  181

Query  289  -TKGVGFIRFDKREEATRAIIALNGTTPSSCTDPIVVKFSNTPGSTSKIIQPQLPAFLNP  347
             +KGVGFIRFD+R EA RAI  LNGT P   ++PI VKF+N P + +K I P   A+L P
Sbjct  182  LSKGVGFIRFDQRVEAERAIQELNGTIPKGSSEPITVKFANNPSNNNKAIPPL--AYLTP  239

Query  348  QLVRRIGGAMHTP------------VNKGLARFSPMAGDML-DVMLPNGLGAAAAAATTL  394
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Sbjct  240  QATRRYGGPIHHPTGRFSTGKAMLAINKGLQRYSPLAGDLLANSMLP---GNAMN-----  291

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SI3: Sequences with the designated promoter regions

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elp2l (2000 bp)

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Am-hsp70 (1001 bp)

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Am-hsp83 (1000 bp)

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attgatttacgtttcttattaaatgttgttctaaatttaatagttttattaaaaaattaatc
ttaactctattttaacacatttttttattcgtataaatttgtttttttttaaattgtatcgg
gaaatattattgcatttattatacaataataaaataatatatatcacatgtaaaaaaaaaaa
aaaaaaataagaaatgcatatataaaatgtttcatatataaattaaaaatagaaatttatat
ttgtgattttatgtaatttttgattgtttaaatttttttttttctaatttaatttttctt
ttttacag

SI4: The mean numbers of *Sf*21-cells and fluorescent *Sf*21-cells per picture that were used to study the promoter activity. The numbers were estimated from pictures taken from N=10 transfections for each promoter construct.

promoter	mean number of cells per picture (±SEM)	mean number of fluorescent cells per picture (±SEM)
Am-actin5c	132±42	16±7
elp2l	143±43	0
Am-hsp70	100±39	10±9
Am-hsp83	112±36	16±6
Dm-hsp70	134±54	13±4
OpIE2	148±42	23±4
CMV	149±33	18±5
IE^{hr5}	148±36	20±6
untransfected	140±39	0

SEM: standard error of the mean

SI5: The mean numbers of *Sf*21-cells per picture detected in pictures that were used to study the impact of temperature shifts.

promoter	temperature	mean number of cells per picture (±SEM)	mean number of fluorescent cells per picture (±SEM)
Am-hsp70	27 °C	131±36	9±3
	34 °C	187±40	12±4
	16 °C	160±45	13±2
Am-hsp83	27 °C	125±51	7±3
	34 °C	128±56	7±4
	16 °C	152±55	8±4
Dm-hsp70	27 °C	130±57	13±5
	34 °C	177±60	17±5
	16 °C	120±58	13±9

SEM: standard error of the mean

SI6: Oligonucleotides that were used in PCRs:

number	name	sequence
#011	elavProm1fw	GATCGAATTCTGATCAGATCGATCGGTCTCAAGATTACGTCAGCATCCAGTTGCGT
#012	elavProm1rev	GATCACCGGTTCTAGATGTGTCCATTCCGTTCGCCAT
#013	elavProm2/3fw	GATCAAGCTTTGACCCACCTGCTAACCTTCGTTG
#014	elav_Prom2/3rev	GATCGGTCTCAATCTGACAATGACTTTCAAAATACAGCGT
#026	*Am-actin5c*_fw_*Hind*III	GATCAAGCTTCACTCCTGATACTGAATATTATCGATG
#027	*Am-actin5c*_rev_*Xba*I	GATCTCTAGACATTTTAAATAAAAAATTATTTTACT
#039	CMV_fw_*Sac*I	GATCGAGCTCTAGTTATTAATAGTAATCAATTACG
#040	CMV_rev_*Xba*I	GATCTCTAGAGATCTGACGGTTCACTAAACCAGCT
#041	cer_fw_*Nhe*I	GATCGCTAGCATGGTGAGCAAGGGCGAGGAGCTGT
#043	cer_rev	GATCCCGCGGCTTGTACAGCTCGTCCATGCCGAGA
#050	IEhr5_fw_*Hind*III	GATCAAGCTTGCTTTACGAGTAGAATTCTACGCGTAAAACACAATCAAGTATGAGTCATA
#051	IEhr5_rev_*Xba*I	GATCTCTAGACTTGTCGCCGCCAGTGTCAACTTGC
#067	*rubia*_fw_*Xba*I	GATCTCTAGAATGGCCTCCTCCGAGGATGTCATCA
#068	rubia_rev_*Sac*II	GATCCCGCGGTCAGGATCCAGCGCCTGTGCTATG
#153	*Dm-hsp70*_fw_*Sac*I	GATCGAGCTCGACAACAACAGTCTTGACAACCTTTACG
#155	*Am-hsp83*_fw_*Sac*I	GATCGAGCTCTTGCCGCGTAATATCAAACACATGGT
#157	*Dm-hsp70*_rev_*Nhe*I	GATCGAGCTCGACAACAACAGTCTTGACAACCTTTACG
#158	*Am-hsp83*_rev_*Nhe*I	GATCGCTAGCCTGTAAAAAAGAAAAATTAAATTAGAAAAAAAAAAAAT
#Mi20	*Am-hsp70*_fw	AGTCAAGCTTTGCGTCACAATTGACGCAAAATGTAC
#Mi22	*Am-hsp70*_rev_*Xba*I	AGTCTCTAGACCATCTTTGCTTTTTTCTTTCTTCTTTGTTCGCTTGGTAAAAATTC

Author´s Contribution

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- Identification and isolation of promoter sequences *Am-actin5c*, *elp2l*, *Am-hsp70* and *Am-hsp83*
- Cloning of promoter sequences into pIZ/V5 vector (except *Am-hsp70*)
- Transfection of plasmids into *Sf*21 cells
- Heat-shock experiments
- Fluorescence analysis and statistics
- Electroporation (together with Nora Gehne and Gerard Leboulle)
- Authoring the manuscript

Manuscript II

Genetic transformation of honeybees (*Apis mellifera*) via transposon mediated integration

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Key words:
Apis mellifera, germline transformation, *piggyBac* transposon

Abstract

The honeybee, *Apis mellifera*, lives in complex social societies, displays a rich behavioral repertoire, caste differentiation and has an interesting mode of sex determination. We have still little understanding of the molecular control of development, physiology and behavior that is associated with the remarkable biology and the social system. Partly this is due to lack of genetic tools to manipulate the function of genes in the honeybee. Here we report on a method which has produced stably integrated and expressed transgenes in the honeybee genome. We established an efficient experimental procedure to achieve a transformation rate of 24 % using a *piggyBac* derived transposon. We showed stable integration in the offspring and found that we can use the marker 6xP3-*rubia* to preselect transgenic, haploid males that can be used for sperm recovery and breeding. We further showed that we can introduce a honeybee derived promoter sequence (*Am-actin5c*) into the genome to activate transcription of the second reportergene *egfp*. By mis-expression or RNAi mediated knockdown of gene transcripts via the transgene we can now constant manipulate genes in entire colonies. This will allow us to study the role of genes in a complex society and the individual member which by social interaction displays such fascinating biology.

Introduction

Honeybees (*Apis mellifera*) live in complex and structured social societies. Their colonies consist of tens of thousands of essentially sterile female workers, one single fertile female, the queen, and several hundreds of male drones (Winston, 1987). The worker/queen caste differentiation is determined by differential feeding (Haydak, 1970) of the early female larvae that effects differences in DNA methylation and in the *epidermal growth factor receptor* (*egfr*) signaling pathway (Kucharski et al., 2008, Kamakura, 2011b). The sexual development is controlled by the *complementary sex determiner* gene (*csd*). Heterozygous *csd* genotypes produce queens/workers and hemizygous genotypes males (Beye et al., 2003b, Gempe et al., 2009a). The collective behaviors of the colony members (Robinson, 1992, Page and Erber, 2002) produce well adapted group phenotypes in a changing environment. For instance the worker bees thermoregulate their nest, collectively defend against diseases and predators, and communicate about the location and quality of food sources via waggle dances (von Frisch, 1967, Wilson, 1971, Michener, 1974, Seeley, 1995). Workers adjust their behaviors to the environment by learning information flexibly (Menzel et al., 2006) and by cognitive performances that were thought to occur only in some vertebrate species (Giurfa et al., 2001).

We have still little understanding of the genetic control of the remarkable features that are associated with the biology of the social system in part, due to the lack of a genetic transformation system. Some insights have been gained by the perturbation of pheromones, endocrine systems and signalling cascades (Robinson et al., 2008, LeBoeuf et al.). This has been done by applying the relevant molecules, molecular analogous or antagonists and by inducing RNAi (Jaycox et al., 1974, Jaycox, 1976, Robinson, 1987, Gempe et al., 2009b, Kamakura, 2011a, Liang et al., 2012, Nissen et al., 2012). In genetic traceable organisms, including the fruit fly and the mouse, gene manipulations by stable genetic transformation have provided fruitful insights into the causal relationship of genes and the development, physiology or behaviour of an organism.

Long generation time and laborious maintenance of reproductive individuals in single honeybee colonies are major obstacles in producing transgenic honeybee lines. On the other hand the possibility of controlled breeding by instrumental insemination (Harbo, 1985a, Harbo, 1985b), the fact that males are haploid and that

a single queen can produce up to 1500 offspring per day (Gary, 1992, Katzav-Gozansky et al., 2001) offer some advantages of the honeybee system.

Transposable elements that have the ability to integrate into different loci of the genome have been successfully applied to produce stable genetic transformation in insects (reviewed in (Atkinson et al., 2001, Fraser, 2012). The *piggyBac* transposon, that was derived from the cabbage looper *Trichoplusia ni* (Cary et al., 1989), was able to transform species of different insect orders with similar frequencies (2-5%) including the silk moth *Bombyx mori* (Tamura et al., 2000), the mediterranean fruit fly *Ceratitis capitata* (Handler et al., 1998) and the sawfly *Athalia rosae* (Sumitani et al., 2003). Expression of flourescent proteins in the eyes or in the central nervous system driven by an artificial promoter, consisting of *Pax6*-response enhancer elements (P3) in combination with a core promoter sequence, named 3xP3, has been used as a transformation marker in a variety of insects (Berghammer et al., 1999, Horn and Wimmer, 2000, Horn et al., 2002).

Here we report stable genetic transformation of the honeybee that will allow further molecular advances in understanding the remarkable biology of this social organism. We established 6xP3-*rubia* as an efficient fluorescent marker integrated via elements of the *piggyBac* transposon into the honeybee genome. We developed injection and rearing methods that allowed us to obtain germline transformed queens at a high frequency. We have also shown via integration that a honeybee specific promoter can drive targeted expression of a second transgene, indicating that our transformations are suitable to manipulate gene functions in the honeybee.

Experimental Procedures

DNA preparation, PCR and sequencing

Genomic DNA was isolated with Phenol/Chloroform extraction (Hunt and Page, 1995) from honeybee larvae in instars 2-5. Plasmid preparation for microinjections was done with Plasmid Midi Kit (Qiagen, Hilden, Germany). Isolated genomic or plasmid DNA was diluted in ddH_2O.

PCR fragments that were subject to cloning were amplified with Phusion High Fidelity polymerase (Finnzymes, Vantaa, Finnland) under standard PCR conditions. Cloned nucleotide sequences were determined by sequencing (MWG Eurofins, Ebersberg, Germany).

PCR conditions for transformation analysis were an initial 94 °C step for 180 s, followed by 35 cycles of 94 °C for 30 s, 54 °C for 40 s, 72 °C for 30 s and then by a 72 °C terminating step for 180 s.

PCR for chromosomal walk was performed as follows: The first PCR for the 5´-integration site was performed with oligonucleotide primer pair #96/#105 and for the 3´-integration site with oligonucleotide primer pair #101/#105. The first PCR was performed under the following conditions: initial 94 °C step for 300 s, followed by 35 cycles of 94 °C for 30 s, 60 °C for 40 s, 72 °C for 60 s and then by a 72 °C terminating step for 180 s. We diluted the resulting PCR products by 1:50 and used this as template for the second PCR. In the second PCR we used oligonucleotide primer pair #102/#103 or #104/102 to amplify the 5´- or the 3´-integration site. The second PCR was performed with an initial 94 °C step for 300 s, followed by 25 cycles of 94 °C for 30 s, 62 °C for 40 s, 72 °C for 60 s and then by a 72 °C terminating step for 180 s. The amplified fragments were cloned into T-overhang vector pGEMT (Promega, Mannheim, Germany) and sequenced (MWG Eurofins, Ebersberg, Germany). Blastn searches were done against the honeybee genome library on http://blast.ncbi.nlm.nih.gov/.

The sequences of the oligonucleotide primers that were used in this study are listed in the supplementary information.

Cloning of marker genes into the vector

We replaced the reportergene *dsRed* with *rubia* gene via *Afl*II/*Eag*I restriction sites in the plasmid pBac [6xP3 *Tc-hsp core dsRed* Express SV40] that was kindly

provided by Dr. Gregor Bucher, Georg August University, Göttingen, (Posnien et al., 2011, Schinko, 2012). Rubia protein is an mRFPmars-like red fluorescence protein with four amino acid differences (Fischer et al., 2004). The nucleotide sequence of *rubia* is set out in the supplementary information. Rubia has in our experience in comparison to DsRed a faster maturation, it is brighter and it is resistant to photobleaching (Otte, unp. data). This cloning produced the plasmid pBac [6xP3-*rubia*] (Fig. 1, Supplementary Fig. 1). To construct the plasmid pBac [3xP3-*rubia Am-actin5c-egfp*] we inserted a multiple cloning site via *Eag*I/*Mss*I restriction sites downstream of *rubia* gene in the plasmid pBac [6xP3-*rubia*] (Fig. 1). We cloned the putative honeybee *Am-actin5c* promoter sequence (Schulte et al., under review) into the multiple cloning site via *Asc*I/*Not*I restriction sites and the *egfp* coding sequence via *Not*I/*Avr*II restriction sites. We inserted via *Eag*I/*Asc*I restriction sites a SV40 polyA site downstream of the *rubia* coding sequence (Fig. 1). The fragments used for cloning were produced by PCR with oligonucleotide primers that introduced restriction sites. Restriction enzymes were obtained from Fermentas (St. Leon-Rot, Germany).

Microinjection and mRNA synthesis

We injected 30 pg plasmid DNA of pBac [3xP3-*rubia*] or pBac [3xP3-*rubia Am-actin5c-egfp*], together with 60-180 pg mRNA (translating the *piggyBac* transposase) per honeybee embryo 0-1.5 hours after egg deposition. The microinjections were performed under a stereomicroscope with an Oxford micromanipulator (SingerInstruments Co., UK) and a microinjector (PLI-100, Medical Systems Corp., Greenvale, NY). The 53 mm long injection pipettes were made from borosilicate capillary tubes (Hilgenberg, Malsfeld, Germany). The tips are rigid and beveled to an angle of 37 °. The inner diameter of the pipette tips was 5 µm. The injection time was 120 ms, the injection pressure was 60 kPa and the balance pressure was 5 kPa. The average amount injected into each embryo was estimated to be 400 pl.

The mRNA was *in vitro* synthesized following basically the instructions provided by the supplier of the mMESSAGE mMACHINE Kit (Ambion, Darmstadt, Germany). We cloned the coding sequence of the transposase gene (derived from phspBac plasmid which was kindly provided by Dr. Gregor Bucher; (Berghammer et al., 1999)) into the pGEMT plasmid (Promega, Mannheim, Germany) downstream of the T7 promoter

site. We linearized the pGEMT [*piggyBac* transposase] plasmid with the *Sal*I restriction enzyme (Fermentas, St. Leon-Rot, Germany). We synthesized transposase mRNA with T7 RNA polymerase *in vitro* that also introduced the 5´ capping structure. We polyadenylated the mRNA with the Poly(A) Tailing Kit and purified them with MEGAclear Kit (both provided by Ambion, Darmstadt, Germany).

The injected embryos were incubated at 34 °C (Beye et al., 2002b) until hatching of the L1 larvae after about 72 hours.

Fluorescence detection in drone heads

We observed red or green fluorescence in pupal drone heads at a wavelength that will detect the red fluorescence of the Rubia or the green one of the EGFP proteins. We used the DsRed filter set (Excitation filter: 546/10 nm, barrier filter: 565 nm LP) and the GFP2 filter set (Excitation filter: 480/40 nm, barrier filter: 510 nm LP) at the Leica MZ FLIII microscope. For high quality pictures drones at pupal stage were decapitated. We fixed the heads over night in PBS with 4 % paraformaldehyde. We washed the fixed heads in PBS. We transferred the heads through a series of 30 %, 50 %, 70 %, 90 %, 96 %, and 100 % ethanol for dehydration. Heads were transferred into 100% methyl salicylat for 2 days which cleared the tissue and took pictures at the Leica MZ FLIII microscope.

PCR-based identification of integration sites

To identify the integration sites of the transposon we performed a PCR-based chromosomal walk (Krieg, 1996). We restricted the genomic DNA of transgenic individuals separately with the blunt end cutting enzymes *Alu*I, *Dpn*I, *Dra*I or *Ssp*I (Fermentas, St. Leon-Rot, Germany) and ligated the DNA fragments to an adapter sequence. This generated different adapter libraries which were used as template in the PCR. The adapter consisted of two oligonucleotides A and A_K (Krieg, 1996) that were annealed by heating 10 µM of each oligonucleotide together up to 98 °C for 5 min and then letting the solution cool down slowly. We performed nested PCRs with adapter libraries that were generated from the transformed progeny of each transgenic queen.

Results

Germline transformed honeybee queens

We combined the coding sequence of Rubia fluorescent protein with the artificial 6xP3 promoter in the *piggyBac* derived vector (Posnien et al., 2011) giving rise to pBac [6xP3-*rubia*] plasmid (Fig. 1A, SI-1). We choose the artificial Pax6 response element 6xP3, a response element that has been widely used to drive the expression of fluorescent marker proteins in the compound eyes or in parts of the central nervous system in a variety of insects (Berghammer et al., 1999, Horn et al., 2002, Posnien et al., 2011).

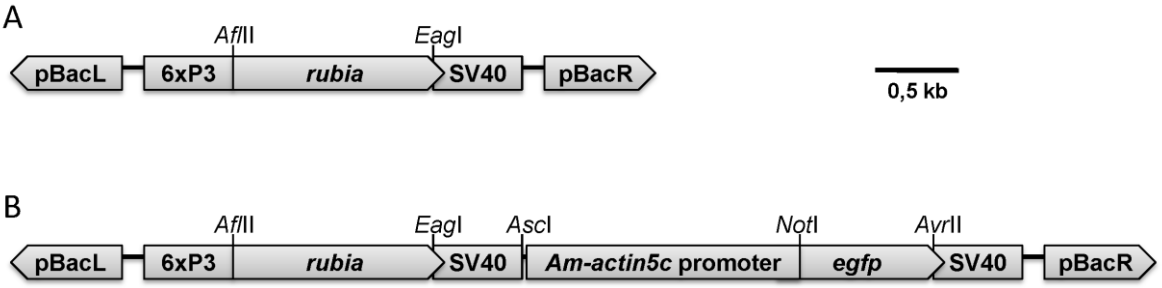

Figure 1: Structural organization of the genetic elements that were transposed into the honeybee genome. A: transposon element with 6xP3-*rubia* marker expression cassette **B:** transposon element with 6xP3-*rubia* marker cassette and *Am-actin5c-egfp* expression cassette. pBacR, pBacL: inverted terminal repeats of the *piggyBac* transposon; 6xP3: Six repeating Pax6 *r*esponse elements upstream of the core promoter of *T. castaneum hsp68* gene (Schinko et al., 2010); *rubia* and *egfp*: reportergenes encoding red or green fluorescent proteins; SV40: SV40 poly adenylation site; *Am-actin5c* promoter*:* 1420 bp sequence derived from *actin5c* promoter region of the honeybee. Letters above denote the restriction sites that were used for cloning. The scale indicates the sizes of the boxes (bp).

We microinjected honeybee embryos 0-1.5 h after egg deposition (Beye et al., 2002a, Gempe et al., 2009b). The embryos were collected with a Jenter queen rearing kit (Karl Jenter, Nürtingen, Germany). Therefore honeybee queens were confined to a plastic comb box that contains removable cell plugs at the bottom of worker cells. The queen usually lays single fertilized eggs at the bottom of each cell. We removed the single cell plugs to which the eggs were attached and fixed the plugs in rows with plasticine onto petri dishes (Beye et al., 2002b) that allowed us to microinject hundreds of embryos with our mircoinjection device (PLI-100, Medical Systems Corp., Greenvale, NY). We injected each embryo with 30 pg of the pBac [6xP3-*rubia*] plasmid DNA and 60-180 pg *in vitro* synthesized transposase mRNA. The embryos were incubated in plastic boxes at 34 °C with 0.5 ml 16 % sulphuric

acid which avoid mould formation. We replaced the acid with water four hours before hatching of the larvae. The hatched larvae (~72 hours after egg deposition) were grafted into queen cell cups that were primed with royal jelly. We led worker bees produce the royal jelly primed cups by transferring young wildtype larvae into the queen cups and by transferring these cups into a queenless colony the day before. We replaced these wildtype larvae with our manipulated ones and transferred them to the queenless colony. After ten days the completed queen cells were removed from the colony and incubated at 34 °C in queen banking cages that we supplied with young worker bees. The emerging queens were placed into small queenless mini mating nucleus hives (Kirchhain nucs; Holtermann, Brockel, Germany). When the queens were eight days old, we treated them on two successive days with CO_2 for seven minutes. This treatment induces laying of unfertilized eggs, eggs which develop into drones (Beye et al., 2003a).

Table 1: Rearing of with pBac [6xP3-*rubia*] germline transformed honeybee queens.

Year	Number of injected embryos	Number of L1 larvae transferred into queen cups	Number of completed queen cells	Number of queens producing offspring	Number of queens producing transgenic offspring	Relative transformation rate+
2011	156	63	33	7	1	14 %
2012	284	133	35	8	3	38 %

+ transformation rate was calculated as the relative proportion of the number of queens that produced transgenic offspring to the total number of queens that produced offspring.

The results of two rearing experiments in the years 2011 and 2012 are shown in Table 1. We improved the injection procedure in 2012 by injecting the embryos dorsal and more posterior, where we suggested the location of the precursor germ cells, to increase the number of germline transformed queens. In the following the results of a single rearing experiment in the year 2012 are presented exemplarily. We injected 284 eggs on two following days and grafted 133 larvae into queen cell cups. We reared 35 complete queen cells in two queenless colonies. 20 (57 %) of the queens hatch from the queen cells. Finally, we obtained eight queens that produced drone offspring and three of them had offspring carrying the transgene (Table 1). 38 % of the queens of the rearing experiment in 2012 produced transgenic offspring suggesting that we established an efficient transformation method. We proved the

integration of the marker expression cassette into the honeybee genome by red fluorescence (Fig. 2 shows an example of offspring of queens 12-07 and 12-31) and PCR analysis (Supplementary Fig. 2 shows an example of offspring of queen 11-59) in the drone offspring (G1) of four queens, one in 2011 and three in 2012 (Table 1). To identify Rubia protein derived red fluorescence in the brain and the compound eyes we looked at heads of males lying in the combs (Supplementary Fig. 3) at pupal stage with a fluorescence microscope. For high quality pictures we fixed the heads with paraformaldehyde and cleared them with methyl salicylat. To identify the transgene in the genome we performed PCRs with oligonucleotide primers that specifically amplify sequences of the transposon elements (Supplementary Fig. 1, Supplementary Fig. 2A) and sequenced the amplified fragments exemplarily. To

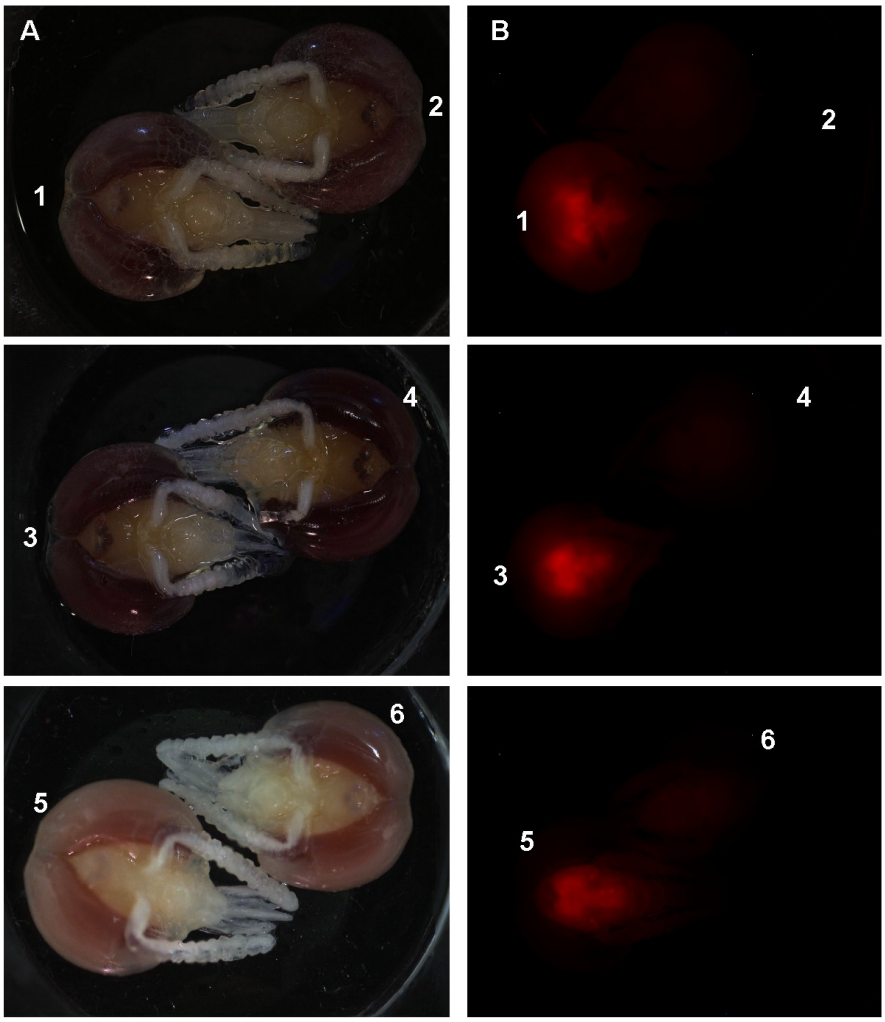

Figure 2: Expression of marker gene *rubia* in the queen's offspring. Heads of genetically transformed (1, 3, 5) and wildtype drones (2, 4, 6) under white light (A) and red fluorescence (B) detection condition. Drones 1 and 3 are derived from queen 12-07 and drone 5 is derived from queen 12-31 (Table 1). Heads were fixed with paraformaldehyde and cleared with methyl salicylat for high quality pictures.

exclude that the amplicons are derived from the injected plasmid we performed a second PCR that amplifies plasmid sequences which are localized partly outside of the transgene construct (Supplementary Fig. 1, Supplementary Fig. 2B). We observed that pupal heads of G1 individuals that carry the marker expression cassette, showed red fluorescence in the inner head, suggesting that *rubia* is expressed in the brain. In contrast, wild type individuals in which the marker gene is absent showed no red fluorescence (Fig. 2, Supplementary Fig. 2A). These results suggest that the 6xP3-*rubia* expression cassette can be used as marker of genetically transformed honeybees.

We analyzed the presence of fluorescence of at least 34 offspring for each of the queens and found that 43 %, 6 %, 30 % and 9 % of the progeny were genetically transformed (Table 2) suggesting that the queens germline is a mosaic of genetically transformed and untransformed cells. All individuals that showed red fluorescence also carried the transgene what we verified by PCR (Supplementary Fig. 2 shows an example of offspring of queen 12-59).

Table 2: Tabular overview of numbers and proportions of transgenic offspring of the different 6xP3-*rubia* germline transformed queens.

# queen	Number of tested individuals	Number of individuals with red fluorescent head	Proportion of individuals with red fluorescent head to tested individuals	Proportion of red fluorescent drones carrying the transgene
11-59	93	40	43 %	100 %
12-05	35	2	6 %	100 %
12-07	37	11	30 %	100 %
12-31	34	3	9 %	100 %

We characterized the integration sites of the transposon in the honeybee genome via a PCR-based chromosomal walk technique (Krieg, 1996). We isolated genomic DNA of G1 individuals carrying the transgene for each queen. We walked by PCR from both sites of the transgene into the flanking genomic regions from which we obtained fragments and sequences spanning the integration sites. The Blastn searches of sequences against the honeybee genome showed that we obtained single integration sites at different chromosomes and genomic positions for each genetically

transformed queen (Table 3). We compared to the reference genome sequence and found that the *piggyBac* mediated transposition repeatedly integrated into a TTAA sequence motif and that the TTAA sequence was apparently duplicated during the transposition event (Table 3). We verified the integration sites by amplifying and sequencing fragments spanning the transgene and the integration sites via PCR with DNA isolates of transformed progeny of each queen (Fig. 3A). For offspring of queen #11-59 we found the same integration site in all tested individuals (N=40; Fig. 3B). These results suggest that the transgene is stably transmitted to the next generation.

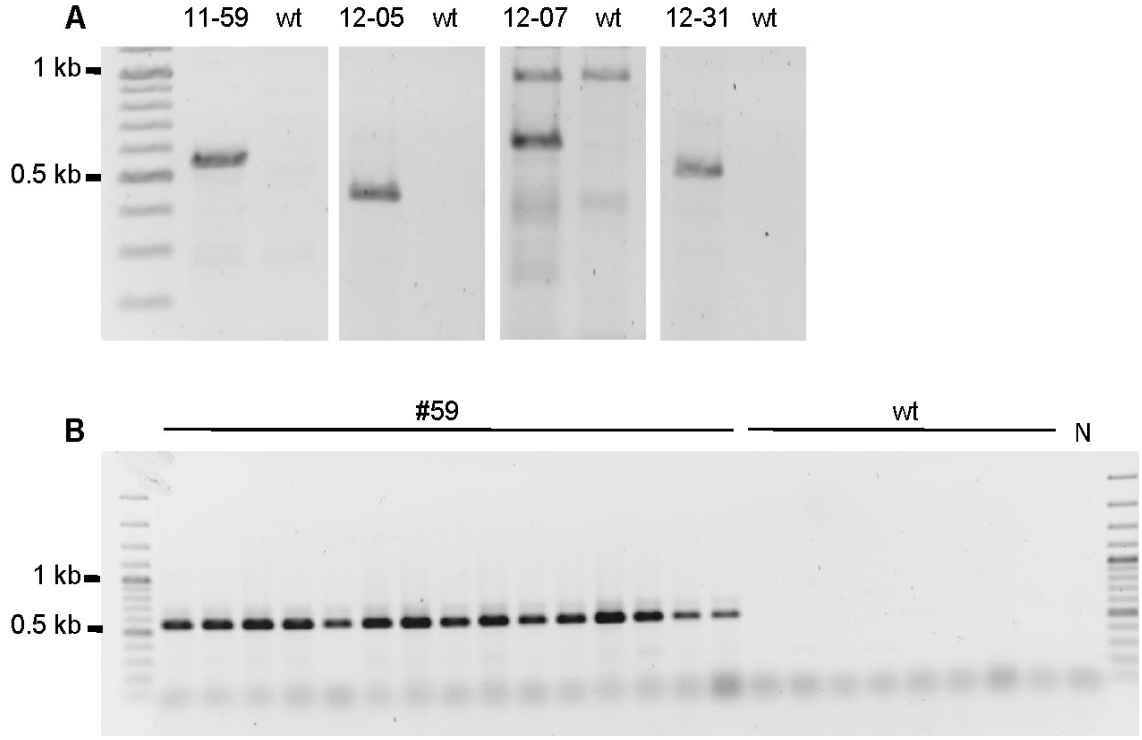

Figure 3: Amplification of queen-specific integration sites of the transposon from genomic DNA. A: Genomic DNA was derived from pools of transgenic offspring of queens 11-59, 12-05, 12-07, and 12-31 and from pools of wildtype individuals (wt). Fragments were amplified by PCR. The sequence of one universal oligonucleotide primer is located in the left inverted repeat (pBacL) of the transgene 6xP3-*rubia*. The sequence of the second primer is located downstream of the integration site and is site specific for the transgene integration site for each of the queens. The amplicons were resolved by agarose gel electrophoresis and stained with ethidium bromide. Primers used: 11-59: #178/#090; 12-05: #206/#090; 12-07: #201/#090; 12-31: #204/#090. **B:** Amplification of the integration site of the transposon from genomic DNA isolates exemplarily from 15 offspring of queen #11-59 that showed red fluorescence in the head (#59) and from eight wildtype drones (wt). The fragments were amplified with oligonucleotide primer pair #178/#090, resolved by agarose gel electrophoresis and stained with ethidium bromide. N: PCR sample without template.

Table 3: Examples of integration sites of the *piggyBac* derived transposon in the honeybee genome.

queen #	pBacR flanking region		pBacL flanking region	position in the genome	
				linkage group	position on the chromosome
11-59	ttcggtttgctttttTTAA	6xP3-*rubia* marker element sequence	TTAAaggatatggttgtaa	LG16	4474602
12-05	tttacataaaatttaTTAA		TTAAataaaattatattaa	LG10	7083928
12-07	ttaacggacggttttTTAA		TTAAttaacggacggtttt	LG1	21160445
12-31	aaaatcatcctaactTTAA		TTAAatactctaaaaataa	LG6	21160552

Targeted gene expression driven by the honeybee promoter *Am-actin5c*

We studied whether we can use honeybee-specific promoter sequences to direct the expression of transgenes. Targeted gene expression via promoter derived sequences in honeybees should allow us to manipulate genes via mis-expression or hpRNA mediated knockdown experiments.

In a previous study we found that the 1420 bp upstream region of the coding sequence of the honeybee *actin5c* gene (*Am-actin5c*) promotes the expression of a reportergene in *Sf*21-cells (Schulte et al., under review). We extended the plasmid pBac [6xP3-*rubia*] by a second expression cassette containing the *Am-actin5c* promoter sequence, the reportergene (*egfp*) and the SV40 polyadenylation site, giving rise to the *piggyBac* vector pBac [6xP3-*rubia Am-actin5c-egfp*] (Fig. 1B).

Table 4: Rearing of with pBac [6xP3-*rubia Am-actin5c-egfp*] germline transformed honeybee queens.

Year	Number of injected embryos	Number of L1 larvae transferred into queen cups	Number of completed queen cells	Number of queens producing offspring	Number of queens producing transgenic offspring	Relative transformation rate[+]
2012	533	190	37	10	2	20%

[+] transformation rate was calculated as the relative proportion of the number of queens that produced transgenic offspring to the total number of queens that produced offspring.

We produced two queens carrying both the 6xP3-*rubia* and the *Am-actin5c-egfp* expression cassette by following the injection and queen raising procedure described above (Table 4). We again analyzed fluorescence in the heads of the G1

offspring and observed green fluorescence in G1 individuals that carry both expression cassettes, but not in individuals that have integrated only the 6xP3-*rubia* expression cassette, or that were wildtype (Fig. 4). We verified the presence of the transgene in the genome of the individuals by PCR (SI-3). This result suggests that our *Am-actin5c* promoter sequence can drive *egfp* expression in genetically transformed honeybees. The EGFP expression is found in the entire head including the antennae. Our result suggests that we can use our method to integrate honeybee derived promoter sequences into the honeybee genome that can direct transgene expression. We think, however, that our results on *Am-actin5c* promoter derived *egfp* expression need further confirmation via western-blot analysis and increased sample size.

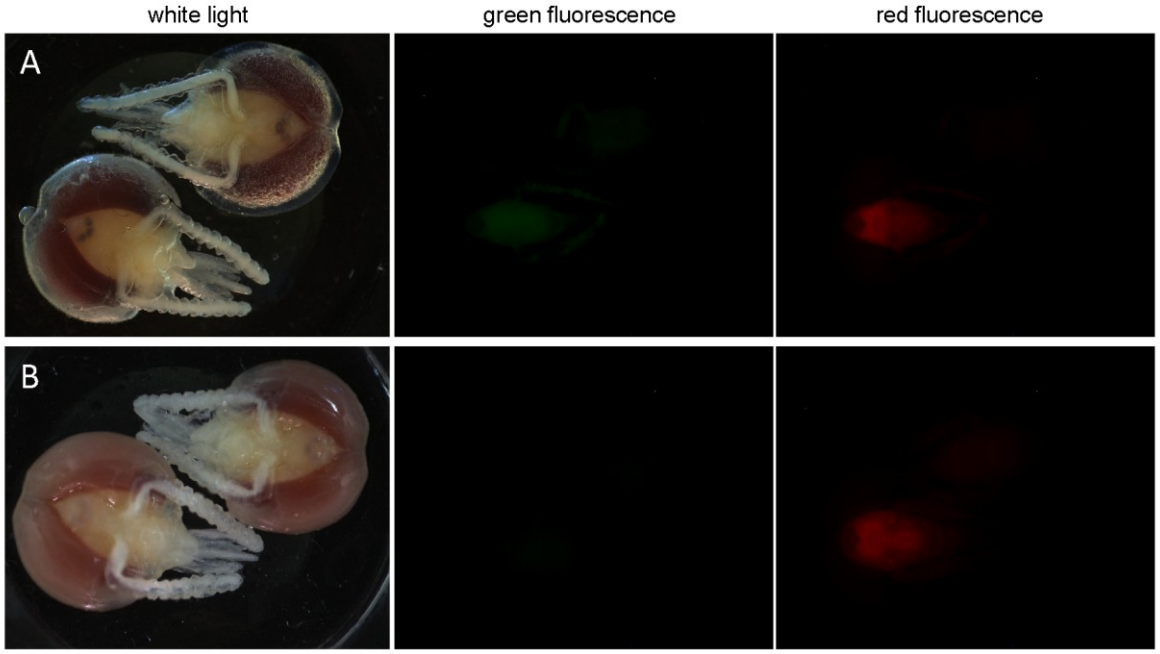

Figure 4: The *Am-actin5c* promoter derived expression of the e*gfp* gene in the queen's offspring. Heads under white light, green and red fluorescence detection conditions. The genetically transformed drones are shown to the left and the wildtype ones to the right of each picture. Heads were fixed with paraformaldehyde and cleared with methyl salicylat. **A:** Drones transformed with [6xP3-*rubia Am-actin5c-egfp*] expression cassettes **B:** Drones transformed with [6xP3-*rubia*] marker cassette. *Am-actin5c*: upstream sequence of honeybee *actin5c* gene. *rubia*: reportergene encoding a red fluorescent protein; *egfp*: reportergene encoding a green fluorescent protein; 6xP3: Six repeating Pax6 response elements in combination with the core promoter of *T. castaneum hsp68* gene.

Discussion

In this study we report on the first genetically transformed honeybee, mediated by a *piggyBac* derived transposon. We present an efficient method that produced genetically transformed queens which stably transmitted their transgene to the next generation at a high frequency (more than 20% of queens produced offspring carrying the transgene). We also showed that we can integrate honeybee-derived promoter sequences which can direct the expression of transgenes. The efficient method of producing transgenic offspring presented, together with instrumental insemination (Laidlaw, 1944, Harbo, 1985a, Harbo, 1985b), sperm freezing techniques (Sawada and Chang, 1964, Collins, 2000, Collins, 2004) and a haploid male system suggest that we can now routinely characterize gene functions via mis-expression of genes or RNAi-induced knockdowns in a social insect.

We showed by fluorescence detection, PCR studies and sequencing that the reportergene is expressed and transmitted to the next generation in 26 % of the generated queens. We further characterized the integration sites by PCR based chromosomal walk and sequencing and showed that the transposon integrated into TTAA sequence motifs in different genomic regions. We also showed stable integration by repeatedly identifying the same integration site in 40 G1 offspring of one queen. We confirmed moreover the expression of the *egfp* gene driven by *Am-actin5c* promoter by the detection of green fluorescence in G1 drones. These males carried the *Am-actin5c-egfp* expression cassette which we confirmed by PCR and sequencing experiments.

Although our sample size in honeybees is still low the repeated finding in three rearing experiments (Table 1, Table 4) suggests that we have achieved on average a relative transformation rate of 24 % which is a substantially higher rate than reported thus far in other insects. *PiggyBac* mediated transformation for *Ceratitis capitata* was 3-5 %, for *Bombyx mori* 2-5 % and for another hymenopteran species *Athalia rosae* 5 % (Handler et al., 1998, Tamura et al., 2000, Sumitani et al., 2003). We speculate that our efficient transformation rate in honeybees is due to the very long syncytial stage of the honeybee that lasts 12 hours (Snodgrass, 1956) at which the injected plasmid DNA and mRNA can spread through the embryo. Active transposons are absent in the honeybee genome (Weinstock et al., 2006) and possibly because of this, an effective transposons defense system (reviewed in (Aravin et al., 2007) is not

required. Alternatively, we provided by injection in comparison to other species larger amounts of transposase due to the considerable size of the honeybee embryo. Irrespective of the cause we hope to further increase the transformation rate by increasing the concentration of transposase in the embryos (Pavlopoulos and Averof, 2005).

We established several technical components that will improve the feasibility of the technique in routine procedures. By injecting into the distal part of the embryo, the mass egg collecting, the improved injection procedure and an optimized queen rearing strategy of the year 2012, we can obtain in a single rearing experiment three germline transformed queens. Not all offspring of the queens were transgenic (6 to 43 %) suggesting that the queens were genetic mosaics. We demonstrated that we can identify the transgenic drone offspring alive by the marker 6xP3-*rubia* at pupal stage in the combs (Supplementary Fig. 3) suggesting that we can preselect those drones which we will later use for sperm recovery and breeding by insemination of a queen. CO_2 treated queens can lay up to hundreds of eggs in larger colonies. Thus we expect to collect semen of dozens of transgenic drones. The sperm of these drones is entirely transgenic as the males are haploid and sperm is produced without meiotic cell division. It is now a routine procedure to inseminate a queen with semen of single drones (Laidlaw, 1944, Hunt and Page, 1995) by which we can produce colonies in which the workers are entirely transgenic. In addition, different studies reported that the semen can be stored and used for later insemination experiments (Sawada and Chang, 1964, Kaftanoglu and Peng, 1984, Collins, 2004). This storage may overcome the difficulty to maintain a line carrying a specific transgene.

The use of the 6xP3-*rubia* maker is in the honeybee restricted to the pupal stage in which the cuticula is not yet sclerotisized and pigmented. The P3 response elements have been widely used in transgenic maker systems of insects including dipteran (*D. melanogaster*), lepidopteran (*B. mori*) and coleopteran (*T. castaneum*) species. (Berghammer et al., 1999, Horn et al., 2000, Horn et al., 2002, Thomas et al., 2002). But in some species, for instance in *Musca domestica* or in *Aedes aegypty*, the marker protein derived fluorescence is quenched completely by the adult eye pigmentation as well (Hediger et al., 2001, Kokoza et al., 2001). In these species the fluorescence can get detected during larval and pupal stages. Alternatively, eye color mutant strains are recommended to screen for marker fluorescence in imagos (Handler et al., 1998, Horn and Wimmer, 2000).

As a proof of concept we showed that we can introduce a honeybee-specific promoter sequence into the honeybee genome to direct transgene expression. We found that the integrated *Am-actin5c* promoter sequence can drive expression of our reportergene in the entire head (although due to the end of the season we were limited in increasing the sample size of transgenic drones). The *actin5c* promoter sequence of *D. melanogaster* has been previously used in experiments to drive constitutive overexpression of transgenes in developmental studies in the fruit fly and in transformation studies in some mosquito species (Chung and Keller, 1990, Pinkerton et al., 2000, Chavous et al., 2001, Nolan et al., 2002). We suggest that we now can use our *Am-actin5c* promoter in experiments in which constitutive downregulation via transcription of hpRNAs (Piccin et al., 2001, Isobe et al., 2004, Dietzl et al., 2007) or mis-expression of genes in worker bees is targeted.

Inserting other honeybee promoters will allow us to drive stage or tissue specific mis-expression and downregulation of gene transcripts. We have tested the honeybee *Am-hsp70* and *Am-hsp83* promoter sequences and showed that they can drive gene expression in lepidopteren *Sf*21 cells and that the expression driven by *Am-hsp83* promoter can get increased during a shift to colder temperatures (Schulte et al., under review). The promoters of the homolog genes have been successfully used to switch on transcription of transgenes by heat shock in other species, including the lepidopteran insects *Bicyclus anynana* and *B. mori* (Monsma et al., 1988, Uhlirova et al., 2002, Ramos et al., 2006, Schinko et al., 2010). For instance the *Dm-hsp70* promoter sequence has been widely used to induce knockdown of genes or express cDNA at specific developmental stages by shifts in the environmental temperature (Monsma et al., 1988, Grotewiel et al., 1998, Lam and Thummel, 2000, Cheng et al., 2001). The use of the Gal4/UAS system (Fischer et al., 1988, Brand and Perrimon, 1993, Duffy, 2002) may allow us to specifically activate genes in worker bees by combining the two interacting elements in the progeny of a transgenic queen and a transformed drone.

Honeybees are economically important pollinators of wild flowers and crop plants. A major threat of honeybees that has implicated the decline of the number of colonies is the ectoparasite *Varroa destructor* (Dainat et al., 2012, Guzman-Novoa et al., 2010). This is in part due to the viruses the mite carry, including deformed wing virus (DWV) and acute bee paralysis virus (ABPV), which have both been implicated in colony collapse disorder (CCD) (Cox-Foster et al., 2007, Yang and Cox-Foster,

2007, Genersch and Aubert, 2010, Guzman-Novoa et al., 2010), a phenomenon in which worker bees from a beehive abruptly disappear. hpRNAs constitutively expressed by the *BmA3* promoter in transgenic *B. mori* have been shown to inhibit the infection by RNA viruses (Isobe et al., 2004, Kanginakudru et al., 2007) which is a powerful strategy to defend against diseases. The strategy is, however, limited in *A. mellifera* since the release of genetically modified honeybees into the environment is prohibited.

The method of producing stable genetic transformations in the honeybee will allow further molecular advances in understanding the remarkable biology of this social organism. We can now deeply study the function of genes in the context of entire colonies including effects on cooperative behaviors giving rise to the highly coordinated group outcomes (Robinson, 1992, Page and Erber, 2002). It will also enhance studies on the individual phenotype, for instance the complex cognition performance or caste as well as sexual development (Giurfa et al., 2001, Beye et al., 2003b, Menzel et al., 2006, Kucharski et al., 2008, Gempe et al., 2009a, Kamakura, 2011b).

Acknowledgements

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Supplementary Information

SI-1: Oligonucleotides that were used in PCRs

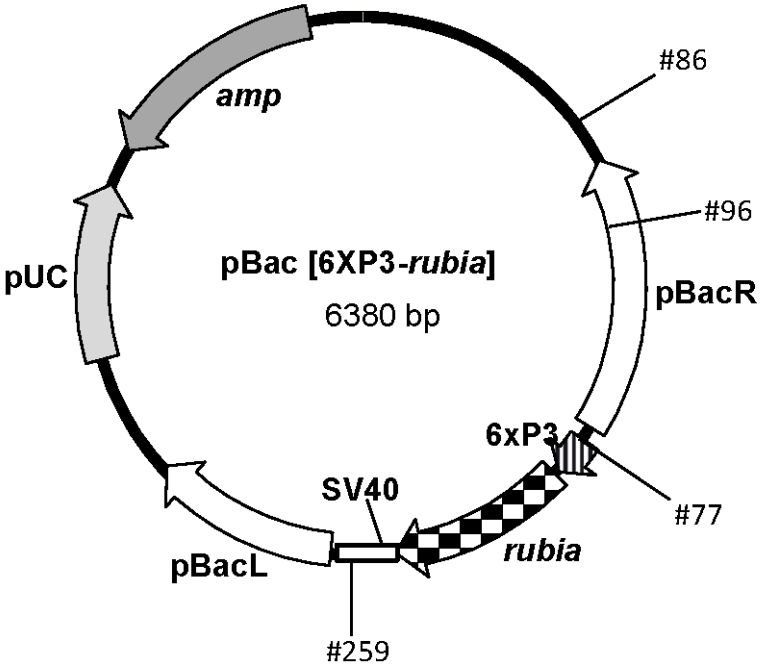
number	name	sequence
#77	pBacR_fw_ClaI	AAACATCGATGTTCCCACTGGCCTG
#79	piggyT_fw_ApaI	GATCGGGCCCCCCGTGAGGCGTGCTTGTCAATGCG
#80	piggyT_rev_NcoI	GATCCCATGGCCCTAGAAAGATAATCATATTGTGAC
#86	EA-piggy_fw	CCATATACGCATCGGGTTGATATCG
#87	EA-piggy_rev	GTGAGCGGATAACAATTTCACACAG
#090	EA-	CATGCGTCATTTTGACTCACGCGG
#96	piggy_out_rev	CAGACCGATAAAACACATGCGTC
#101	Adapter out	CCATCCTAATACGACTCACTATAGGGC
#102	Adapter in	ACTCACTATAGGGCTCGAGCGGC
#103	marathon5in2	GCGTCAATTTTACGCATGATTATCTTTAACG
#104	marathon3out2	CGTCAATTTTACGCAGACTATCTTTCTAGG
#105	marathon3in2	CAAGAATGCATGCGTCAATTTTACGCAG
#178	#59_rev	ACTCAACCTAATTAACCAACTATTTCAAC
#201	12-07 3´	GACTCAATAAAACGAGACCC
#204	12-31 3´	cgacatcgcgcttcgtgtcgtcg
#206	12-05 3´rev	CTTTCTCTTTCAATCAATTAATCGAGC
#259	Fastbac_seqR	CTACAAATGTGGTATGGCTGATTATGATC

SI-2: Nucleotide sequence of rubia gene

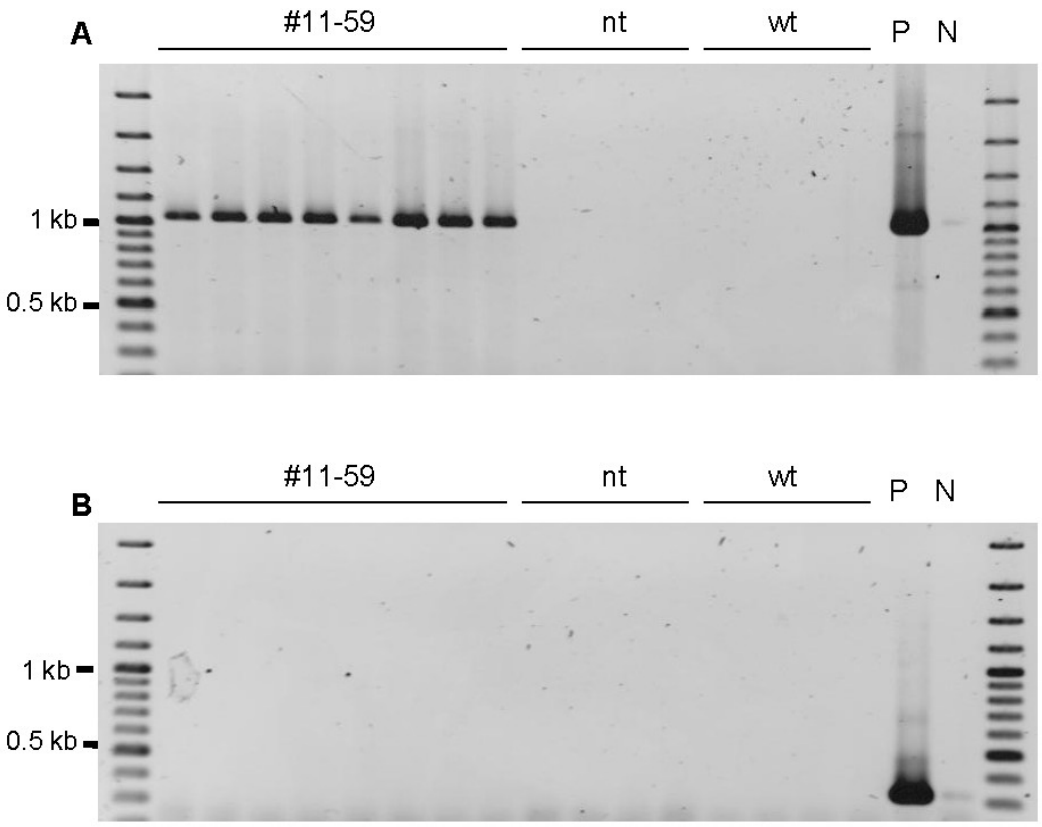
atggcctcctccgaggatgtcatcaaagagtttatgagatttaaggtcaagatggagggaag
cgtcaacggacacgagttcgagattgagggagaaggagaaggccggccttacgagggcacac
aaaccgctaagctcaaggtcacaaaaggaggacccctccccttctcctgggatattctgagc
cctcagttccagtacggaagcaaagcctatgttaaacaccctgccgacatccctgactatct
gaagctctccttccctgaaggcttcaagtgggagagattcatgaacttcgaggacggaggcg
tggtgacagtcacacaagatagcaccctccaggacggagagtttatttataaggtgaaactc
agaggaaccaacttcccctccgatggccctgtcatgcaaaaaaaaacaatgggatgggaagc
ctccaccgagagaatgtatcctgaggatggcgctctgaaaggcgaaattaaaatgagactga
aactcaaagacggaggacactacgatgccgaggtcaaaacaacctacaaggccaagaaacaa
gtgcagctgcctggcgcctacatgactgatattaaactcgacattatcagccataatgggga
ctacaccatcgtggaacaatatgagagagctgagggcagacatagcacaggcgctggatcct
ga

SI-3: Tabular overview of numbers and proportions of red fluorescent offspring of the 6xP3-*rubia Am-actin5c-egfp* germline transformed queens.

# queen	Number of tested individuals	Number of individuals with red fluorescent head	Proportion of individuals with red fluorescent head per tested individuals	Proportion of red fluorescent drones carrying the transgene
actin15	27	1	4 %	100 %
actin22	35	2	6 %	100 %



Supplementary Figure 1: Structural organization of the *piggyBac*-derived vector plasmid pBac [6XP3-*rubia*]. pBacR, pBacL: inverted terminal repeats; 6xP3: Six repeating Pax6 response elements in combination with the core promoter of *T. castaneum hsp68* gene; *rubia*: reportergene encoding a red fluorescent protein; SV40: SV40 poly adenylation site; *amp*: ampicilin resistance gene: #77/#259 and #86/#96 denote the designated binding sites of oligonucleotide primers used to detect the transgene (primers #77/#259) and to detect sequences that lay partly outside of the transgene (primers #86/#96) by PCR. The PCR with oligonucleotide primers #77 and #259 amplifies a 1040 bp long fragment (Supplementary Fig. 2A). PCR with oligonucleotide primer pair #86/#96 amplifies a 313 bp long fragment (Supplementary Fig. 2B).



Supplementary Figure 2: Detection of transgenes in the DNA isolates of G1 individuals by PCR. Example from transgenic offspring of queen 11-59 in comparison to not transgenic offspring of queen 11-59 (nt) and wildtype drones (wt) **A:** The fragments were amplified with oligonucleotide primers #77 and #259. Amplified fragment of the transgene is spanning 6xP3 promoter, *rubia* reportergene and a part of SV40 poly adenylation site (Supplementary Fig. 1). The amplified fragment has a size of 1040 bp. Fragments were amplified by PCR, resolved by agarose gel electrophoresis and stained with ethidium bromide. **B:** Detection of vector plasmids in the DNA isolates of G1 individuals and wildtype drones by PCR. The fragments were amplified with oligonucleotide primers #86 and #96. Amplified fragment is spanning a part of the left inverted repeat and the flanking plasmid sequence of pBac [6xP3-*rubia*] and has a size of 313 bp (Supplementary Fig. 1). Fragments were amplified by PCR, resolved by agarose gel electrophoresis and stained with ethidium bromide. Fragment identity was verified exemplarily by sequencing of the amplified fragments. P: PCR sample with pBac [6xP3-*rubia*] plasmid DNA as template; N: PCR sample with no template.

Supplementary Figure 3: Photograph of male offspring of a transformed queen lying alive in cells of a comb. Comb was taken out of a Kirchhain nuc and the single cells were uncapped. Drones can get screened for Rubia marker fluorescence and will afterwards develop as usual.

Author´s Contribution

Genetic transformation of honeybees (*Apis mellifera*) via transposon mediated integration

Journal: PlosONE (under review)
Impact Factor: 3.7
1st author

Author´s contribution: 95%

- Establishing the transformation an rearing system
- Injections (except *Am-actin5c* injections)
- Rearing of transgenic queens and offspring
- Confirmation experiments
- Authoring the manuscript

III Zusammenfassung

Um die Funktionen zu charakterisieren, die Gene in ihrem entwicklungs- oder verhaltensbiologischen Kontext einnehmen, müssen diese Gene in einem Organismus gezielt exprimiert oder ihre Transkription verhindert werden.

In der vorliegenden Arbeit wurde eine Methode zur stabilen Gentransformation der Honigbiene *Apis mellifera* mittels eines *piggyBac*-basierten Transposons etabliert. Im Zuge dessen wurde eine Methode zur Aufzucht von transgenen Königinnen und deren Nachkommen entwickelt. Dabei wurde die Expressionskassette 6xP3-*rubia* als effizienter Marker für transformierte Honigbienen eingesetzt und die Transformation mittels PCR, Fluoreszenz des Markerproteins Rubia und Sequenzierung der Integrationsstellen im Genom nachgewiesen. Es konnte gezeigt werden, dass das integrierte Transposon stabil an die nächste Generation weitergegeben wird. Bei der Generierung von transgenen Honigbienen konnte eine Transformationsrate von 26% erzielt werden. Diese Rate ist im Vergleich zu anderen Spezies, die durch ein *piggyBac*-basiertes Transposon transformiert wurden, bis zu zehnmal höher.

Um Gentranskripte innerhalb transgener Tiere zu exprimieren oder abzubauen, werden speziesspezifische Promotoren benötigt. In der vorliegenden Arbeit konnten Promotorkandidaten für konstitutive, temperaturinduzierbare und gehirnspezifische Genexpression in der Honigbiene identifiziert werden. In *Sf*21-Insektenzellkultur konnte gezeigt werden, dass die Promotorsequenzen der Honigbienengene *Am-actin5c, Am-hsp70 und Am-hsp83* die Expression des Reportergens *rubia* initiieren. Es wurde zudem gezeigt, dass die Expression von *rubia* unter Kontrolle des Promotors *Am-hsp83* durch einen Kälteschock erhöht werden kann. In einem Elektroporationsexperiment konnte weiterhin gezeigt werden, dass der Promotor des Honigbienengens *elp2l* in lebenden Zellen des Bienengehirns Genexpression initiiert.

Abschließend wurde der Promotor *Am-actin5c* mit dem etablierten Transformationssystem kombiniert und so erste transgene Honigbienen generiert, in denen ein honigbienenspezifischer Promotor die Expression des Reportergens *egfp* vermittelt.

Das etablierte Transformationssystem bietet die Möglichkeit in zukünftigen Experimenten Gene zu identifizieren, die die einzigartigen entwicklungs- und verhaltensbiologischen Eigenschaften der Honigbiene kodieren.

IV Summary

The honeybee *Apis mellifera* displays a rich behavioural repertoire, social organisation, caste differentiation and has an interesting mode of sex determination, but we still know little about the underlying genetic programs.

In the present work I established a *piggyBac* transposon-mediated system to transform the honeybee and a method to raise transgenic honeybee queens and their offspring. I inaugurated the expression cassette 6xP3-*rubia* as an efficient fluorescent marker for honeybee transgenesis and confirmed the transformation via PCR, Rubia-derived marker fluorescence and by identifying the integration sites of *piggyBac* in the honeybee genome. I found that the integrated transposon is stable transmitted to the next generation. I achieved a transposition rate of 26 % which is substantially higher than in other species that were transformed with a similar system. The transposition rate displays the proportion of queens with transgenic progeny within all fertile queens.

As an initial step towards transgene expression, I characterized honeybee promoters that can drive constitutive, tissue specific and cold shock induced gene expression. I identified the promoter sequences of *Am-actin5c*, *elp2l, Am-hsp83* and *Am-hsp70* and showed that, except for the *elp2l* sequence, the identified sequences were able to direct reportergene expression in *Sf*21-cells. I showed further that gene expression driven by the *Am-hsp83* promoter is increased after shift to a lower temperature. I further demonstrated via electroporation experiments that the putative neuron specific *elp2l* promoter sequence can direct gene expression in the honeybee brain. The identification of these promoter sequences is an important step towards the study of gene functions with transgenic honeybees.

In a final experiment I combined a honeybee specific promoter with the honeybee transformation system and generated first transgenic individuals in which *Am-actin5c* promoter drives the expression of the reportergene *egfp*.

The established transformation system will enable us to identify genes that encode the fascinating features and behaviours of the honeybee. It will allow further molecular advances in understanding the remarkable biology of this social organism.

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