The first confirmation that Insects synthesize Cytokinins: Cytokinin metabolite and gene expression profiling following functional manipulations of tRNA IPT genes in *Drosophila melanogaster*

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ABSTRACT:

The first confirmation that Insects synthesize Cytokinins: Cytokinin metabolite and gene expression profiling following functional manipulations of tRNA IPT genes in *Drosophila melanogaster*

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Using Drosophila deficiency (Df) and Over Expression (OE) (GAL4/UAS>dCas9-VPR; sgRNA) gene systems, it was demonstrated that Dmel_CG31381 and Dmel_CG11089 are functional tRNA isopentenyltransferase (EC 2.5.1.8) genes (tRNA IPT1 and IPT2) critical to the first committed step in insect cytokinin biosynthesis. IPT Df mutants showed significant decreases in total CK levels and IPT1/IPT2 transcript levels compared to parent lines. IPT OE mutants showed significant increases in total CK levels and IPT1/IPT2 transcript levels compared to parent lines. Further, endogenous CK analyte levels and qPCR relative fold gene expression of Dmel_CG31381 and Dmel_CG11089 (tRNA IPT1 and IPT2) genes demonstrated expression patterns with functional confirmation corresponding to the predicted IPT mutant variants.

The functional confirmation of tRNA IPT1 and IPT2 as the first committed step was further supported by the bioinformatic detection of putative gene homologs to corroborate seven remaining enzyme transcripts supporting the novel description of a CK biosynthesis pathway in insects.

KEYWORDS: Drosophila, insects, gall, cytokinins, functional genetics, gene expression, analyte detection, mass spectrometry, biosynthesis, tRNA IPT, tRNA degradation.

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1. CHAPTER ONE: Introduction

Cytokinin biosynthesis by insects has long been hypothesised as a mechanism by which endophytic phytophagous insects like gall formers and leaf miners can control plant growth by synthesizing their own native phytohormones (Mapes and Davies 2001, Straka et al. 2010, Tanaka et al. 2013, Gutzwiller et al. 2015, Takei et al. 2015, Andreas et al. 2020). Newer hypotheses posit further that exophytic phytophagous insects possess these same mechanisms (Brütting et al. 2018, Andreas et al. 2020). This thesis investigates whether insects have the metabolic and genetic ability to create the cytokinin (CK) phytohormones. The ability for synthesis of these signal molecules would allow interspecies biochemical communication across kingdoms with broad implications. CKs biosynthesised by plants and the potential for CKs biosynthesised by insects must be clearly differentiated to build a better understanding of the interkingdom signaling between insects and plants. Also to better understand the roles of CK in insects that do not interact with live plants.

1.1. Importance of plant-insect interactions

Humanity relies heavily on plants for the air we breath to the food we eat. The United Nations' Food and Agriculture Organization estimates a loss of $220 billion or 20 to 40 percent of global crop production to pests, each year. Insects are estimated to cause $70 billion of this loss. The strongest allies in the fight against pests are pesticides. Currently pesticides are indispensable to agriculture and without them there would be estimated losses of 78% of fruit production, a 54% of vegetable production, and a 32% of cereal production (Tudi et al. 2021). The biochemical approach to pesticides has been the

same since the 20th century. These compounds affect the nerve function of insects inhibiting success and attacking their physiology (Umetsu and Shirai 2020). To better address physiology of insects, one must first gain a better understand how they interact and communicate with plants.

Insects are heavily reliant on host plants in fulfilling their life cycles. This reliance brings into question whether insects can biochemically communicate with their hosts. Decades of research support that a special group of endophytic insects, living inside plant tissues, can synthesize phytohormones to produce macrohabitat growths on plants and sustain their lifecycle (Mapes and Davies 2001, Straka et al. 2010, Tanaka et al. 2013, Gutzwiller et al. 2015, Takei et al. 2015, Andreas et al. 2020). Other theories suggest that an endosymbiont bacterial-insect relationship exists wherein bacteria are producing phytohormones (Giron et al. 2007, Zhang et al. 2017) although bacterial species identification has come up empty handed, and a common symbiont has yet to be discovered (Hammer et al. 2021)

However, new theories propose that all plant eating insects can synthesis phytohormones as a currency in exchange for food and habitat. They suggest that phytophagous insects are farming plant tissue by manipulating the plant's own native compounds as to maintain foreign diplomacy and asylum (Brütting et al. 2018, Andreas et al. 2020). Previous work shows that phytophagous insects contain phytohormones; yet, to date, it has yet to be shown that they possess the genetic machinery to functionally synthesize them.

1.2. Cytokinins

Cytokinins (CKs) are classed as phytohormones that influence many aspects of plant growth and development including sink/source relations, apical dominance, embryonic development, shoot initiation, and cell division and differentiation (Mok and Mok 2001, Kieber and Schaller 2018). CKs have been detected endogenously in bacteria (Kisiala et al. 2013), fungi (Morrison et al. 2016a, 2016b), protists (Noble et al. 2014, Malinkowski et al. 2016), nematodes (Siddique et al. 2015), insects (Andreas et al. 2020), mammals (Seegobin et al. 2018), and even humans (Aoki et al. 2019). Hypotheses implicate CKs as inter-kingdom signaling molecules for cross talk with host plants (Stirk and van Staden 2010, Andreas et al. 2020) and in other models consider their role to be endogenous and unrelated to plants (Seegobin et al. 2018, Aoki et al. 2019).

Plants have two well established CK biosynthesis pathways that begin with either de novo isopentenyl transferase (IPT) or a tRNA IPT. In other organisms, like those referenced above, the presence and role of enzymes in pathway modeling is commonly compared to those pathways described in plants: Both pathways encompass the enzymes, regulatory genes, and specific CKs products (Sakakibara et al. 2006).

Many interkingdom studies focus on CK concentrations measured through development and among tissue types of modelled systems often categorizing CK profile changes across timepoints or through spatial localization. Such profiles are like fingerprints or signatures composed of distinct types (ie. *cis* vs. *trans*) and forms (ie: nucleotide, riboside, freebase) of CKs detected. Profile patterns often emerge showing an abundance of certain active forms or unique types with changes detected in differences between host plant tissues or when compared to an insect visitor. This makes pinpointing

cause-and-effect a difficult feat. If a visitor produces the same compounds as its host, how can one be sure who is the producer? Therefore, detecting the presence/absence of these compounds and comparing the functional activity of the genetic pathway that determines their origin is fundamental to understanding their causal role.

1.3. Plant-Insect Galls

Plant galls are tumor-like growths formed on the plant in the presence of specific insects. When formed by bacteria, fungi, or nematodes these galls are considered infectious. In insects, egg deposition (oviposition) and/or boring of newly emerged larvae accompanied by the release of insect-born CKs is believed to initiate gall formation through a flourish of cell division and differentiation within the subepidermal cells in leaves, roots, shoots or stems (Raman 2011). This is most common for insects that live inside plant organs (endophytic insects) such as gall forming and leaf mining insects (Mapes and Davies 2001, Giron et al. 2007, Straka et al. 2010, Tanaka et al. 2013, Gutzwiller et al. 2015, Takei et al. 2015, Zhang et al. 2017, Andreas et al. 2020).

For more than two decades, CKs have been examined as initiation factors in cecidogenetic behaviors or insect-plant gall and green island leaf mining formation. Both plant and insect tissues measured show quantitative phytohormone dynamics between host and inducer, across many insect-plant systems (Mapes and Davies 2001, Giron et al. 2007, Straka et al. 2010, Tanaka et al. 2013, Gutzwiller et al. 2015, Takei et al. 2015, Zhang et al. 2017). Insect -plant galls are phenotypically complex plant organs formed at meristematic undifferentiated or partially differentiated stem cell-rich reactive sites. They provide food, shelter, and protection for the insect. Over time, insects are

developmentally encapsulated in the growth of a rich nutrient sink layer replete with sugars and proteins and further encased by callus parenchyma providing protection to facilitate their life cycle (Barnewall et al. 2012, Nogueira et al. 2018, Schultz et al. 2019, Andreas et al. 2020).

Phytohormone detection in insects and insect-to-plant transport has been well documented but no genetic mechanisms for insect CK biosynthesis have been discovered (Mapes and Davies 2001, Straka et al. 2010, Yamaguchi et al. 2012, Tanaka et al. 2013, Takei et al. 2015, Zhang et al. 2017, Andreas et al. 2020). CKs are not unique to gall inducing insects alone and are indeed abundant and widespread among phytophagous insect species (Brütting et al. 2018, Andreas et al. 2020). The detection of CKs in a survey of endophytic or sedentary insects like gall formers, leaf miners, and stem borers versus exophytic or free-living phytophagous insects widens the investigation of their potential roles (Brütting et al. 2018, Andreas et al. 2020). Gall forming insects contain unique CK signatures, often dominated by trans-zeatin (tZ) and N^6-isopentenyladenine (iP) in massive quantities, when compared to non-gall forming insect. The endogenous CK patterns observed when comparing plant gall tissue to insect tissue is presumedly attributed play a role in the growth of plant galls. On the other hand, non-gall producing insects possess much lower-level patterns, perhaps, as a currency in exchange for food in the form of sustained feeding sites and habitat (Andreas et al. 2020). This supports the hypothesis that all phytophagous insects are using CK to farm plant tissue by synthesising the plant's native compounds. Yet the biosynthetic origins of insect CKs are debated and often proposed to be of bacterial-insect endosymbiont origin (Giron et al. 2007, Kaiser et al. 2010, Body et al. 2013, Gutzwiller et al. 2015, Zhang et al. 2017).

RNA Seq transcripts of *Rhus chinensis* (Mill) gall tissues induced by aphid *Schlechtendalia chinensis* compared transcriptomes of galled vs control leaves and found four isoforms of the two-component response regulator ARR, which plays an important role in CK signal transduction. Of these, the gene expression ratio of three genes (Unigene10325, CL3428.Contig1 and Unigene18453) was highly active in Gall vs. Control Leaf (Wang et al. 2017). These findings provide further support that CKs detected in insect tissues are active in their host plant, where they induce and sustain plant-gall growth as interkingdom signalling molecules.

The effector protein hypothesis has often been seen as an alternative to the phytohormone production hypothesis. This theory posits that effector proteins are the initiation factors insects produce to induce gall formation in their plant hosts (Aggarwal et al. 2014, Zhao et al. 2016, Wang et al. 2018). Other work has made attempts to induce galls using CKs as initiation factors and although gall-like growth was observed in plant tissues, phenotypic consistency to insect galls were not observed (Bartlett and Conner 2014). Recent work by Hirano et al. (2024-PrePrint), used microinjections of different combinations of candidate gall initiation factors and claim to be the first to induce a phenotypically similar gall on the host plant, without the insect being present. They first synthesised CAP peptide from transcripts isolated from the horned gall aphid. The trials compared many different combinations of candidate initiation factors but was said to be successful using synthesised Cysteine-rich secretory proteins, Antigen 5, and Pathogenesis-related 1 proteins, or CAP peptide in combination with a cocktail of CKs mirroring those detected in the insects' tissues. The combination of both CAP peptide and CK profile as initiations factors that claims to have generated the first synthetically

induced insect-plant gall without the insect being present. This work supports both the protein effector and phytohormone hypotheses.

It is not yet understood whether a unique insect CK profile signature can be paired with a specific plant species gall type. Insect-born CKs may have several rolls in plant physiology such as cell division to grow their galls, delaying senescence to keep the plant alive, and cell dedifferentiation to sustain their feeding sites. Therefore, consequential consideration must be given for a diversified role of these unique phytohormones in a multifaceted relationship between insects and the supporting host plants (Mapes and Davies 2001, Giron et al. 2007, Straka et al. 2010, Tanaka et al. 2013, Gutzwiller et al. 2015, Takei et al. 2015, Zhang et al. 2017, Andreas et al. 2020).

1.4. Cytokinins in Insects

To date, CK detection in insects and insect-to-plant transport has been well documented but no genetic mechanisms for insect CK biosynthesis have been discovered (Mapes and Davies 2001, Straka et al. 2010, Yamaguchi et al. 2012, Tanaka et al. 2013, Takei et al. 2015, Zhang et al. 2017, Andreas et al. 2020). Results from a wide survey of plant-gall producing - and related non-gall producing insects - show that all phytophagous insects possess CKs. Gall producing insects contain massive quantities of unique CK combinations to cause the growth of plant tumors. Whereas non-gall producing insects possess much lower-level patterns as a currency in exchange for food in the form of sustained feeding sites and habitat (Andreas et al. 2020). This supports the hypothesis that all phytophagous insects are using CK to farm plant tissue by manipulating the plant's native compounds. Yet the biosynthetic origins of insect CKs are debated and often attributed to bacterial endosymbionts in the insect.

Thus, this thesis investigates the extent to which insects have a genetic ability to create CKs. *Drosophila melanogaster* provides a powerful model system to study the presence and genetic regulation of CK biosynthesis genes in insects.

1.5. Cytokinin Biosynthesis Pathway in Insects

CK biosynthesis is not unique to plants as various CK pathway are known to exist in bacteria (Akiyoshi et al. 1984, Powel and Morris 1986, Sakakibara et al. 2005, Barash and Manulis-Sasson 2007, Pertry et al. 2010, Samanovic et al. 2015, Seo et al. 2016, Uniyal et al. 2022) fungi (Hinsch et al. 2015, Morrison et al. 2016b) and Dictyostelium (Akoi et a. 2020). Through plants, which are the most well studied, emerging pathway modeling often compares with the two well established CK biosynthesis pathways: de novo isopentenyl transferase (IPT) and tRNA degradation IPT. CK's are adenine derived compounds. Both pathways synthesise CK molecules from either ATP, ADP, or AMP (de novo IPT) or tRNA degradation (tRNA IPT) (Sakakibara et al. 2006).

In plants, the tRNA IPT model is often associated with cis-isomer cytokinin forms whereas the de novo model is attributed to trans-isomer production (Sakakibara et al. 2005, Sakakibara et al. 2006). Most bacteria possess the tRNA degradation pathway (Frébortová and Frébort 2021). However, the emergence of CK pathways in other organisms has shown that trans-type CKs can be biosynthesised from a tRNA IPT pathway given the correct ordered modification, adenine substrate, and prenyl donor are present such as those from fungi (Morrison et al. 2016b) and Dictyostelium (Akoi et a. 2020).

The presence of these phytohormone biosynthesis pathways in non-plant species is an important discovery and brings into question; why are they present and what they

are doing? Some hypotheses implicate CKs as inter-kingdom signaling molecules for cross talk with host plants (Stirk and van Staden 2010, Andreas et al. 2020) whereas other model systems consider their role to be endogenously bioactive and unrelated to plants (Seegobin et al. 2018, Aoki et al. 2019).

A recent study by Mooi et al. 2024 used a bioinformatic pipeline approach to examine the transcriptome of 670 hexapod species, made up of mostly insects, and were able to verify that ~80% of these insects possess candidate CK biosynthesis genes. None of the insects surveyed were gall formers, however given the widespread detection of CKs across several genus of phytophagous insects (Brütting et al. 2018, Andreas et al. 2020), this works supports the theoretical presence of a CK biosynthesis pathway in all Insecta. The widespread detection of CKs (Andreas et al 2020) and CK biosynthesis genes in multiple insect species (Mooi et al. 2024) supports the unproven assertion that all insects make their own CKs and possess a CK biosynthesis pathway.

Drosophila melanogaster is a model organism insect system with a publicly available reference genome and a collection of mutant genetic lines that have been extensively researched and developed. I have identified a candidate CK biosynthesis gene in Drosophila. This gene meets the high similarity to standard to tRNA IPT 1. The candidate is currently classified as CG31381/CG11089 uncharacterized protein has no known function in Drosophila but has predicted function in the modification of tRNA within the mitochondria (NCBI-Gene [2024-07-20], Supplementary Table 2). These analogous genes in many other eukaryotes, are currently classified for their role in isopentenylation of cytoplasmic and mitochondrial tRNAs (Konevega et al. 2006, Chimnarounk et al. 2009). Isopentenylation at A37 (i^6 A37) by tRNA IPT is well know

for regulating the efficiency and fidelity of protein synthesis and has even been described in insects. Three splicing isoforms of BmIPT1-BmIPT3 have been identified in Bombyx mori (silkworm) for the vital roll in silk spinning, normal growth, and metamorphosis. This groundbreaking studied looked at the over expression and knockdown of BmIPT and detected tRNA bound i^6 A37, in insects. However, the focus was tRNA IPT role in B. mori protein synthesis and the functional biosynthesis of CKs was never investigated (Chen et al. 2018).

tRNA IPT has been overlooked for its functional role is CK biosynthesis by insects. The first committed step for CK biosynthesis pathway in insects requires tRNA substrate accompanied by a prenyl donor for adenine modification in the cytosol of insects where the prenyl donor is made available by the mevalonate pathway (MVA) (Bellés et al. 2005, Tarkowska and Strnad 2018). Therefore, CG31381/CG11089 gene function should control the production of the enzyme that catalyzes the first committed pathway step as a keystone in the production of CK metabolites. If the expression of this gene is affected, the production of the enzyme should then influence the ability to biosynthesize CK substrates for further modification.

CG31381/CG11089 genes are a candidate IPT1 and IPT2 keystone CK biosynthesis genes, and the bioinformatic support of all other subsequent enzyme-gene transcripts (Mooi et al. 2023) and endogenous CK detection in insect tissues (Andreas et al 2020) support the further pursuit to discover a CK pathway in insects.

1.6. Drosophila as a Genetic Model

The Drosophila melanogaster model system provides an opportunity for a closed experiment involving CK analysis that absents environmental influences normally associated with insects sampled from natural environments. There have been extensive scientific collaborations and efforts to understand Drosophila genomics and genetics. With the use of advanced molecular techniques, many groups have produced repositories of Drosophila mutant variants (Roote and Russell 2012). Such techniques have produced stock lines with deletion, deficiency, overexpression, under-expression, knockdown, and knockout mutations (Jennings 2013). Mutagenesis of embryos results in variable gene mutations including insertion or excision of specific targets in the genome to disrupt a gene of interest (GOI) or insert and deploy a genetic regulator such as an upstream activator sequence like the yeast specific transcription activator protein (GAL4-UAS) system, RNA interference (RNAi), and a nuclease-dead Cas9 fused to a transcriptional activator (Cas9. VPR) (Jennings 2013). These molecular mechanisms provide the basis for researching loss-of-function (LOF) or gain-of-function (GOF) studies in Drosophila. Techniques and tools used to generate these stock lines and complex mating schemes/crosses will be discussed in detail below:

Drosophila deficiency lines and balancer crosses are two important genetic tools used in Drosophila research to study gene function, chromosome stability, and karyotype. They are commonly employed to analyze the effects of gene deletions and to maintain stable stocks of genetically modified flies (Miller et al. 2019). Deficiency lines are strains of flies in which specific chromosomal segments have been deleted. These deletions remove one or more genes and their regulatory regions, allowing researchers to study the

effects of gene loss on phenotypes and biological processes. Deficiency lines are generated through a process called "chromosome engineering," in which specific regions are deliberately inserted to either remove or disrupt the gene function and/or arrangement. The resulting strains are screened to identify those that carry targeted mutations (Roote and Russell 2012). Deficiency lines are named based on the region of the chromosome that is affected. For example, a deficiency line named Df(2R)Exel1580 represents a deletion on the right arm of the second chromosome. These lines can be valuable tools for mapping genes, identifying genetic interactions, and studying gene function by analyzing the phenotypic consequences of gene loss.

To perform a balancer cross, a fly carrying a balancer chromosome is crossed with a fly of interest, such as one carrying a mutation or transgene. The resulting offspring inherit one copy of the balancer chromosome, which helps maintain the stability of the desired genetic elements. This allows for selective propagation and study of the fly's genetic modifications of interest without the risk of losing them after recombination. Drosophila deficiency lines, when used in conjunction with balancer chromosomes, facilitate the study of gene function by allowing researchers to examine the effects of specific gene deletions or LOF. The use of balancers helps maintain the stability of these lines and prevents unintended genetic changes (Roote and Russell 2012).

By combining Gal4-driven gene activation, the Drosophila Gal4-UAS-Cas9 transcriptional activator genetic cross allows for precise control over gene expression for genes of interest in specific cells or tissues of the fruit fly. Gal4-driven gene activation further be paired with transgenic RNAi lines (Trip) to generate knockdown reagents. These powerful tool offers a way to investigate LOF and GOF gene function, study

developmental processes, and explore the consequences of gene manipulation in a controlled and tissue-specific manner (Kaufman 2017). (For more information on balancers, deficiency, Gal4-UAS, Cas9-VPR, and RNAi Gal4-Trip; see Supplementary: Background on Drosophila Genetic Toolbox)

These genetic variants provided an opportunity to compare gene expression of the candidate CK biosynthesis gene to the predicted increase or decrease CK metabolite levels in these insect lines. Using expression levels and CK signatures obtained from over expression, knock down, and deficiency mutant lines offer support for the function of candidate CK biosynthesis genes and a CK biosynthesis pathway in insects.

Specific mating schemes generate overexpression, knockdown, and deficiency of the candidate CK biosynthesis gene (tRNA IPT genes) CG31381/CG11089. The crossing of stock lines either increase or decrease activation elucidating the function through the gene expression and CK metabolite analysis of CK biosynthesis in Drosophila. A bioinformatic comparison confirmed high sequence homology of two tRNA isopentenyl transferase (tRNA IPT) tRNA IPT1 and IPT2 genes, coding for the keystone enzyme required for biosynthesis and further confirmed the presence of six other putative enzymes necessary for insect CK biosynthesis pathway. Further, preliminary investigations (data not shown here) indicate Drosophila shows species specific CK signatures patterns characteristic among wild types and parental lines representative of a simple low-level baseline for analysis when introducing genetic variants. Lastly, several Drosophila mutant lines including over-expression and deficiency of the uncharacterized candidate CK biosynthesis genes are predicted to increase or decrease the activation of homologous tRNA-IPT genes in Drosophila, after which CK biosynthesis can be

monitored and compared in these lines. CK profile signatures and gene expression levels obtained from parental lines and F1 cross mutant lines revealed a CK biosynthesis pathway is functional in insects.

We hypothesized that CG31381 and CG11089 would be keystone insect tRNA IPT1 and IPT2 genes that initialize CK biosynthesis in insects. We predict that Drosophila IPT mutants will produce CK metabolite and IPT gene transcript levels that reflect their expected mutation type, when compared to parent lines. To address the hypothesis and prediction the thesis was broken into five research objectives:

1.7. Research Objectives

1. Generate mutant variants that contain CK biosynthesis candidate gene by crossing Drosophila stock lines to produce offspring generations with expected mutations in candidate CK biosynthesis gene including: overexpression, deficiency, and knockdown.

2. Collect mutant variants tissue, based on phenotype selection marks, for RNA and CK extraction.

3. Extract RNA for rtPCR, and qPCR analysis on mutant variants to obtain data for expression of CK biosynthesis candidate gene.

4. Extract CKs for HPLC-(ESI)-MS/MS analysis on mutant variant to obtain data for CK production.

5. Compare function and significance of candidate CK biosynthesis gene in insect through comparison of expected changes in CK levels relative to CK biosynthesis gene transcripts (over expression/over production of CKs and deficiency + knockdown/under production of CKs).

Results of this thesis represent the first molecular evidence that demonstrates insects can biosynthesize cytokinin plant hormones. As such it represents a fundamental step in building a better understanding of insect physiology and the biochemical relationships between insects and plants.

2. CHAPTER TWO: Methods

2.1. D. melanogaster bioinformatics and stock line genetics:

CK biosynthesis enzymes transcripts were bioinformatically characterized as putative enzymes capable of endogenous CK production and metabolism in D. melanogaster through BLASTp search (Table 1). Amino acid and nucleotide transcripts revealed seven key enzymes capable of both forward and reverse CK synthesis and metabolism including: tRNA delta (2)-isopentenylpyrophosphate transferase (also known as tRNA isopentenyltransferase), cytochrome P450 mono-oxygenase, tRNA-2-methylthio-N6-dimethylallyladenosine synthase, adenosine kinase, and purine nucleoside phosphorylase, 5'ribonucleotide phosphohydrolase, and adenine phosphoribosyltransferase. Reported enzymes contained conserved domain architecture enabling prospective function for CK biosynthesis in *D. melanogaster*. Pairwise alignments generated using BLASTp showed homology with enzyme encoded gene clusters. Sequence orthologs were comparable for all seven enzymes with several other species. All enzymes were identified with high sequence homology and classed in GenBank Homologe Database. (Table 1, Supplemental Table 6-11).

It is important to note that tRNA isopentenyltransferase catalyzes the pathway's first committed step and all other enzymes including: cytochrome P450 mono-oxygenase, tRNA-2-methylthio-N^6-dimethylallyladenosine synthase, purine nucleoside phosphorylase, adenosine kinase are the five key enzymes involved in the forward biosynthesis of CKs (Akiyoshi et al. 1984, Sakakibara et al. 2005, Sakakibara et al. 2006, Kamada-Nobusada and Sakakibara 2009, Spíchal 2012, Morrison et al. 2015a, Akoi et a. 2020, Frébortová and Frébort 2021)

Functional Category	Gene (Dmel#)	Peptide length (AA)	Current Description	EC Reference
tRNA isopentenyltransferase	Dmel_CG31381 Dmel_CG11089	477	-Uncharacterized protein -Homologous (predicted) tRNA isopentenyltransferase 1	(EC: 2.5.1.75)
Cytochrome P450 mono-oxygenase	Dmel_CG4163	503	-Uncharacterized protein -Homologous probable cytochrome P450	(EC: 1.14 -,-) (Cyp303a1)
Adenosine Kinase;	Dmel_CG11255	345	Adenosine kinase	(EC 2.7.1.20)
Purine nucleoside phosphorylase	Dmel_CG16758	396	Purine nucleoside phosphorylase	(EC 2.4.2.1)
5’ribonucleotide phosphohydrolase	Dmel_CG3362	300	5'-nucleotidase, cytosolic IIIB	(EC 3.1.3.5)
Adenine phosphoribosyltransferase	Dmel_CG18315	182	Aprt Adenine phosphoribosyltransferase	(EC 2.4.2.7)
tRNA-2-methylthio-N6-dimethylallyladenosine synthase	Dmel_CG2904	1746	Enables protein phosphatase 1 binding activity	(EC: 2.8.4.3)

Table 1: Putative gene candidates of CK biosynthesis orthologs identified in Drosophila melanogaster BLASTp hits. EC number denoted with -,- indicate family domain. AA - amino acid.

Dmel_CG31381 and homologue Dmel_CG11081 are currently classed as an uncharacterized protein with predicted tRNA isopentenyltransferase activity (tRNA IPT) (Table 1, Supplemental Table 6). Dmel_CG31381/CG11081 hold high homology to other tRNA isopentenyltransferase 1 genes identified as homologs including TRIT1 *H. sapiens*, TRIT1 *B. taurus*, Trit1 *M. musculus*, Trit1 *R. norvegicus*, TRIT1 *G. gallus*, trit1 *X. tropicalis*, trit1 *D. rerio*, AgaP_AGAP000639 *A. gambiae*, gro-1 *C. elegans*, MOD5 *S. cerevisiae*, KLLA0C07359g *K. lactis*, AGOS_AER341W *E. gossypii*, tit1 *S. pomb*e, MGG_04857 *M. oryzae*, IPT2 *N. crassa*, IPT2 *A. thaliana*, and Os01g0968700 *O. sativa* (Supplemental Table 6). *Drosophila melanogaster* stock lines were selected based on their relavence to CG11089/CG31381 mutations. Reared parent lines and F1 crosses were generated with presumed CG11089/CG31381 mutations to be sampled for both qPRC and CK analysis. Comparisons between gene expression levels and phytohormone (CK) levels were used to confirm significant influence on GOF via over expression resulting in over production of CKs and LOF through deficiency and knockdown resulting in under production of CKs.

Drosophila Crosses		Genetic Variants of F1 Crosses	
Stock # and Genotype	Stock # and Genotype	Mutation	Phenotype Selection Marker
24344 w[1118]; Df(3R)BSC318/TM6C, Sb[1] cu[1]	6326 w[1118]	Deficiency in CG11089/CG31381	Sb[1] Stubby bristles on back in adults
79824 y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TOE.GS02365}attP4 0	67048 w[*]; P{w[+mC]=UAS3xFLAG.dCas9.VPR}attP40; P{w[+mC]=tubP-GAL4}LL7/T(2;3)TSTL14, SM5: TM6B, Tb[1]	Overexpression in CG11089/CG31381	Cy curled wings in adults, Hu[1] extra bristles on the shoulders in adults, Tb[1] Tubby or short/fat body in larvae and pupae
58121 y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMJ22058}att P40	5138 y[1] w[*]; P{w[+mC]=tubPGAL4}LL7/TM3, Sb[1] Ser[1]	Knocked down expression of CG11089/CG31381	Sb[1] Stubby bristles on back in adults

Table 2: Drosophila stocks, genotypes, IPT1/2 mutation/chemotypes and phenotypic selection marker used for scoring trait inheritance in F1 offspring

2.2. Rear and Cross Drosophila Stock lines:

Drosophila stock lines categorized in Table 2 (Bloomington Drosophila Stock Center) were reared to adult maturity at 21°C, 60% RH, and 12/12 LD photoperiod on Nutri-Fly® BF media (Genesee Scientific, Santiago, California, Cat #: 66-112) a Bloomington recipe and crossed based on mating scheme to produce the predicted scorable phenotypes and candidate CK genetic mutation prior to sampling (Table 2, Figure 1, Figure 2).

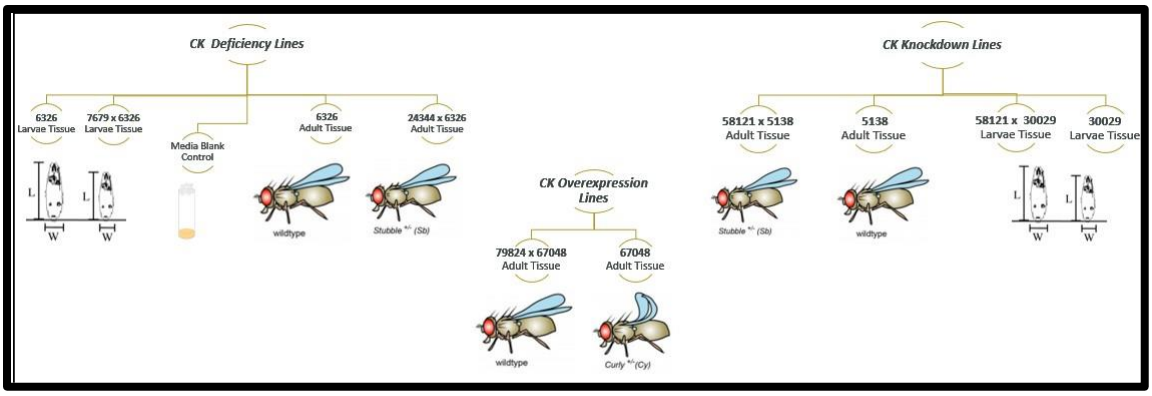

Figure 1: Mating schemes of potential Dmel_CG31381 and Dmel_CG11089 genes variants originally selected as candidates (IPT1/IPT2 mutants for comparison. Mutation type, Bloomington stock line number, and phenotypic marker are shown for each mating scheme.

2.3. Sample Drosophila Tissues:

Reared adults of F1 crosses were selected and sampled based on the presence or absence of scorable phenotypic selection markers as an indication for the presence of specific CG11089/CG31381 mutations (Table 1). Adults were anesthetised using FlyNap anesthesia (Genesee Scientific). Cultured individuals were viewed under a dissection microscope, scored, sorted, and pooled based on scorable phenotypes.

Figure 2: Dissection microscopy images of scorable phenotypic selection marker mutations for Dmel_CG31381 and Dmel_CG11089 genes mutants. Including Male (left) and female (right), fly stock #, genotypes: (1) 67048 w[*]; P{w[+mC]=UAS3xFLAG. dCas9.VPR}attP40; P{w[+mC]=tubP-GAL4}LL7/T(2;3)TSTL14, SM5:TM6B, Tb[1], (2) 79824 y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TOE.GS02365}attP40, (3) 24344 w[1118]; Df(3R)BSC318/TM6C, Sb[1] cu[1] (4) 6326* w[1118].

Tissues of the same genotype were pooled to allow for sufficient tissue mass (give mass range) required for replication and subsequent analysis. Media control samples were taken from fresh uncultured media and spent cultured media for phytohormone analysis in replicates of n=5 at 0.1 g to differentiate exogenous introduction or secretions. Fly tissues collected were pooled based on n=5 biological replicates at 0.1 g of larvae and adult fly tissue. All fly tissue types were collected in duplicate and stored at -80ºC for gene expression and phytohormone analysis.

2.4. Gene Expression of Drosophila:

Total RNA was isolated from 25 flies per genotype as per RNeasy Mini Kit (#74106, Qiagen) manufacturer's protocol. NanoDrop™ One (#ND-ONE-W, ThermoFisher Scientific) was used to quantify total RNA. CDNA synthesis was

conducted using 2 µg per sample for retrotranscription reaction with the GB-ScrIPT III 1st Strand, as per the manufacturer's protocol.

Standard PCR primers were first designed to validate CG11089/CG31381 (IPT1/IPT2) target specificity and product size before adapting to qPCR application. cDNA sequences were compared using NCBI Global Alignment tool pack to evaluate sequence homology and alignment scores. CG31381/tRNA IPT1 has a sequence length of 1783bp and CG11089/tRNA IPT2 4434 bp. Although, CG31381 is a much smaller sequence, the tRNA isopentenyltransferase 1 conserved domain architectures are contained within both cDNA sequences (Supplementary table 4). Based on 1783bp sequence length of CG31381/tRNA IPT1 there is 100% sequence homology within CG11089/tRNA IPT2 4434 bp including the conserved domains for tRNA isopentenyltransferase 1. Meaning, IPT1 and IPT2 cDNA genes are indistinguishable from a targeting diagnostic standpoint (Supplementary table 4). Therefore, primers were designed to target both cDNA sequences simultaneously. Primer sequences and technical specifications can be seen in Supplementary table 5.

For standard PCR primer optimization, cDNA from retrotranscribed RNA was targeted to confirm primer specificity and PCR product before completing qPCR analysis. Samples were amplified in triplicate (n=3) and standard PCR conditions were used (Supplementary Table 5). PCR products were visualised through agarose gel electrophoresis. Drosophila amplified IPT1/IPT2 PCR product was run on 1.5% [m/v] gel in 0.5X TBE running buffer at ~90V for 60 min using DynaView nucleic acid stain. Samples were loaded with 10 µl DNA and 8µl loading dye with a low mass 100bp ladder used for sizing fragments.

qPCR primer design used identical forward and reverse primers while later a TaqMan probe was designed to work in conjunction with this primer pair. Aligned primer and probe complementation can be seen in Supplementary Table 6. Custom TaqMan™ Gene Expression Assay, FAM (Catalog number: 4331348) by Thermo -Applied Biosystems was used to develop and optimize the Real Time PCR (qPCR) assay.

Gene expression profiling through qRT-PCR reactions was designed and optimized with primers to target CG11089/CG31381 exons including TaqMan chemistry. Expression was measured on QuantStudio 5 Real-Time PCR Systems (Applied Biosystems) as per default settings. Threshold cycle numbers were optimized by the Applied Biosystems software. Previously validated qRT-PCR reference gene probe Rpl32 was be used as a reference for normalization based on the mean values of commonly used Drosophila gene (Ponton et al. 2011, Spurrier et al. 2018). ΔΔCt methods were used to determine fold changes in expression levels between F1 mutant variants vs. parental lines as controls (Livak and Schmittgen, 2001).

2.5. Phytohormone extraction and analysis by HPLC-(ESI)-MS/MS:

A modified version of the protocol described by Quesnelle and Emery (2007) and Farrow and Emery (2012) was used for the CK extraction. Insect samples were suspended in Bieleski #2 extraction buffer (methanol:water:formic acid [15:4:1,v/v/v]). Samples were spiked with 10 ng of each deuterated internal standard CKs, including: (Nucleotides) $^{2}H_{5}$[9RMP]Z,$^{2}H_{3}$[9RMP]DHZ, $^{2}H_{6}$[9RMP]iP; (Ribosides) $^{2}H_{5}$[9R]Z, $^{2}H_{3}$[9R]DHZ, $^{2}H_{6}$[9R]iP; (Free bases) $^{2}H_{3}$DHZ, $^{2}H_{6}$iP; (Glucosides) $^{2}H_{5}$ZOG, $^{2}H_{7}$DHZOG, $^{2}H_{5}$ZROG, $^{2}H_{7}$DHZROG, $^{2}H_{5}$Z9G, $^{2}H_{3}$DHZ9G, $^{2}H_{5}$iP7G; (Methylthiols)

$^{2}H_{5}$2MeSZ, $^{2}H_{5}$2MeSZR, $^{2}H_{6}$2MeSiP, $^{2}H_{6}$2MeSiPR; (Aromatic cytokinins) $^{2}H_{7}$BA, $^{2}H_{7}$BAR (OlChemim Ltd., Olomouc, Czech Republic), and homogenized (ball mill, RetschMM300; 5min/25Hz) at 4°C with sterile zirconium oxide grinding beads (Comeau Technique Ltd., Vaudreuil-Dorion, Canada).

The samples were allowed to extract passively overnight (approximately 12 hours) at 20°C. Pellets were removed by centrifugation (Thermo Scientific; Model Sorvall ST16, Ottawa, Canada; 10 min at 10,000 RPM), the supernatant extracts collected, and samples re-extracted with 1 mL extraction buffer at -20°C for 30 min. Supernatants were pooled and dried in a speed vacuum concentrator at 35°C (Thermo Scientific; Model Savant SPD121P, Ottawa, Canada). Extraction residues were reconstituted in 0.2 mL of 1M formic acid (pH 1.4) to ensure complete protonation of all CKs. Each extract was purified on a mixed mode, reverse-phase, cation-exchange cartridge (Waters; Oasis MCX 2cc; columns, Mississauga, ON, Canada). The process was conducted on a vacuum manifold. Columns were activated with 1 mL of HPLC grade methanol and equilibrated using 1 mL of 1M formic acid (pH 1.4). After equilibration, each sample was loaded and washed with 1 mL of 1M formic acid (pH 1.4) followed by 1 ml of HPLC grade methanol. CKs were eluted based on their chemical properties. The nucleotide fraction (NTs) was eluted using 1 mL of 0.35 M ammonium hydroxide, free bases (FBs), ribosides (RBs), methylthiols (METs), and glucosides (GLUCs) were retained on the column based on charge and hydrophobic properties and, thus, these were eluted last using 1 mL of 0.35 M ammonium hydroxide in 60% methanol. All samples were evaporated to dryness in a speed vacuum concentrator at 35°C and stored at -20°C until further processing.

NTs were dephosphorylated using 3 units of alkaline phosphatase (New England BioLabs, alkaline phosphatase calf intestine, Whitby, Ontario, Canada) in 1 mL of 0.1 M ethanolamine-HCl (pH 10.4) for 12 hours at 37°C (Emery et al. 2000). The resulting RBs were dried in a speed vacuum concentrator at 35°C. Samples were re-constituted in 0.3 mL double distilled Milli-Q water for further purification on a reversed-phase C18 column (Canadian Life Sciences; C18, 2cc; columns, Peterborough, ON, Canada). Columns were activated using 0.6 mL HPLC grade methanol and equilibrated with 1.2 mL double distilled Mill-Q water. The samples were loaded onto the C18 column and allowed to pass through the column by gravity. The sorbent was washed with 0.6 mL of double distilled water and resultant ribosides eluted using 1 mL HPLC grade methanol. All sample eluents were dried in vacuo at 35°C and stored at -20°C until further processing. Dried CK samples were re-constituted in 1.5 mL of starting conditions buffer (acetic acid:acetonitrile:water [0.08:5.0:94.92, vol/vol/vol]) (Kisiala et al. 2019, Andreas et al. 2020).

2.6. Drosophila CK profiling by HPLC-MS/MS analysis:

Drosophila sample CKs quantification was conducted through UHPLC-(ESI)-HRMS/MS (Kisiala et al. 2019). A 25 µl sample was injected into the Thermo Ultimate 3000 UHPLC coupled to a Thermo Q-Exactive™ Orbitrap mass spectrometer equipped with a heated electrospray ionization (HESI) source (Thermo Scientific, San Jose, CA, USA). Compounds were separated using a reversed-phase C18 column (Kinetex 2.6 µm C18 100 A, 2.1 × 50 mm; Phenomenex, Torrance, CA, USA). All hormone fractions were eluted with a multistep gradient of component A: B-Pure water with 0.08% acetic acid mixed with component B: acetonitrile with 0.08% acetic acid at a flow rate of 0.4

mL/min. The initial conditions were 5% B increasing linearly to 10% B over 2 min followed by an increase to 95% B over 6.5 min; 95% B was held constant for 1.5 min before returning to starting conditions for 5 min; total run time was 15 min.

The eluate was introduced into the Orbitrap HESI source (capillary temperature of 250°C) and analysed using parallel reaction monitoring (PRM) at a resolution of 35,000. CKs were analysed in positive ion mode. The HESI source was operated with sheath gas, 30 arbitrary units; auxiliary gas, 8 arbitrary units; max spray current, 100 µA; auxiliary gas heater temperature, 450 °C; S-lens RF level, 60 and spray voltage 3.9 kV. The PRM parameters included the following: automatic gain control (AGC), 1×10^6; maximum injection time (IT), 128 ms; *m/z* 1.2 isolation window and normalized collision energy (NCE) individually optimized for each CK analyte (Kisiala et al. 2019, Andreas et al. 2020).

2.7. Data Management and Statistical Analysis:

Gene expression data was analyzed using QuantStudio 5 Real-Time PCR Systems (Applied Biosystems) and relative fold gene expression or delta-delta CT (2^-ΔΔ Ct) values were calculated in Microsoft excel for *Drosophila melanogaster* newly emerged adults including parent lines and crosses. (means ±SE, n=5 biological and mean for n=3 technical replicates). Data were statistically evaluated using ANOVA and monitored by the LSD test using GraphPad Prism version 10.2.3 for Windows. The results were considered significant when the P value was < 0.05. Phytohormone profile data was analysed using Analyst (v 1.5) software (AB Sciex, Framingham, MA, USA), to calculate

peak area based on qualified recovery of 2H-labelled internal standards. Concentrations of total cytokinin was calculated in Microsoft excel based on [pmol/g fresh weight] isolated from *Drosophila melanogaster* newly emerged adults including parent lines and crosses based on means ±SE, n=5 biological replicates. Data were statistically evaluated using Two Way ANOVA with Tukey Post Hoc multiple comparisons test using GraphPad Prism version 10.2.3 for Windows. The results were considered significant when the P value was < 0.05.

3. CHAPTER THREE: Results

3.1. tRNA IPT Mutants

Of the potential stock lines acquired and crosses described in methods (Figure 1, Table 2), three mating schemes were generated as candidate Dmel - CG31381/CG11089 (IPT1/IPT2) mutants (Figure 3): 1) CG31381/CG11089 (IPT1/IPT2) knockdown RNA interference (RNAi) (58121x5138), parental lines 58121 transgenic RNAi line (Trip) and 5138 Gal4 driver line (Gal4). 2) CG31381/CG11089 (IPT1/IPT2) heterozygous deletion - deficiency (22344x6326), stock balancer line (6326 $^{w\ [1118]}$), and in vivo balancer line (24344 $^{w\ [1118]}$) and 3) CG31381/CG11089 (IPT1/IPT2) over expression mutant (67048x79824) assessed against parental lines: Gal4-UAS (67048), and TOE line (79824).

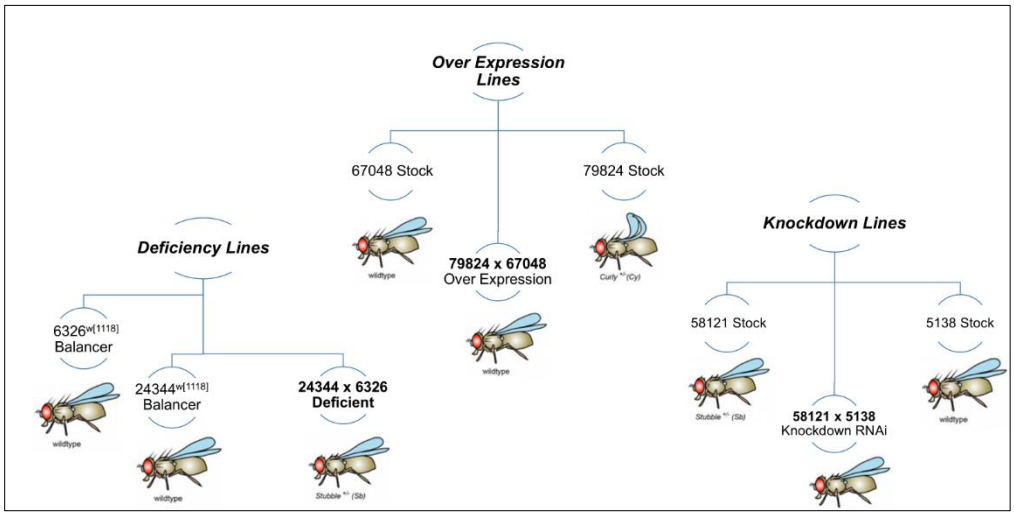

Figure 3: Mating schemes of sampled Dmel_CG31381 and Dmel_CG11089 genes variants cultured and selected as candidates IPT1/IPT2 mutants for comparison. Mutation type, Bloomington stock line number, and phenotypic marker are shown for each mating scheme. (n=5 biological replicates for CK analysis and n=5 biological replicates and n=3 technical replicates for gene expression.

Drosophila deficiency line carry a heterozygous deletion of both CG31381 and CG11089 genes. Since Drosophila is diploid, this implies that one version of both genes has been deleted from the genome and one version remains. If a full deletion (homozygous/knock out) of these genes occurs, the result is lethal. To avoid recombination and loss of the deleted gene, the deficient line is crossed with a balancer line and resulting F1 generation carry a balancer chromosome. The balancer chromosome is denoted as w [1118] and can be noted in the respective genotypes (Table 2,) whereas the sister chromosome carrying the deletions is marked and expresses a scorable phenotype (Figure 3). Deficiency lines decrease expression of candidate insect tRNA IPT1/IPT2 genes capable of initializing CK biosynthesis. Conversely, over expression

stock expresses a guide RNA that can be used to direct a nuclease-dead Cas9 fused to a transcriptional activator (Cas9.VPR) to the space in between CG11089/CG31381 to activate transcription. Expression of this guide RNA is ubiquitous and Cas9.VPR so stocks express Cas9.VPR under the control of UAS (Upstream Activating System) when GAL4 is present. Therefore, activation of this overexpression mechanism occurs in our reared F1 generations crosses containing a scorable phenotype (Figure 3) (Jennings 2013).

3.2. Gene Expression

RT-qPCR was used to examine relative fold gene expression levels in Dmel - CG31381/CG11089 (IPT1/IPT2) genes in knockdown RNAi line (58121x5138) and parental line (58121). Parental lines (58121) transgenic RNAi line (Trip) and (5138) Gal4 driver line (Gal4) were cultured independently whereas knockdown RNAi (58121x5138) were crossed, and mutant selected based on their phenotypic marker (Figure 1, Table 2) from the same culture vials. The purpose of this was to compare the potential knocked down expression of IPT1/IPT2 to both the original parent lines used to generate the knockdown. The knockdown RNAi line (58121x5138) exhibited a 0.81-fold expression representing a 20% relative fold change in gene expression compared to Trip (58121) at 1.01-fold expression; however, the decrease was not considered significant (Supplementary Figure 10, Supplementary Table 12). RNA extraction negatives and no template controls showed no amplification as expected.

RT-qPCR was used to examine relative fold gene expression levels in Dmel - CG31381/CG11089 (IPT1/IPT2) genes in heterozygous deletion - deficiency (22344x6326), stock balancer line (6326 $^{w\ [1118]}$), and in vivo balancer line (24344 $^{w\ [1118]}$). Stock balancer line (6326 $^{w\ [1118]}$) was cultured independently whereas in vivo balancer (24344 $^{w\ [1118]}$) and (24344x6326) were selected based on their phenotypic marker from the same culture vials. The purpose of this was to compare the deficient expression of IPT1/IPT2 to both the original stock balancer line used to generate the deficiency line and in vivo control (24344 $^{w\ [1118]}$) carrying the same balancer chromosomes as (6326 $^{w\ [1118]}$), both absent of the IPT1/IPT2 deletion.

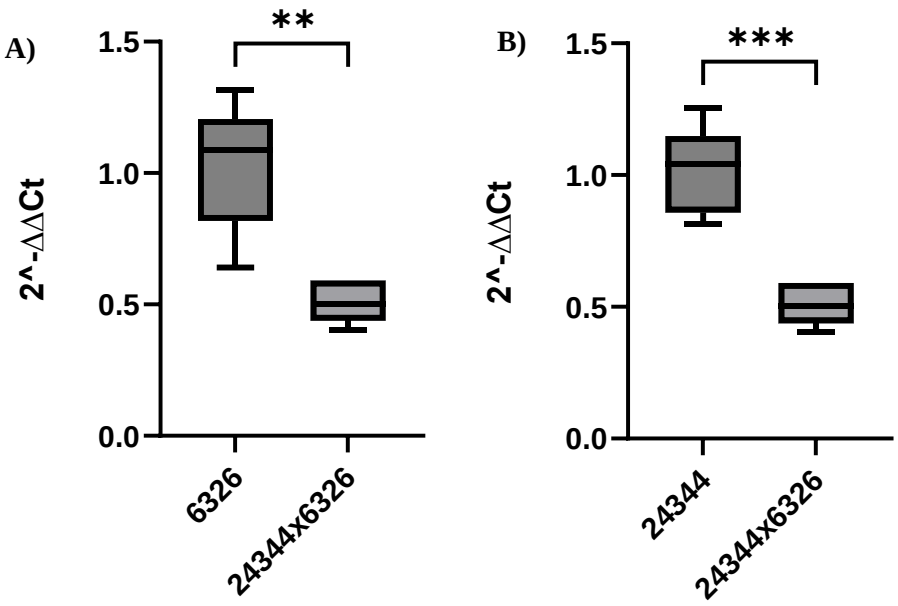

Figure 4: IPT1/IPT2 Def - relative fold gene expression (2^-ΔΔ Ct) of *Drosophila melanogaster* adults including parent lines and crosses: Including A) deficiency (22344x6326) against stock balancer line (6326 $^{w\ [1118]}$) and B) deficiency (22344x6326) against in vivo balancer line (24344 $^{w[1118]}$). Where means ±SE, n=5 biological and mean for n=3 technical replicates were used including unpaired T test comparisons: Alpha 0.05 - P Value ** 0.0021 Alpha 0.05 - P Value ** 0.0003.

Expression of IPT1/IPT2 differed in both above mentioned comparisons when using the delta-delta CT (2^-ΔΔ Ct) 'control vs. treated' relative fold gene expression method with endogenous control gene Rpl32 for normalization. Comparing transcript levels of (6326 $^{w\ [1118]}$) stock balancer line against (24344x6326) deficiency line, there was more than a 50% decrease in expression with a mean value of 1.03-fold expression to 0.51-fold expression, respectively. Expression was significantly lower (P Value = 0.0021) in (24344x6326) deficiency as demonstrated by an unpaired T test comparison (Fig 4 A, Supplementary Table 12).

Similarly, when comparing transcript levels of (24344 $^{w\ [1118]}$) in vivo balancer line against (24344x6326) deficiency line, there was a 50% decrease in expression with a mean value of 1.01-fold expression to 0.51-fold expression, respectively. Expression was significantly lower (P Value = 0.0003) in 24344x6326 deficiency as demonstrated by an unpaired T test comparison (Fig 4, B). RNA extraction negatives and no template controls showed no amplification as expected.

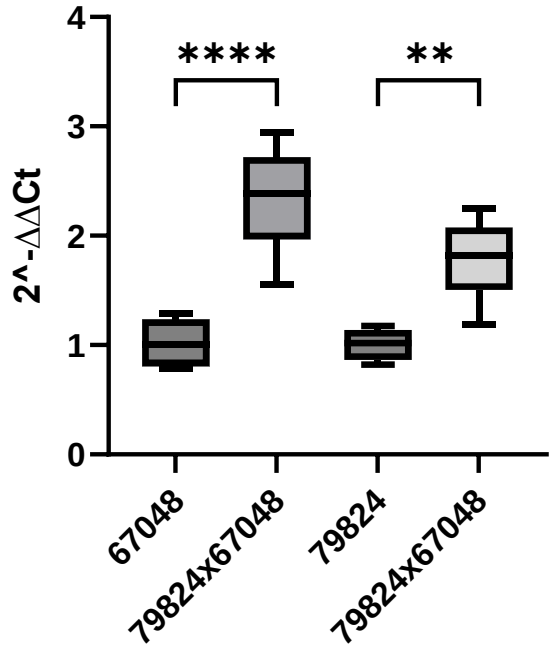

Figure 5: IPT1/IPT2 OE - relative fold gene expression (2^-ΔΔ Ct) of *Drosophila melanogaster* adults including parent lines and crosses: Including: Gal4-UAS Driver Line (67048) against IPT Over Expression (67048x79824) and Trip Over Expression Line (79824) against IPT Over Expression (67048x79824). Where means ±SE, n=5 biological and mean for n=3 technical replicates were used including a One-way Anova Multiple Comparisons: Alpha 0.05 - P Value **** 0.0001 and ** 0.0086.

RT-qPCR was used to examine relative fold gene expression levels in Dmel - CG31381/CG11089 (IPT1/IPT2) genes in over expression mutant (67048x79824) compared to both parental lines: (67048) Gal4-UAS driver line (Gal4-UAS), and (79824) trip over expression line (TOE). Parental lines (67048 and 79824) were cultured separately and over expression (67048x79824) crosses were selected based on their phenotypic marker from their own culture vials. The purpose of this was to compare the expression of IPT1/IPT2 to both parent lines using the delta-delta CT (2^-ΔΔ Ct) ‘control

vs treated' relative fold gene expression method with endogenous control gene Rpl32 for normalization.

Expression of IPT1/IPT2 differed in both above mentioned comparisons when comparing transcript levels of Gal4-UAS line (67048) to over expression (67048x79824), there was more than a 100% difference in expression with a mean value of 2.35-fold expression to 1.09-fold expression, respectively. Expression was significantly higher (P Value = 0.0001) in (67048x79824) over expression as demonstrated by a One-way Anova using multiple comparisons (Figure 5). Correspondingly, when comparing transcript levels of (79824) TOE line to over expression (67048x79824), there was approximately an 80% difference in expression with a mean value of 1.01-fold expression to 1.80-fold expression, respectively. Expression was significantly higher (P Value = 0.0086) in (67048x79824) over expression as demonstrated by a One-way Anova using multiple comparisons (Figure 5). RNA extraction negatives and no template controls showed no amplification as expected.

3.3. Cytokinin Profiles

CK profiling was conducted in all tRNA IPT mutants discussed in section 3.1. including comparisons between 3 separate candidate Dmel - CG31381/CG11089 (IPT1/IPT2) mutations. 1) CG31381/CG11089 (IPT1/IPT2) RNA interference (RNAi) (58121x5138), parental lines (58121) transgenic RNAi line (Trip) and (5138) Gal4 driver line (Gal4). 2) CG31381/CG11089 (IPT1/IPT2) heterozygous deletion - deficiency

(22344x6326), stock balancer line (6326 $^{w\ [1118]}$), and in vivo balancer line (24344 $^{w[1118]}$) and 3) CG31381/CG11089 (IPT1/IPT2) over expression mutant (67048x79824) assessed against parental lines: Gal4-UAS driver line (67048), and trip over expression line (79824).

A method similar to Kisiala et al. (2019) was used to scan for 25 different CKs using reverse-phase C18 (UHPLC-MS/MS) chemistry. Interestingly, it was common that the same 9 distinct CK analytes were detected as CK signatures in all parental lines and mutant crosses tested (Table 3 and Table 4). From these signatures, total CK concentration and a comparison between trans-zeatin (tZ) and N^6-isopentenyladenine (iP) forms were calculated and presented here in order compare against corresponding gene expression level and investigate tRNA IPT CK biosynthesis activity. Concentrations of total cytokinin [pmol/g fresh weight] isolated from Drosophila were compared between IPT1/IPT2 - RNAi knockdown (58121x5138) and parental lines (58121) Trip line and (5138) Gal4 line. Interestingly, 58121x5138 RNAi knockdown line showed more than a 10X higher total CK levels with a mean value of 1033 pmol/gFW to 180 pmol/gFW found in (5138) Gal4 line, and with a mean value of 174 pmol/gFW to (58121) Trip line (Supplementary Figure 6, Supplementary Figure 7).

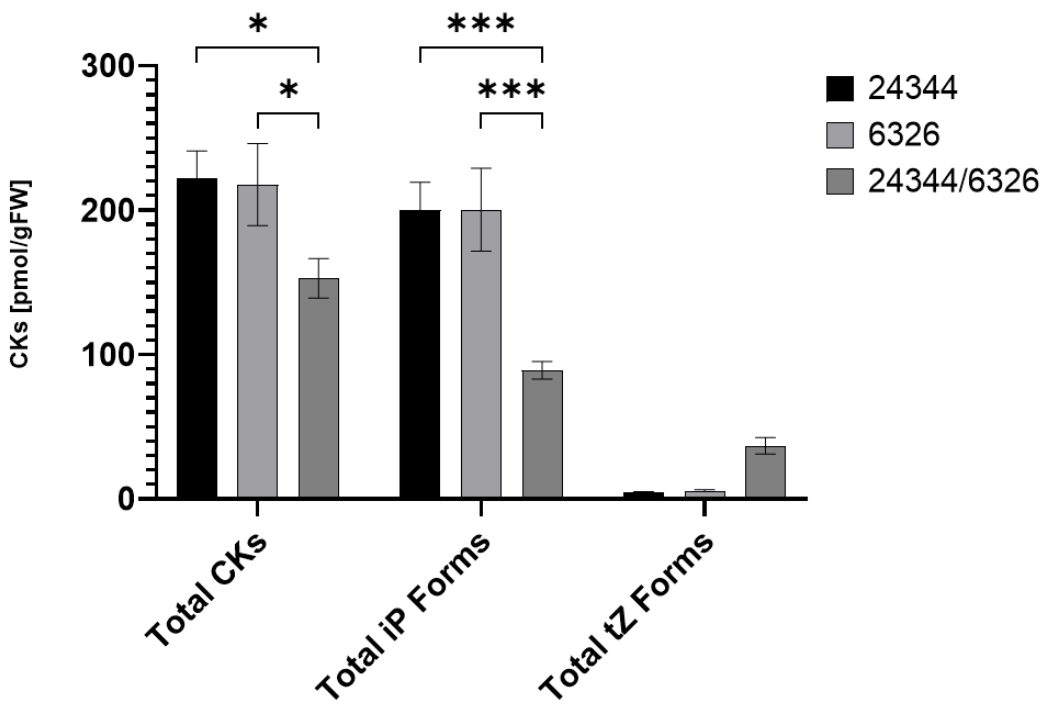

Figure 6: IPT1/IPT2 Def - total cytokinin concentrations [pmol/g fresh weight] isolated from *Drosophila melanogaster* adult parent lines and cross. Including in vivo balancer line (24344 $^{w\,[1118]}$), stock balancer line (6326 $^{w\,[1118]}$), and deficiency (22344x6326) (means ±SE, n=5 biological replicates). Table Analyzed: Def - CKs Groups Two-way ANOVA, Ordinary Alpha 0.05 Source of Variation % of total variation P value <0.0007 *** P value <0.0012 *.

Concentrations of total cytokinin [pmol/g fresh weight] isolated from Drosophila were further compared between (6326 $^{w[1118]}$) stock balancer line against (24344x6326) deficiency line, showing more than a 30% difference in total CK levels with a mean value of 218 pmol/gFW to 153 pmol/gFW, and a 32% difference in total CK levels, between in vivo balancer line (24344 $^{w[1118]}$) against (24344x6326) deficiency line, with a mean value of 222 pmol/gFW to 153 pmol/gFW, respectively. Total CKs was significantly lower (P Value = 0.0012) in 24344x6326 deficiency compared to both (6326 $^{w[1118]}$) stock

balancer line and in vivo balancer line (24344/6326 $^{w[1118]}$) as demonstrated by a Two-way ANOVA (Fig 6).

Total iP forms to tZ forms were examined to investigate whether there was a relationship between IPT deficiency mutations and tRNA IPT CK biosynthesis pathway dynamics. Similarly, when total CKs were split up into iP and tZ forms, it is apparent that iP forms make up the majority of the CK pool, for non-IPT mutant line. Such as comparing only, iP forms between (6326 $^{w[1118]}$) stock balancer line against 24344x6326 deficiency line, there was 2.25X more iP forms with a mean value of 200 pmol/gFW to 89 pmol/gFW, and notably the exact same difference in iP forms, between in vivo balancer line (24344 $^{w[1118]}$) against 24344x6326 deficiency line, with a mean value of 200 pmol/gFW to 89 pmol/gFW, respectively. Total iP forms were significantly lower (P Value = 0.0007) in (24344x6326) deficiency compared to both in vivo balancer line (24344 $^{w[1118]}$) and stock balancer line (6326 $^{w[1118]}$) as demonstrated by a Two-way ANOVA (Fig 6). Notably, tZ forms did not show a significant difference but interestingly, (24344x6326) deficiency contained more tZ overall and most of which is composed of tZ freebase (Table 3).

Drosophila melanogaster				
		24344 (Def)	24344 $^{w\ [1118]}$	6326 $^{w\ [1118]}$
FB	DZ	n.d.	n.d.	n.d.
	tZ	36.86±5.78	0.21±0.13	0.84±0.16
	cisZ	n.d.	n.d.	n.d.
	iP	17.95±2.95	7.69±1.06	6.58±1.23
RB	DZR	n.d.	n.d.	n.d.
	tZR	n.d.	2.74±0.78	3.08±0.70
	cisZR	n.d.	n.d.	n.d.
	iPR	n.d.	25.61±2.75	16.16±0.45
NT	DZR	n.d.	n.d.	n.d.
	tZR	1.27±0.63	1.79±0.59	n.d.
	cisZR	n.d.	n.d.	n.d.
	iPR	71.17 ±3.25	166.63±18.40	177.59±28.80
MET	MeSZ	19.66±2.75	5.53±0.76	3.28±1.22
	MeSiP	2.02±0.70	1.93±0.34	3.81±0.77
	MeSZR	5.15±1.98	3.53±0.54	1.04±0.11
	MeSiPA	n.d.	6.75±0.74	3.59±0.38

Table 3: IPT1/IPT2 Def - cytokinins profiles detected in Deficient (24344 Def), in vivo balancer line (24344 $^{w\ [1118]}$) and (6326 $^{w\ [1118]}$) stock balancer line of *D. Melanogaster* expressed as pmol/g fresh weight (means ±SE, n=5) and n.d. represents not detected.

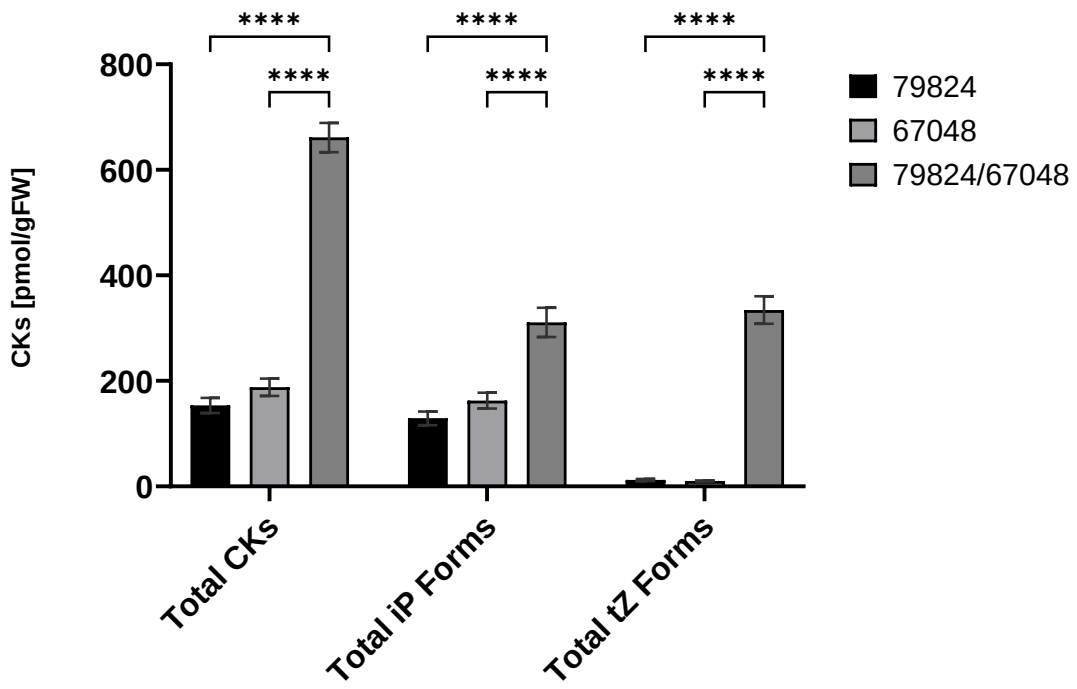

Figure 7: IPT1/IPT2 OE - total cytokinin concentrations [pmol/g fresh weight] isolated from *Drosophila melanogaster* adult parent lines and cross: Including: Gal4-UAS Driver Line (67048), Trip Over Expression Line (79824), and IPT Over Expression (67048x79824) (means ±SE, n=5 biological replicates). Table Analyzed: OE- CKs Groups Two-way ANOVA, Ordinary Alpha 0.05 Source of Variation % of total variation P value <0.0001 ****.

Concentrations of total cytokinin [pmol/g fresh weight] isolated from Drosophila were compared between of Gal4-UAS line (67048) to over expression (67048x79824). Comparisons show 3.75X more total CKs detected with a mean value of 196 pmol/gFW to 737 pmol/gFW, respectively. Correspondingly, when comparing total CK levels of (79824) TOE to over expression (67048x79824), there was 4.82X more total CKs detected with a mean value of 153 pmol/gFW to 737 pmol/gFW, respectively. Total CKs

was significantly higher (P Value = 0.0001) in (67048x79824) over expression compared to (67048) and 79824 as demonstrated by a Two-way ANOVA (Fig 7).

Total iP forms to tZ forms were again compared to investigate whether there was a relationship between IPT over expression mutations and tRNA IPT CK biosynthesis pathway dynamics. Conversely, when total CKs were split up into iP and tZ forms, it is clear that iP forms make up the majority of the CK pool, parental line Gal4-UAS line (67048) and TOE line (79824). When comparing only, iP forms between over expression (67048x79824) to Gal4-UAS line (67048), there was 3.7X more iP forms with a mean value of 737 pmol/gFW to 196 pmol/gFW, and 4.8X more iP forms with a mean value of 737 pmol/gFW to 153 pmol/gFW difference in iP forms, when comparing over expression (67048x79824) to TOE line (79824), respectively. Total iP forms was significantly higher (P Value = 0.0001) in over expression (67048x79824) compared to trip over expression (79824) and Gal4-UAS line (67048) as demonstrated by a Two-way ANOVA (Fig 7). Notably, tZ forms show a significant difference (P Value = 0.0001) in over expression (67048x79824) compared to TOE (79824) and Gal4-UAS line (67048) and interestingly, the highest tZ levels measured in this study most of which is composed of tZ freebase (Table 4).

Drosophila melanogaster				
		79824x67048 (OE)	79824	67048
FB	DZ	n.d.	n.d.	n.d.
	tZ	323.33±24.39	0.79±0.23	0.74±0.21
	cisZ	n.d.	n.d.	n.d.
	iP	44.15±5.93	4.33±0.81	3.07±0.62
RB	DZR	n.d.	n.d.	n.d.
	tZR	10.81±2.36.	3.30±0.53	5.18±0.92
	cisZR	n.d.	n.d.	n.d.
	iPR	44.96±8.64	15.50±1.42	25.27±4.33
NT	DZR	n.d.	n.d.	n.d.
	tZR	n.d.	8.09±1.59	4.27±0.44
	cisZR	n.d.	n.d.	n.d.
	iPR	221.72±18.17	109.30±12.19	134.23±10.58
MET	MeSZ	7.36±1.21	3.54±0.45	4.84±0.88
	MeSiP	1.43±0.53	3.87±0.75	4.15±0.48
	MeSZR	5.75±0.48	1.64±0.26	1.97±0.40
	MeSiPA	1.71±0.17	2.99±0.38	4.13±0.48

Table 4: IPT1/IPT2 OE - cytokinins profiles detected in (79824x67048 OE), parent line (79824) and parent line (67048) of *D. Melanogaster* expressed as pmol/g fresh weight (means ±SE, n=5) and n.d. represents not detected.

Cytokinins profiles were compared between Drosophila tissue and media types to rule out the possibility that CKs detected in insect tissues could be a result of bioaccumulation of consumptions. A further comparison was made between blank (new) and spent (used) media to consider both bioaccumulation and secretion as effects (Supplementary Fig 12). Interestingly overall CKs detected in both media were unique compared in Drosophila tissues. Distinctive CK metabolites like cisZR found in both media types that were not detected in Drosophila tissues (Table 3, Table 4, Supplementary Fig 12). Further distinctive CK metabolites like MesZ not detected in fresh media were present in spent media (Supplementary Fig 12).

CHAPTER FOUR: DISCUSSION

The purpose of this study was to determine if CG31381 and CG11089 are functional IPT1 and IPT2 genes involved in CK biosynthesis and regulation in Drosophila. To address this question concordant experiments compared genetic variants and checked for the expected increase or decrease in IPT gene expression, and any corresponding changes in CK analyte production. This work is the first to show that CG31381 and CG11089 are functional IPT1 and IPT2 genes involved in CK biosynthesis and regulation in Drosophila. As such it is the first time CK synthesis has been shown to occur in an insect.

3.4. tRNA IPT Mutants

Mating schemes described (Table 2, Figure 2) were used to generate candidate Dmel - CG31381/CG11089 (IPT1/IPT2) mutations. 1) CG31381/CG11089 (IPT1/IPT2) RNA interference (RNAi) (58121x5138), parental lines 58121 transgenic RNAi line (Trip) and 5138 Gal4 driver line. 2) CG31381/CG11089 (IPT1/IPT2) heterozygous deletion - deficiency (22344x6326), stock balancer line (6326 $^{w\ [1118]}$), and in vivo balancer line (24344/6326 $^{w\ [1118]}$) and 3) CG31381/CG11089 (IPT1/IPT2) over expression mutant (67048x79824) assessed against parental lines: Gal4-UAS driver line (67048), and trip over expression line (79824).

Mutant Line screening for continued IPT mutant candidacy use include the RNAi (58121x5138), Trip (58121), and Gal4 (5138) lines which did not produce the expected chemotype for decreased CK production and IPT1/IPT2 gene expression. This was apparent when comparing gene expression levels (58121x5138) RNAi line to parent

lines. Although, 58121x5138 RNAi line was lower than parent line 58121 (Supplementary Figure 10, Supplementary Table 12) the difference between mutant and control line was not significant. Second, the CK profiles of 58121x5138 RNAi knockdown line showed the opposite effect as expected and produced more than a 10X higher total CK levels than 5138 parental line and 58121 parent line (Supplementary Figure 6, Supplementary Figure 7). These results were unexpected and the opposite of the candidate phenotype. The mating schemes and expected phenotypes have been screen for their expected genotypes but mating schemes are theoretical and have never been tested for CK genes and analyte production. This may be a result of an unknown CK recovery mechanism or possibly an error in Drosophila reagent production. Therefore, future investigation would be required to interpret the unexpected outcome from these trials. Both candidates for overexpression and deficiency of CK biosynthesis gene (tRNA IPT genes) CG31381/CG11089 mutant lines served to elucidate the function gene expression and CK metabolite profile of CK biosynthesis in Drosophila.

3.5. Gene Expression

CG31381 and CG11089 sequences were compared using NCBI Global Alignment tool pack to evaluate sequence homology and alignment scores. Based on 1783bp sequence length of CG31381/tRNA IPT1 there is 100% sequence homology within CG11089/tRNA IPT2 4434 bp including the conserved domains for tRNA isopentenyltransferase 1 (Supplementary table 4). Standard PCR primers designed to validate CG11089/CG31381 (IPT1/IPT2) target specificity and product size of 183bp

(Supplemental Figure 4) before adapting to TaqMan qPCR. Therefore, primers used for standard PCR were adapted to qPCR and Taqman probe was design to target the intron of RNA transcript sequences simultaneously. Primer sequences and technical specifications can be seen in Supplementary table 5. It is important to consider that assays used such as RNA extraction with DNAase digestion, gDNA WiperMix incubation prior rt-PCR were used to prevent the chance of DNA contamination influencing qPCR downstream detection. A multiplex qPCR approach was used to ensure that qPCR TaqMan probes of IPT1/2 were run with endogenous control gene Rpl132, to ensure normalized availability of template cDNA for each of the targets within the reaction. This approach is not required but is best practice for gene expression (Livak et al 2001, Roote and Russel 2012).

RNA transcript levels quantified using Rt-qPCR (TaqMan qPCR IPT1/2 gene probes normalized with Rp132 gene) showed a significant relationship between CG31381/CG11089 transcript levels and the deficiency and over expression of tRNA IPT activity (Fig 4, Fig 5). This was further supported by CK analyte signatures obtained from these parental and mutant lines via UHPLC-(ESI)-HRMS/MS methods (Fig 6, Fig 7) demonstrating a direct correlation between gene expression and total CK levels measured in corresponding mutants. Overall, these data (Fig 4, Fig 5, Fig 6, Fig 7) provide valuable insights into the differences in gene expression between the groups and highlights the biological consequences of the tRNA IPT mutation in controlling CK biosynthesis in Drosophila. This information is crucial for understanding the underlying mechanisms and potential future applications of this genetic system.

3.6. Cytokinin Profiling

Increased CK production was observed in IPT overexpression (79824/67048), when compared to parental control lines (Figure 7). Whereas decreased CK production was observed in the deficiency (24344x6326), when compared to parental control lines (Figure 6), indicating that the genetic mechanisms of these mutant lines lead to a significant regulatory control of the pathway's first committed step in tRNA IPT cytokinin biosynthesis. Further, Drosophila shows species specific CK signatures patterns characteristic among parental lines (139 -222 pmol/g FW, Table 3, Table 4, Figure 6, Figure 7, Supplemental Figure 6) representative of a simple low-level baseline for analysis when introducing genetic variants. It is noteworthy that deficiency (24344x6326) is a heterozygous deletion of the IPT1 and IPT2 genes, meaning that there are normally 4 version in the Drosophila diploid genome and full deletion of these genes is lethal. Therefore, the expected phenotype decreased CK production and IPT transcript levels is difficult to predict. A 50% reduction could be estimated for both metrics. However, deficiency (22344x6326) showed approximately 30% decrease in CK production and 50% decrease in IPT transcript levels compared to both stock balancer ($6326^{w\,[1118]}$) and in vivo balancer ($24344^{w\,[1118]}$) lines (Figure 4, Figure 6).

Drosophila CK signatures are proportionally composed of trans-zeatin (tZ) and N^6-isopentenyladenine (iP), and no dihydro-zeatin (DZ) or cis-zeatin cZ forms of CKs (Table 3 and 4). IPT overexpression (79824x67048) produced the highest measure of tZ, a freebase form and, in plants, a highly bioactive CK, (Romanov et al 2006) suggesting that tRNA IPT overexpression in insects induces activation of cascading downstream

enzymatic biosynthesis activity that could be beneficial to the insect. Further, gall forming insects contain similar CK signatures, often dominated by tZ and iP (Mapes and Davies 2001, Straka et al. 2010, Yamaguchi et al. 2012, Tanaka et al. 2013, Takei et al. 2015, Zhang et al. 2017, but in much higher quantities, when compared to non-gall forming insect (Andreas et al 2020). The endogenous CK levels in plant gall forming insect tissue is presumedly attributed play a role in the growth of plant galls. Non-gall forming phytophagous insects that feed directly on plant tissues, have been hypothesised to use low level CKs as a currency in exchange for food in the form of sustained feeding sites and or habitat (Andreas et al. 2020). While Drosophila produces lower level CK signatures (Figure 6 and 7), it is important to consider their role in the organism's life history. Drosophila is unique from other gall forming and non-gall forming phytophagous insects because it does not rely on a growing plant tissue for a feeding site. Wild Drosophila can feed on the surfaces of live fruits, leaves, plant secretions, and aphid honeydew but often during preoviposition choose to feed on decaying fruits, vegetables and other decaying materials. Therefore, plant interaction is not obligate but rather their feeding style is facultative (Broderick and Lemaitre 2012). This supports the observation of low level CKs detected (139 -222 pmol/g FW, Table 3, Table 4, Figure 6, Figure 7, Supplemental Figure 6) observed across all parental lines/excluding IPT mutant during experimental rearing. It is also noteworthy the media comparisons between blank and spent media showed different compositions than Drosophila tissues as well as higher CK levels in spent media than fresh, lending to the possibility that secretion of CKs by Drosophila back into the media as a potential explanation (Table 3, Table 4, Supplementary Fig 12). This further supports the hypothesis that all phytophagous insects

likely can produce CKs as a means of farming their plant host tissue by synthesising the plant's own native compounds. Therefore, it is possible the conservation of a CK biosynthesis gene pathway in lab-reared Drosophila is a result of result circumstance rather than necessity. However, it is important to consider that CK biosynthesis in Drosophila/Insecta is not exclusively related to plant interactions but may play a role in the insect's own physiology. 24344 deficiency lines possess a heterozygous deletion of CG31381/CG11089 because full homozygous deletions are lethal. Although these gene may be multifactor and regulate other process, such as post transcriptional modification in isopentenylation of cytoplasmic and mitochondrial tRNAs (Konevega et al. 2006, Chimnarounk et al. 2009, Chen et al. 2018), it is likely that CKs may be endogenously bioactive in Drosophila and important to their own physiological and regulatory process. As previously identified, CKs can be categorized as both inter-kingdom signaling molecules for cross talk with host plants (Stirk and van Staden 2010, Andreas et al. 2020) and in other organisms their role endogenous and unrelated to plants (Seegobin et al. 2018, Aoki et al. 2019).

3.7. Cytokinin Biosynthesis Pathway

The tRNA degradation CK biosynthesis pathway, shown in Figure 8, produces isoprenoid CKs in *D. melanogaster* via (1) tRNA isopentenyltransferase (*EC 2.5.1.8) responsible for N-prenylation of adenosine monophosphates (AMP) at the N6-terminus with dimethylallyl diphosphate (DMAPP). The dimethylallyl moiety in DMAPP is transferred to the amino group of A37 in certain tRNAs, resulting in the formation of i^6A (Xie et al. 2007). This non –hydroxylated tRNA substrate preylated with DMAPP forms

the iPRP-tRNA construct. i^6A tRNA is methylthiolated with S-Adenosyl L-methionine and a thiol group by the enzyme EC: 2.8.4.3 (tRNA-2-methylthio-N6-dimethylallyladenosine synthase), forms 2MesiPR-tRNA. (3) iPRP-tRNA is catalyzed by cytochrome P450 mono-oxygenase (EC: 1.14 -,-), allowing for the conversion of tZRP-tRNA and 2MesiPR to 2MesZPR. These reactions are interconverted and therein reversible. Noteably, Cyp303a1 is identified with higly conserved functional domains to CYP450 (Supplmental Table 7). All tRNA bound CKMP are naturally degraded to MP forms. (4) 5'-nucleotidase (EC 2.4.2.1) formaly know as 5'ribonucleotide phosphohydrolase (EC 3.1.3.5) catalyses dephosphorylation of nucleotides, iPRP, tZRP, 2MesiPR, and 2MesZPR. (5) Resulting iPR and tZR forms are further catabolised by adenosine nucleosidase (EC 3.2.2.7) to produce iP, tZ, 2MeSiP, and 2MeSZ freebases. (6) Adenosine kinase catalyses dephosphorylation of nucleotides, iPRP and tZRP and is capbable of reversible interconversion . (7)Reversibly, purine nucleoside phosphorylase catalyses the reaction of the 5'-ribonucleotide with the addition of a phosphate allowing for interconversion of tZR and iPR to tZRP and iPRP.(8) Adenine phosphoribosyltransferase catalyzes a phosphoribosyl transfer from PRPP to adenine, forming AMP and releasing pyrophosphate (PPi), thus allowing for direct interconversion of tZ and iP to tZRP and iPRP. Other candidate enzymes such as adenosine nucleosidase was detected only with low sequence pairwise percentage identity and CK phosphoribohydrolase 'Lonely guy: LOG' showed no significant similarities through enzyme encoded BLASTp sequence analysis and was not identified through homologene identity and therefore not characterized herein.

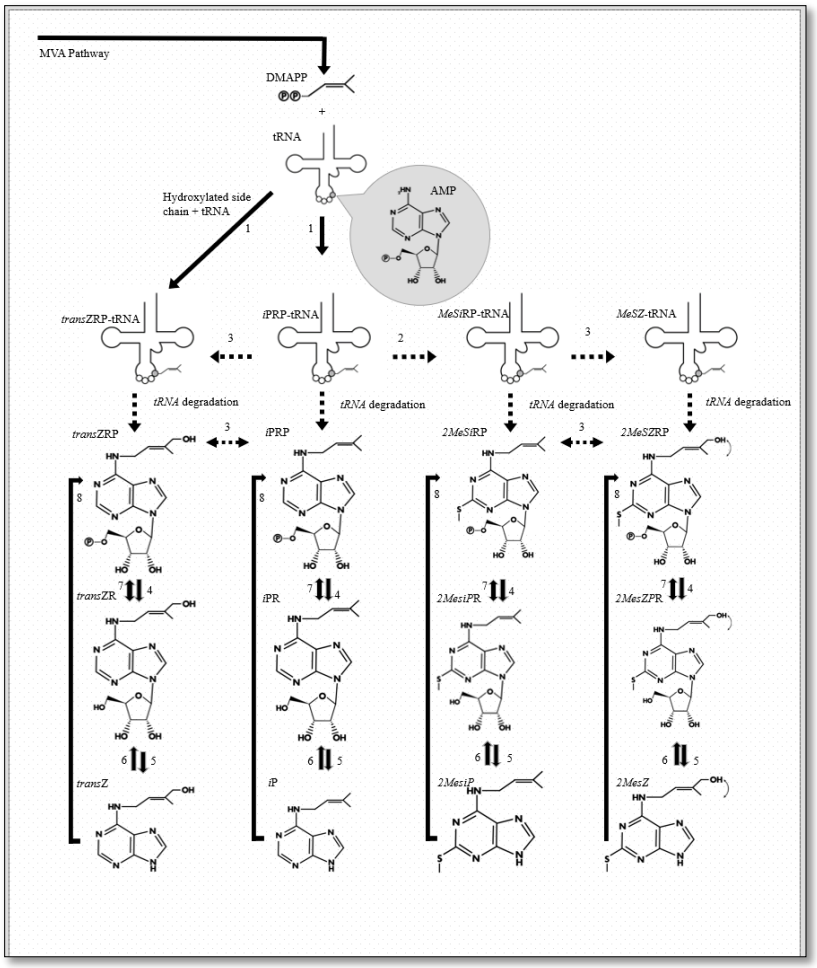

Figure 8: A proposed tRNA Degradation CK biosynthesis pathway in Insecta. Numbers represent putative candidate enzymes as follows: 1. tRNA delta (2)-isopentenylpyrophosphate transferase; (*EC: 2.5.1.75) –formerly known as tRNA isopentenyltransferase (*EC 2.5.1.8). 2. tRNA-2-methylthio-N6-dimethylallyladenosine synthase (EC: 2.8.4.3). 3. cytochrome P450 mono-oxygenase (EC 1.14). 4. 5'-nucleotidase (EC 2.4.2.1) - formaly known as 5'ribonucleotide phosphohydrolase (EC 3.1.3.5). 5. adenosine nucleosidase (EC 3.2.2.7). 6. adenosine kinase (EC 2.7.1.20). 7. purine nucleoside phosphorylase (EC 2.4.2.1). 8. adenine phosphoribosyltransferase (EC 2.4.2.7). CKs named represent those detected in Drosophila tissues. Numbers represent CK biosynthesis enzyme. (*EC represents functionally characterized enzymes and EC represent bioinformatically putative enzymes. Both with orthology identified based on searches in the *Drosophila melanogaster* genome).

It is important to consider that, in plants, the tRNA-IPT model is often associated with cis-isomer (cZ) CK forms whereas the de novo model is attributed to trans-isomer (tZ) production (Sakakibara et al. 2005, Sakakibara et al. 2006). However, a tRNA IPT tZ conjugate pathway is possible given the correct ordered modification, adenine substrate, and prenyl donor are present such as those from fungi (Morrison et al. 2016b) Dictyostelium (Akoi et a. 2020), and insect (Mooi et al. 2024).

CKs biosynthesized by insects is a fundamental step in understanding the biochemical relationships between insects and plants. The pathway signifies insects may be farming plant tissue by manipulating the plants own native compounds as to maintain foreign diplomacy and refugee asylum. This research has shown that insects have the genetic ability to create CKs and conjointly compare the CK metabolite profile to investigate their potential role in biochemical crosstalk. This work will be the first to categorize an insect genetic pathway to create, a phytohormone, cytokinin (CK). A functional genomics approach has confirmed the high sequence homology of two tRNA isopentenyl transferase (tRNA IPT) tRNA IPT1 and IPT2 genes, coding for the keystone enzyme required for biosynthesis and further bioinformatic comparison confirmed the presence of six other enzymes necessary for an insect CK biosynthesis pathway (Table 1, Supplemental Table 6-11), although these six other enzymes were only inferred, and functional genomics confirmation was not performed.

The characterization of keystone enzymes tRNA IPT1/IPT2 by functional confirmation of CK biosynthesis through Drosophila screening provides an opportunity to further investigate the accompanying CK biosynthesis enzymatic function associated with the modification of CK isoprenoid conjugates. In this research, genetic alignments

used ortholog enzyme encoded gene clusters identified based on searches in the *Drosophila melanogaster* reference genomes to name putative enzmes involved in the modification of CK isoprenoid conjugates to construct a modified tRNA degradation pathway for the Drosphila/Insecta (Figure 8) and the CKs named in this model reflect those detected in Drosophila tissues (Supplmental Table 6-12, Table 3 and Table 4). However, other putative homologs can be considered for future investigations. A recent survey by Mooi et al. (2024) examined the transcriptome of 670 hexapod species, made up of mostly insects, and were able to verify that ~80% of these insects possess candidate CK biosynthesis genes. This suggests that insects and other hexapods likely possess the ability to biosynthesize phytohormones as a mode of inter-species biochemical crosstalk.

The detection of tZ and iP forms of CK in extremely high quantities is common to gall forming (Mapes and Davies 2001, Straka et al. 2010, Yamaguchi et al. 2012, Tanaka et al. 2013, Takei et al. 2015, Zhang et al. 2017, Andreas et al. 2020). Drosophila CK analyte detection containing a similar tZ and iP analyte profiles but in much lower quantities (Table 2, Table 3). This work is therefore likely to open investigations into tRNA IPT functional genomics and quantitative transcript studies in gall forming insects like *Eurosta Solidaginis* and other model plant gall forming insects.

3.8. Conclusions:

For over two decades, cytokinin has been detected in insects and the plants that the support them (Mapes and Davies 2001, Straka et al. 2010, Yamaguchi et al. 2012, Tanaka et al. 2013, Takei et al. 2015, Zhang et al. 2017, Andreas et al. 2020). In fact, it couldn't be ruled out that microbiota within the insects were responsible for the detected CKs. Until now, there has been no genetic mechanism to support cause and effect. Although the functional confirmation of tRNA IPT1 and IPT2 is novel, these findings contribute to the growing body of evidence that CKs, once considered a phytohormone, are interkingdom signaling molecules and regulated by endogenous biosynthesis pathways across different kingdoms of life, like those found in Bacteria (Akiyoshi et al. 1984, Powel and Morris 1986, Sakakibara et al. 2005, Barash and Manulis-Sasson 2007, Pertry et al. 2010, Samanovic et al. 2015, Seo et al. 2016, Uniyal et al. 2022) Fungi (Hinsch et al. 2015, Morrison et al. 2016b) and Protista (Akoi et a. 2020). Future work provides the prospects for exciting insight to investigate the complex biochemical communication granting promising opportunities to understand how and why insects and plants communicate with one another.

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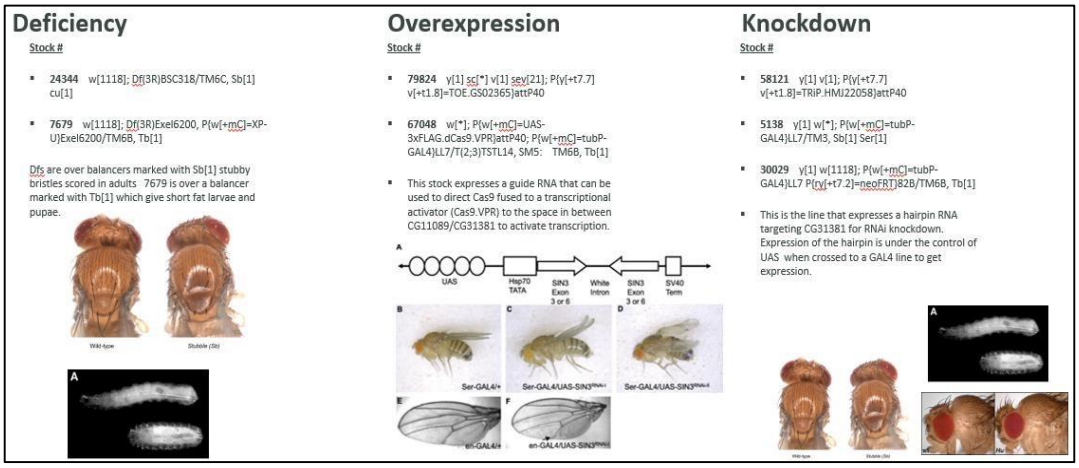
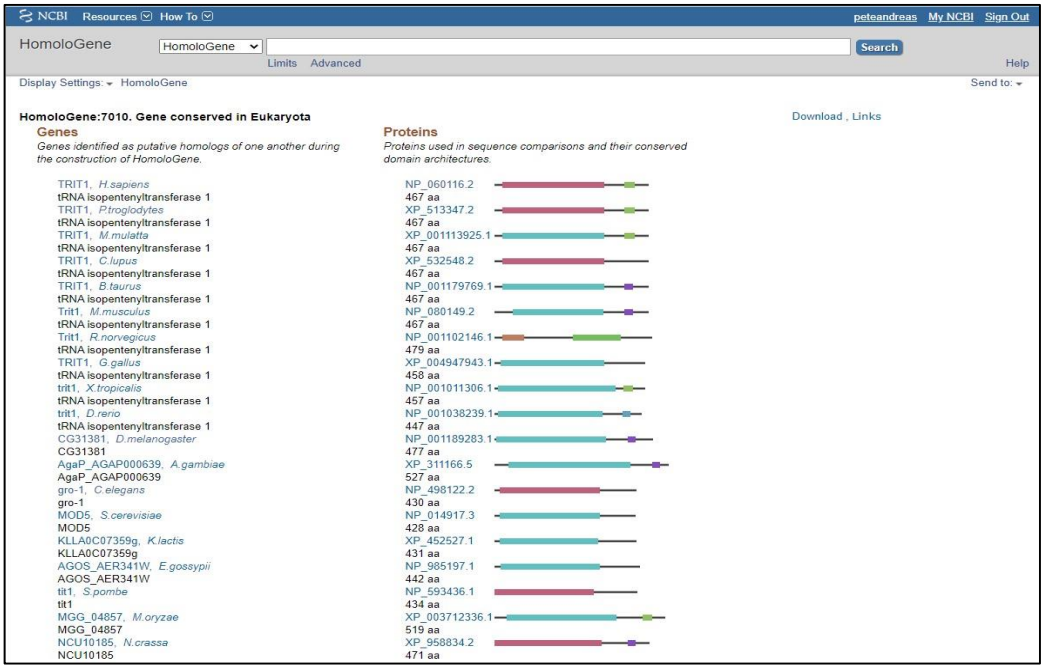
5. Supplementary Tables and Figures:

Drosophila Crosses		**Genetic Variants of F1 Crosses**	
Stock # and Genotype	Wild-type* or Balancer Stock # and Genotype	Mutation	Phenotype Selection Marker
24344 **w[111** **8];** **Df(3R)BSC318/TM6C, Sb[1] cu[1]**	6326* w[1118]	Deficiency in CG11089 /CG31381	Sb[1] Stubby bristles on back in adults
7679 **w[1118]; Df(3R)Exel6200, P{w[+mC]=XP-U}Exel6200/TM6B, Tb[1]**	6326* w[1118]	Deficiency in CG11089 /CG31381	Tb[1] Tubby or short/fat body in larvae and pupae
79824 **y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TOE.GS02365}attP40**	67048 w[*]; P{w[+mC]=UAS3xFLAG.dCas9.VPR}attP40; P{w[+mC]=tubP-GAL4}LL7/T(2;3)TSTL14, SM5: TM6B, Tb[1]	Overexpression in CG11089 /CG31381	Cy curled wings in adults, Hu[1] extra bristles on the shoulders in adults, Tb[1] Tubby or short/fat body in larvae and pupae
58121 **y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMJ22058}attP40**	5138 y[1] w[*]; P{w[+mC]=tubPGAL4}LL7/TM3, Sb[1] Ser[1]	Knocked down expression of CG11089 /CG31381	Sb[1] Stubby bristles on back in adults
58121 **y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMJ22058}attP40**	30029 y[1] w[1118]; P{w[+mC]=tubP-GAL4}LL7	Knocked down expression of CG11089 /CG31381	Tb[1] Tubby or short/fat body in larvae and pupae

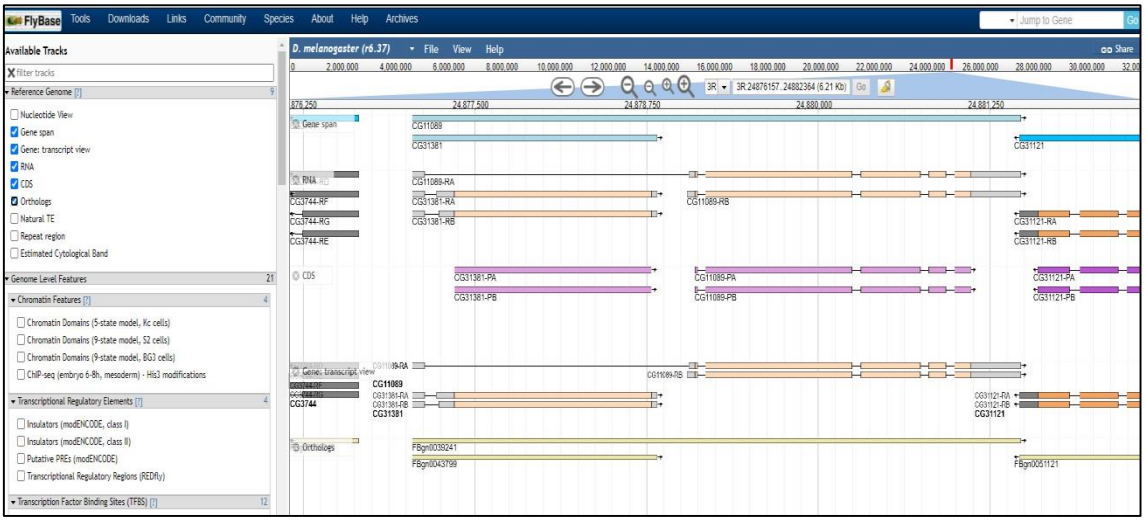
Supplementary Table 1: Drosophila candidate stock lines cross to produce suspected mutation/phenotype and breeding marker used for scoring trait inheritance in F1 offspring.



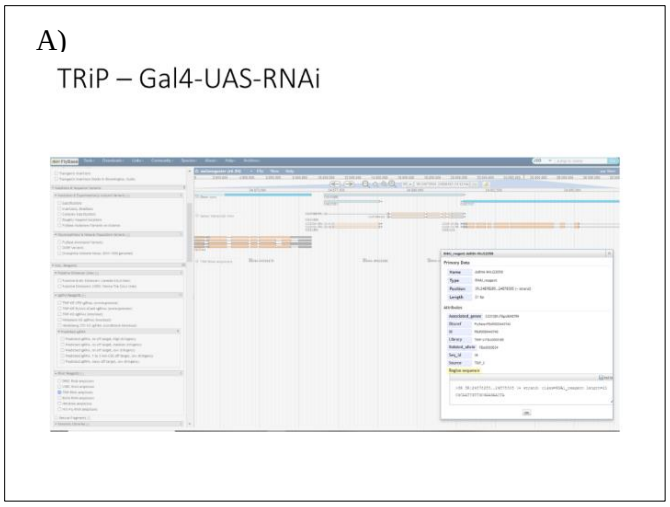
Supplementary Figure 1: Scorable phenotypic marker mutations used during genetic crosses for selection of Dmel_CG31381 and Dmel_CG11089 genes mutants. Including mutation type, fly stock #, genotypes name, and function.

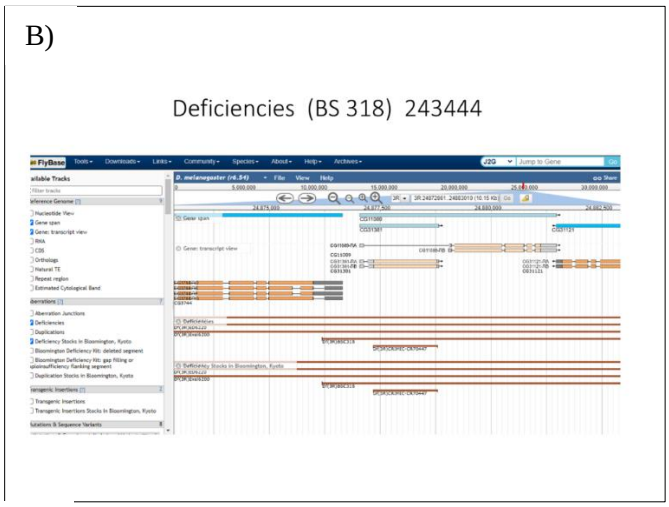


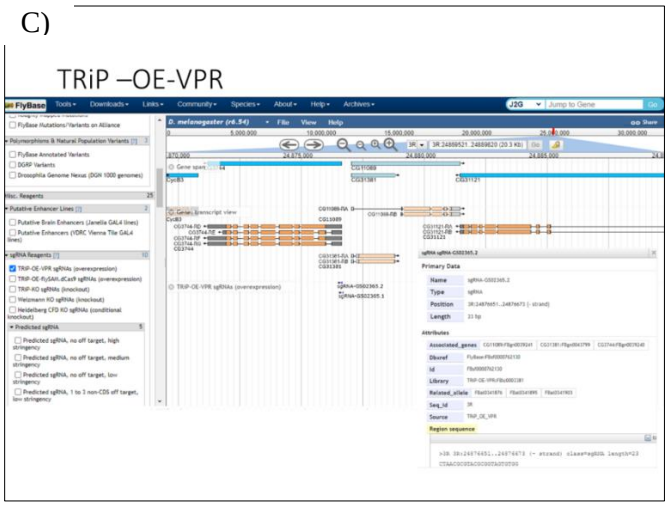
Supplementary Table 2: Dmel_CG31381 and genes identified as putative homologs of one another, and proteins used in sequence comparisons through conserved domain architectures. Based on conserved domains located in tRNA isopentenyltransferase 1. Source: Homologene [Internet] NCBI database image. Jan.11.2022. https://www.ncbi.nlm.nih.gov/gene/192528



Supplementary Table 3: Dmel_CG31381 and Dmel_CG11089 genes showing identified homology of one another and proteins used in sequence comparisons through conserved domain architectures. Based on conserved domains located in tRNA isopentenyltransferase 1. Source: Gene [Internet]. Flybase database image. NCBI (linked) Jan.11.2022







Supplementary Figure 3: Flybase stock line mutations generate Dmel_CG31381 and Dmel_CG11089 genetic variant tissues:A) Knockdown – RNAi, B) Deficiency, C) Over expression. Based on conserved domains located in tRNA isopentenyltransferase 1. Source: Gene [Internet]. Flybase database image (linked) Jan.11.2022.

Supplementary Table 4: Pairwise alignment of CG11081 (Query) and CG31381 (cDNA transcipts), using NCBI Global Alignment tool pack.

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Query ID: lcl|Query_87721 Length: 4434
Sequence ID: Query_87723 Length: 1783
Range 1: 1 to 1783

<td>1783/4434(40%)</td>
<td>2651/4434(59%)</td>
Query  1
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Sbjct  61
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Query  121
AATAGGTTTATTTTTTAGCAAATAATTAACTTAATATACGCCAATTGCAAACAGAATACC  180

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Query  421
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Query  481
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Query  541
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Query  841
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Query  901
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Query  1021
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1783
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Query 1981
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Query 2761
GCTCTACACGCAGCTGGCCAAGCTTCCACTGACGGTCCTCAATGCCTCGCCGGGATTTAT

Query 2821
CAACCTGTGCGACGCCCTCAACGGTTGGCAGTTGGTTAGGGAACTGAAGAAGGCTCTGCA

Query 2881
GCTGCCGGCGGCCACCAGCTTTAAGCACGTTTCTCCAGCGGGTGCGGCTGTTGGAGTGCC

Query 2941
TCTAAATCCGGCACAGGCCAAGCTGTGCATGGTGGATGATCTGTACGAGCAGTTGACACC

Query 3001
TCTGGCAACGGCCTATGCTCGTGCCCGTGGAGCCGACCGCATGAGCTCCTTCGGCGACTT

Query 3061
TGTGGCCCTCTCTGATGTGTGCGATGTTGTGACGGCCAGGATAATCTCGCGAGAGGTGTC

Query 3121
CGATGGTATTATTGCCGCGGGTTATGAGCCCGAAGCTCTGGAGATCCTGAAGAAAAAGAA

Query 3181
GAATGGCGGCTACTGTATACTGCAGGTAGGATTGATGCTTTAAGGTCATCTGTCCTTGAT

Query 3241
TATTGAGTTTAAATTTTAATACTTTTAGATGGACCCCAACTACGAACCCTCCGCGGTGGA

Query 3301
GCGCAAGACTATTTTTGGTCTGACGCTGGAGCAGAAGCGCAACGATGCTGTCATCGACGC

Query 3361
CTCGCTCTTTTCCAATGTGGTGAGCAAGCGTGGTCCTCTGCCCGAGGTGGCTGTGCGGGA

Query 3421
TCTGATCGTGGCCACCATTGCCTTGAAGTACACTCAGAGCAACTCCGTGTGCTACGCCCG

Query 3481
CGATGGCCAGGTCGTGGGCATCGGTGCCGGCCAGCAGTCGAGGATTCACTGCACCCGCTT

Query 3541
GGCGGGCGAGAAGGCGGACAACTGGTGGCTGCGTCAGCATCCCAGCGTGGCCGGCATGAA

Query 3601
GTTCAAGGCTGGTGTGAAGCGGGCGGAGATCTCTAATGCAATCGACAATTACGTAAACGG

Query 3661
AACTGTGGGCAAGGACATGCCTTTGTCGCAGTTCGAGGGAATGTAAGTTGTAGAGATTTT

Query 3721
TCACATATTTGCAGACCCTAACCACACTCTTCTCTATATAGGTTCGATAAGGCACCAGCC

Query 3781
CAACTGACCAGTGAGCAAAAAGTGGAATGGTTAAAGCAGCTGAGTGGCGTGGCCTTGGGA

Query 3841
TCCGATGCCTTCTTCCCCTTCCGTGATAATATCGATCGCGCTAGCCTGGTGAGTTAGTTA

Query 3901
GCTAGTATTTTTGAGCTTCTTTTTTTAAATTGCCCAAATATTCTTTCAGAGCGGCGTCTC

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Query  3961
CTACATAGCCAGTCCCGCAGGATCCACCAATGATGCTGGTGTCATTGCCGCCTGCGATGA

Query  4021
GCACGGTATCATCATGGCCCATACCAATCTGCGATTGTTCCACCATTAGGTTCAACACAC

Query  4081
TCTTGAGTATACGATTCATTCCCAGTTTTTAAGCTCTTTCTTTGATTTCCATAAAGTTCA

Query  4141
ACTTTAAATCCCAACTTCTCAAGTCGAATACATAAGAGAATTGTAAATAATTCTCAATTC

Query  4201
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Query  4261
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Query  4321
CATTTGTAGCGTTTTAAGTATTTTTATAGAAACACCCTGTTAATCAAACCCCATCAAACT

Query  4381  GCCTTATGACCATTCCAAGCTCAATAAAACCACTTGCCTTTAGTTAGTTAAACG
4434
```

Supplementary Table 5: Forward and reverse standard PCR primer pair for fragment confirmation of IPT1/IPT2 homologous sequence template site (CG31381/CG11081)

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%
	Self complementarity	Self 3' complementarity					
Forward primer	CTCACTTTCGTAACGCAGCA	Plus	20	537	556	58.86	
	50 5	1					
Reverse primer	GCATTCAGTTCGGCATCCTT	Minus	20	723	704	58.9	
	50 2	0					
Product length	187 bp						

Supplementary Table 5: qPCR gene targets tRNA –IPT1/IPT2 homologous sequence with forward and reverse primers denoted in red and taqman probe sequence denoted in blue. Note: forward and reverse primers are identical to standard PCR primers and replicate a qPCR product size of 187 bp within the conserved domain of tRNA isopentenyltransferase 1

5′----> 3′

GTAGTTGGCAACGCGGTGCTTGTATATTCCGGCAGAATTTTGTGTGGTTCCACTATTTCC
CTTTAAAACTCTACCATTATTTTTCGATTTCACAAAAAACAGCTGACGGCAACAAGTGGT
TCGGTCCCCATCGGAATACACGTGCTCAAAACGTGTGGGTTTTATTTGCCTTAATTGACT
TAAATTCACTCGCAATAAGTGGAAATGATTCGAAAGGTGCCGCTAATTGTAGTCCTGGGC
TCCACGGGCACCGGAAAGACGAAACTGTCTTTGCAACTGGCCGAACGCTTCGGAGGAGAA
ATAATCAGCGCTGACTCCATGCAGGTTTACACCCACCTGGACATCGCCACCGCCAAGGCA
ACCAAGGAGGAGCAGTCCCGGGCACGACATCATCTACTGGACGTGGCCACACCGGCCGAA

5′CTCACTTTCGTAACGCAGCA CCTTGGCGAGCAGGCG
CCCTTCACAGTCACTCACTTTCGTAACGCAGCACTGCCCATTGTGGAGCGCCTGCTCGCC

CTCC
AAGGACACTTCTCCGATTGTGGTGGGCGGCACGAATTACTACATAGAATCCCTACTTTGG

GATATTCTGGTTGACTCGGATGTCAAGCCGGACGAAGGCAAACATTCGGGGGAGCATCTT
CGTAAGTCAAGCCGTAGGAA ‘5
AAGGATGCCGAACTGAATGCTTTGTCCACCCTCGAGCTGCATCAGCACCTTGCCAAGATC

GACGCAGGTAGTGCCAACCGTATTCACCCCAACAACCGGCGCAAGATCATCCGGGCTATC

GAAGTGTATCAGAGCACCGGGCAGACTTTGAGCCAGATGCTGGCGGAACAGCGGGCACAG

CCGGGAGGAAACCGCCTGGGTGGACCCCTTCGCTATCCACACATCGTTCTCCTTTGGTTG
CGTTGCCAGCAGGATGTTCTAAACGAGCGATTGGATTCCCGCGTAGATGGCATGCTGGCC
CAAGGGCTGCTCCCTGAACTACGACAGTTTCACAATGCCCACCATGCTACCACTGTGCAA
GCCTATACGTCGGGAGTTCTGCAGACGATTGGCTACAAGGAGTTTATTCCCTATCTGATC
AAGTACGACCAGCAGCAGGACGAAAAGATAGAGGAGTACCTCAAAACCCATAGTTACAAG
CTGCCAGGCCCAGAAAAACTGAAAGAAGAAGGTCTTCCAGATGGCTTGGAACTCCTACGC
AATTGTTGCGAAGAACTAAAGTTAGTCACTCGCCGATACTCAAAGAAGCAGCTGAAGTGG
ATCAACAATCGATTCCTGGCCAGCAAAGATCGTCAAGTGCCGGATCTCTACGAACTGGAC
ACCAGTGATGTGTCAGCTTGGCAGGTGGCAGTCTACAAGCGGGCAGAGACCATCATAGAA
AGCTATCGAAACGAAGAGGCTTGCGAGATACTACCAATGGCCAAGCGGGAGCATCCTGGA
GCGGATTTGGATGAGGAGACTAGCCATTTTTGTCAAATATGCGAACGGCATTTCGTTGGG
GAGTACCAATGGGGACTGCATATGAAGTCCAACAAACACAAGCGAAGAAAGGAGGGACAG
CGCAAGCGGCAAAGGGATCACGAAACAATGCTCTCAACGGATCTAGCGAAGAAGCAAAAG
GAGGAGAAAGAGGAGGCAGGAAAGGCGGAGACTCAGCCACCACCCAGCCGAGTCAATGAT
ACTGATAAGGCAATGTAACACTAGACGCGGCTTGGCAATAAATGAACCTACGTAAATTTG 3′

Supplementary Table 6. Pairwise Alignment Scores including Dmel_CG31381and genes identified as putative homologs of one another, and proteins used in sequence comparisons through conserved domain architectures. Based on conserved domains located in tRNA isopentenyltransferase 1. Bold row indicates highest percent identity.

D. melanogaster	**CG31381**	**Identity (%)**	
Species	Gene Symbol	Protein	DNA
vs. H.sapiens	TRIT1	43.3	50.4
vs. P.troglodytes	TRIT1	44.2	50.9
vs. M.mulatta	TRIT1	43.6	50.6
vs. C.lupus	TRIT1	43.3	50.8
vs. B.taurus	TRIT1	43.1	50.2
vs. M.musculus	Trit1	45.5	51.9
vs. R.norvegicus	Trit1	43.9	50.2
vs. G.gallus	TRIT1	44.4	53.1
vs. X.tropicalis	trit1	45.1	52.9
vs. D.rerio	trit1	43.2	52.2
vs. A.gambiae	**AgaP_AGAP000639**	**54.2**	**57.6**
vs. C.elegans	gro-1	36.2	44.6
vs. S.cerevisiae	MOD5	36.1	45.6
vs. K.lactis	KLLA0C07359g	33.8	43.8
vs. E.gossypii	AGOS_AER341W	37.5	47.5
vs. S.pombe	tit1	36.7	44.9
vs. M.oryzae	MGG_04857	37.0	47.5
vs. N.crassa	NCU10185	36.7	48.2
vs. A.thaliana	IPT2	31.1	44.2
vs. O.sativa	**Os01g0968700**	**34.8**	**45.8**

Supplementary Table 7. Pairwise Alignment Scores including Dmel_Cyp303a1 gene and genes identified as putative homologs of one another, and proteins used in sequence comparisons through conserved domain architectures. Based on conserved domains located in p450 (pfam00067) Cytochrome P450 and p450 (cl12078) Cytochrome P450. Bold row indicates highest percent identity.

D. melanogaster	**Cyp303a1**	**Identity (%)**	
Species	Gene Symbol	Protein	DNA
vs. D.rerio	LOC100148115	31.6	43.4
vs. D.rerio	cyp2x10.2	31.7	43.6
vs. D.rerio	cyp2x12	31.4	43.9
vs. D.rerio	cyp2x6	31.5	44.8
vs. D.rerio	cyp2x7	30.4	44.2
vs. D.rerio	cyp2x8	31.9	45.6
vs. D.rerio	cyp2x9	33.7	45.5
vs. D.rerio	wu:fd49b10	31.9	44.3
vs. A.gambiae	**AgaP_AGAP010077**	**49.4**	**56.6**

Supplementary Table 8. Pairwise Alignment Scores including Dmel_CG11255 gene and genes identified as putative homologs of one another, and proteins used in sequence comparisons through conserved domain architectures. Based on conserved domains located in adenosine kinase; Provisional. Bold row indicates highest percent identity.

D. melanogaster	**CG11255**	**Identity (%)**	
Species	Gene Symbol	Protein	DNA
vs. H.sapiens	ADK	52.8	55.4
vs. P.troglodytes	ADK	52.8	55.3
vs. M.mulatta	ADK	52.8	55.3
vs. C.lupus	ADK	52.2	55.5
vs. B.taurus	ADK	52.2	55.4
vs. M.musculus	Adk	52.2	56.4
vs. R.norvegicus	Adk	52.5	57.1
vs. G.gallus	ADK	50.4	56.2
vs. X.tropicalis	adk	51.6	53.3
vs. D.rerio	adka	50.1	57.1
vs. C.elegans	**R07H5.8**	**52.9**	**56.0**
vs. A.thaliana	ADK1	47.5	55.5
vs. A.thaliana	ADK2	47.1	54.5
vs. O.sativa	Os02g0625500	50.1	55.3
vs. O.sativa	**Os04g0518000**	**50.7**	**53.9**

Supplementary Table 9. Pairwise Alignment Scores including Dmel_CG16758 gene and genes identified as putative homologs of one another, and proteins used in sequence comparisons through conserved domain architectures. Based on conserved domains located in Purine nucleoside phosphorylase (EC 2.4.2.1). Bold row indicates highest percent identity.

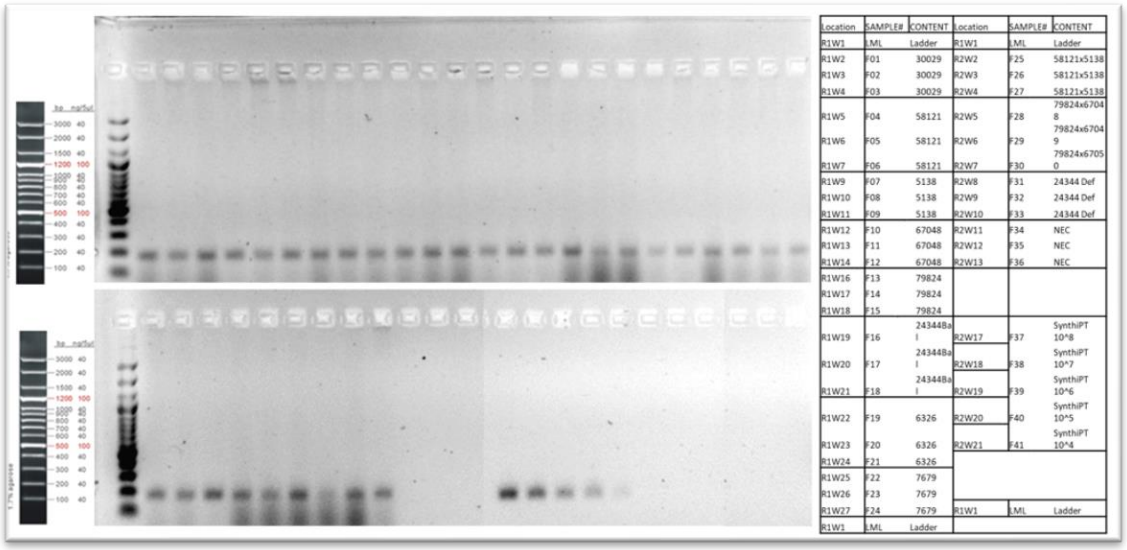
D. melanogaster	**CG16758**	**Identity (%)**	
Species	Gene Symbol	Protein	DNA
vs. H.sapiens	PNP	51.2	55.4
vs. P.troglodytes	PNP	51.2	55.1
vs. M.mulatta	LOC710245	50.9	55.0
vs. C.lupus	PNP	50.2	54.8
vs. B.taurus	LOC790312	47.9	54.0
vs. B.taurus	PNP	51.4	55.4
vs. M.musculus	Pnp	51.9	54.7
vs. M.musculus	Pnp2	51.2	54.3
vs. R.norvegicus	Pnp	51.0	55.7
vs. G.gallus	PNP	51.4	57.5
vs. X.tropicalis	pnp	50.7	54.5
vs. A.gambiae	AgaP_AGAP005944	54.7	63.6
vs. A.gambiae	**AgaP_AGAP005945**	**60.6**	**67.5**
vs. C.elegans	K02D7.1	44.3	50.1
vs. S.cerevisiae	PNP1	43.8	49.0
vs. K.lactis	KLLA0C16621g	44.7	48.5
vs. E.gossypii	AGOS_ACR198W	39.9	45.2
vs. S.pombe	**SPAC1805.16c**	**46.6**	**51.0**

Supplementary Table 10. Pairwise Alignment Scores including Dmel_CG3362 gene and genes identified as putative homologs of one another, and proteins used in sequence comparisons through conserved domain architectures. Based on conserved domains located in 5'-nucleotidase (EC 2.4.2.1) also known as 5'-ribonucleotide phosphohydrolase. Bold row indicates highest percent identity.

D. melanogaster	**CG3362**	**Identity (%)**	
Species	Gene Symbol	Protein	DNA
vs. H.sapiens	NT5C3B	39.5	52.4
vs. P.troglodytes	NT5C3L	39.5	52.4
vs. M.mulatta	LOC708104	44.3	55.1
vs. C.lupus	NT5C3B	40.6	53.7
vs. B.taurus	NT5C3L	40.3	53.2
vs. M.musculus	Nt5c3b	40.6	53.1
vs. R.norvegicus	Nt5c3b	40.6	53.6
vs. G.gallus	NT5C3L	41.0	52.8
vs. X.tropicalis	nt5c3b	35.5	49.7
vs. A.gambiae	AgaP_AGAP010681	62.8	65.0
vs. A.thaliana	AT2G38680	36.1	43.5
vs. O.sativa	**Os03g0648900**	**37.1**	**47.7**

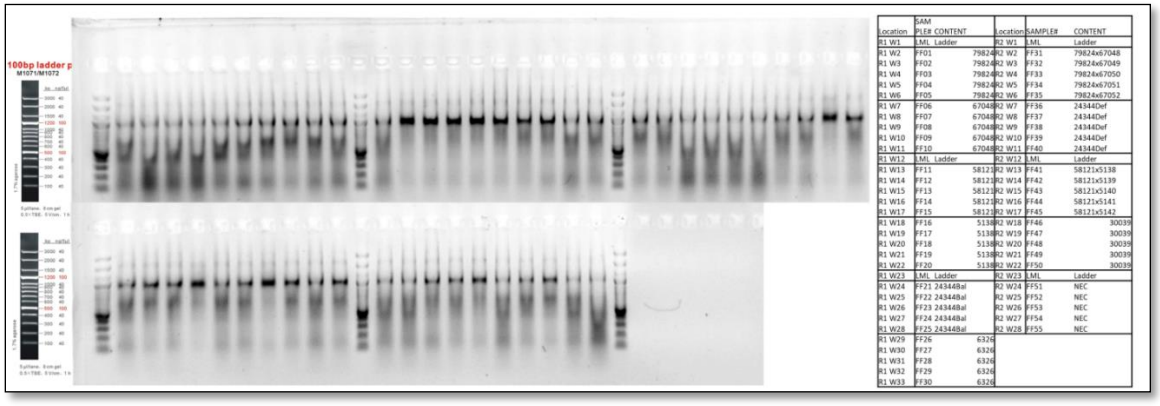
Supplementary Table 11. Pairwise Alignment Scores including Dmel_CG18315gene and genes identified as putative homologs of one another and proteins used in sequence comparisons through conserved domain architectures. Based on conserved domains located in Adenine phosphoribosyltransferase (EC 2.4.2.7). Bold row indicates highest percent identity.

D. melanogaster	**CG18315**	**Identity (%)**	
Species	Gene Symbol	Protein	DNA
vs. H.sapiens	APRT	46.6	53.0
vs. P.troglodytes	APRT	46.6	53.2
vs. M.mulatta	APRT	47.7	54.0
vs. C.lupus	APRT	49.4	55.1
vs. B.taurus	APRT	48.3	54.5
vs. M.musculus	Aprt	47.2	53.0
vs. R.norvegicus	Aprt	49.1	53.7
vs. G.gallus	APRT	50.0	52.8
vs. X.tropicalis	aprt	46.1	49.1
vs. D.rerio	aprt	51.1	54.0
vs. A.gambiae	AgaP_AGAP005723	54.4	59.1
vs. C.elegans	T19B4.3	45.1	52.0
vs. S.cerevisiae	APT1	45.8	50.4
vs. K.lactis	KLLA0B01309g	41.3	49.5
vs. E.gossypii	AGOS_AER325W	45.8	51.4
vs. S.pombe	apt1	45.4	50.2
vs. M.oryzae	MGG_17399	46.8	53.9
vs. N.crassa	NCU02090	46.1	53.5
vs. A.thaliana	APT1	41.1	48.3
vs. O.sativa	**Os12g0589100**	**44.8**	**52.1**



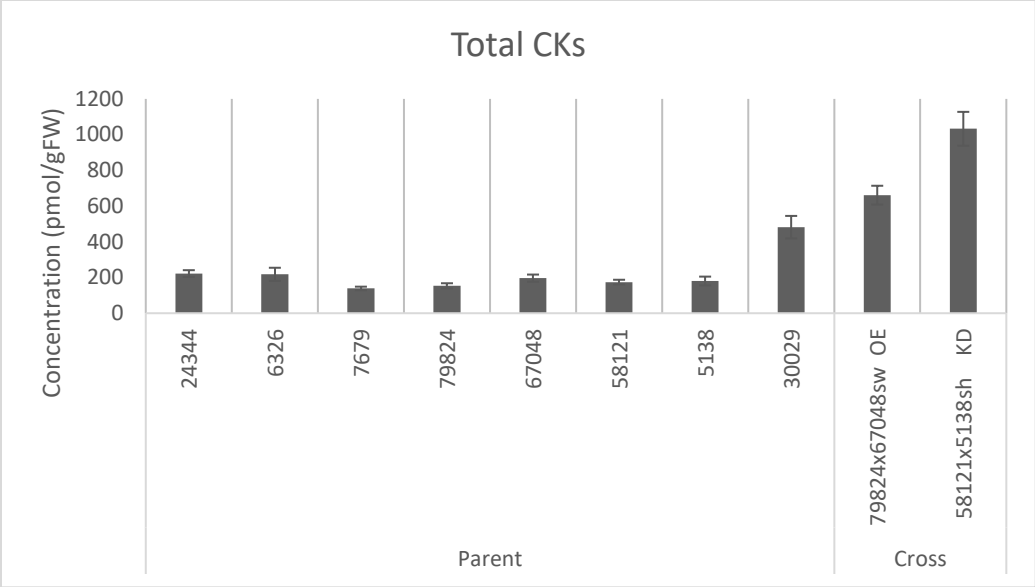
Location	SAMPLE#	CONTENT	Location	SAMPLE#	CONTENT
R1W1	LML	Ladder	R1W1	LML	Ladder
R1W2	F01	30029	R2W2	F25	58121x5138
R1W3	F02	30029	R2W3	F26	58121x5138
R1W4	F03	30029	R2W4	F27	58121x5138
R1W5	F04	58121	R2W5	F28	79824x67048
R1W6	F05	58121	R2W6	F29	79824x67049
R1W7	F06	58121	R2W7	F30	79824x67050
R1W9	F07	5138	R2W8	F31	24344 Def
R1W10	F08	5138	R2W9	F32	24344 Def
R1W11	F09	5138	R2W10	F33	24344 Def
R1W12	F10	67048	R2W11	F34	NEC
R1W13	F11	67048	R2W12	F35	NEC
R1W14	F12	67048	R2W13	F36	NEC
R1W16	F13	79824			
R1W17	F14	79824			
R1W18	F15	79824			
R1W19	F16	24344Bal	R2W17	F37	SynthiPT 10^8
R1W20	F17	24344Bal	R2W18	F38	SynthiPT 10^7
R1W21	F18	24344Bal	R2W19	F39	SynthiPT 10^6
R1W22	F19	6326	R2W20	F40	SynthiPT 10^5
R1W23	F20	6326	R2W21	F41	SynthiPT 10^4
R1W24	F21	6326			
R1W25	F22	7679			
R1W26	F23	7679			
R1W27	F24	7679	R1W1	LML	Ladder
R1W1	LML	Ladder			

Supplementary Fig 4: Agarose gel Electrophoresis Drosophila amplified PCR product run on 1.5% [m/v] gel in 0.5X TBE running buffer at ~90V for 60 min using DynaView nucleic acid stain. Samples were loaded with 10 µl DNA and 8µl Loading Dye. A Low Mass 100bp ladder was used for sizing fragments.

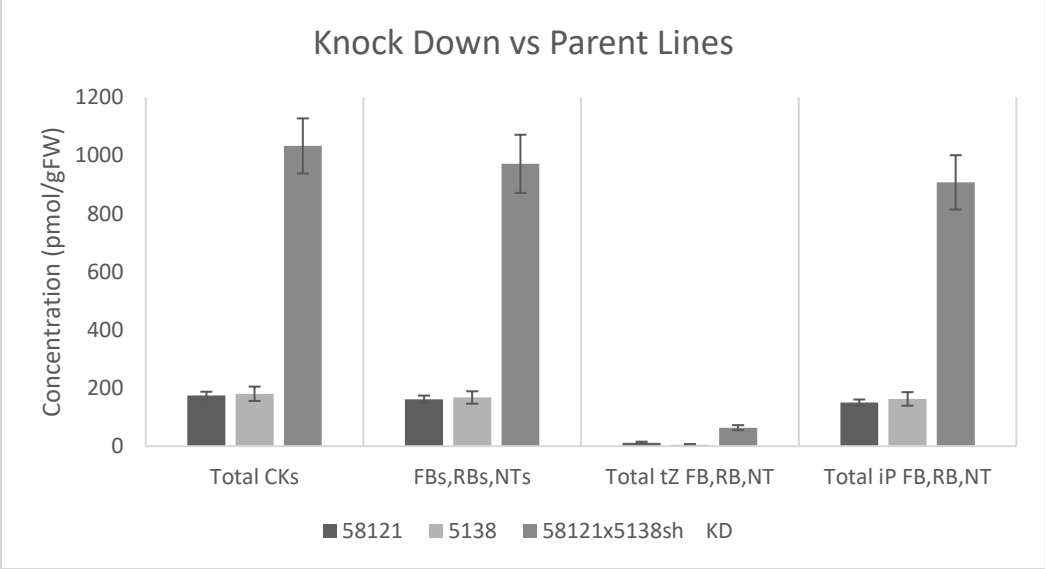


Location	SAMPLE#	CONTENT	Location	SAMPLE#	CONTENT
R1 W1	LML	Ladder	R2 W1	LML	Ladder
R1 W2	FF01	79824	R2 W2	FF31	79824x67048
R1 W3	FF02	79824	R2 W3	FF32	79824x67049
R1 W4	FF03	79824	R2 W4	FF33	79824x67050
R1 W5	FF04	79824	R2 W5	FF34	79824x67051
R1 W6	FF05	79824	R2 W6	FF35	79824x67052
R1 W7	FF06	67048	R2 W7	FF36	24344Def
R1 W8	FF07	67048	R2 W8	FF37	24344Def
R1 W9	FF08	67048	R2 W9	FF38	24344Def
R1 W10	FF09	67048	R2 W10	FF39	24344Def
R1 W11	FF10	67048	R2 W11	FF40	24344Def
R1 W12	LML	Ladder	R2 W12	LML	Ladder
R1 W13	FF11	58121	R2 W13	FF41	58121x5138
R1 W14	FF12	58121	R2 W14	FF42	58121x5139
R1 W15	FF13	58121	R2 W15	FF43	58121x5140
R1 W16	FF14	58121	R2 W16	FF44	58121x5141
R1 W17	FF15	58121	R2 W17	FF45	58121x5142
R1 W18	FF16	5138	R2 W18	FF46	30039
R1 W19	FF17	5138	R2 W19	FF47	30039
R1 W20	FF18	5138	R2 W20	FF48	30039
R1 W21	FF19	5138	R2 W21	FF49	30039
R1 W22	FF20	5138	R2 W22	FF50	30039
R1 W23	LML	Ladder	R2 W23	LML	Ladder
R1 W24	FF21	24344Bal	R2 W24	FF51	NEC
R1 W25	FF22	24344Bal	R2 W25	FF52	NEC
R1 W26	FF23	24344Bal	R2 W26	FF53	NEC
R1 W27	FF24	24344Bal	R2 W27	FF54	NEC
R1 W28	FF25	24344Bal	R2 W28	FF55	NEC
R1 W29	FF26	6326			
R1 W30	FF27	6326			
R1 W31	FF28	6326			
R1 W32	FF29	6326			
R1 W33	FF30	6326			

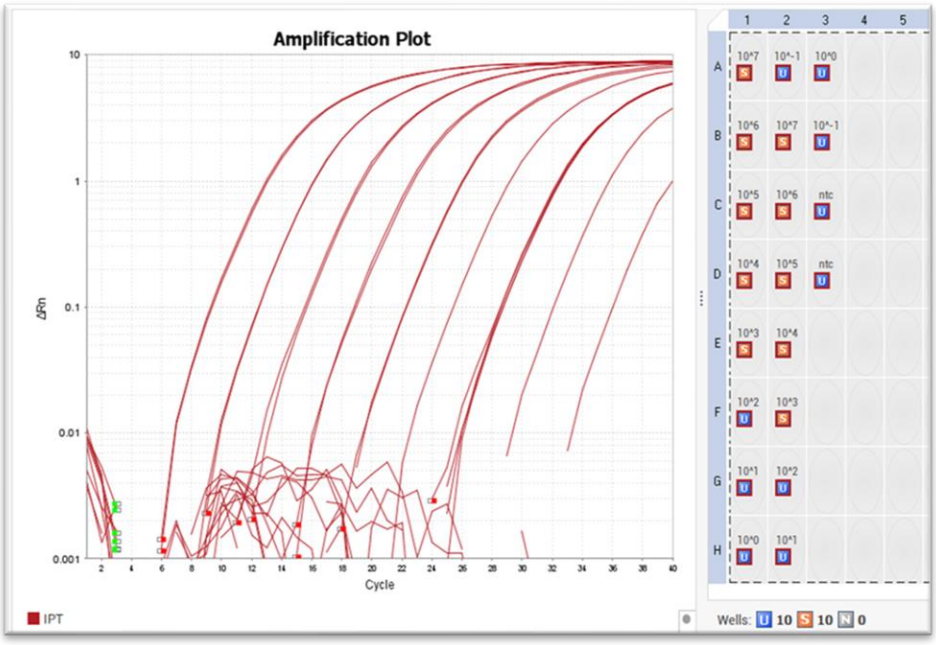
Supplementary Fig 5: Agarose gel Electrophoresis Drosophila extracted RNA run on 1.5% [m/v] gel in 0.5X TBE running buffer at ~90V for 60 min using DynaView nucleic acid stain. Samples were loaded with 2 µl RNA and 8µl Loading Dye. A Low Mass 100bp ladder was used for sizing fragments.



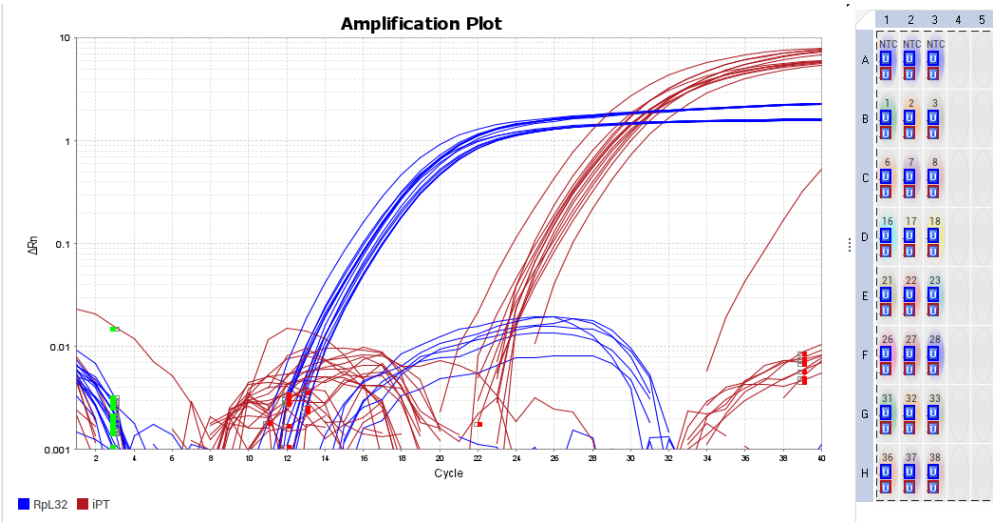
Supplementary Fig 6: Concentrations of total cytokinin [pmol/g fresh weight] isolated from *Drosophila melanogaster* newly emerged adults including parent lines and crosses. (means ±SE, n=5 biological replicates).



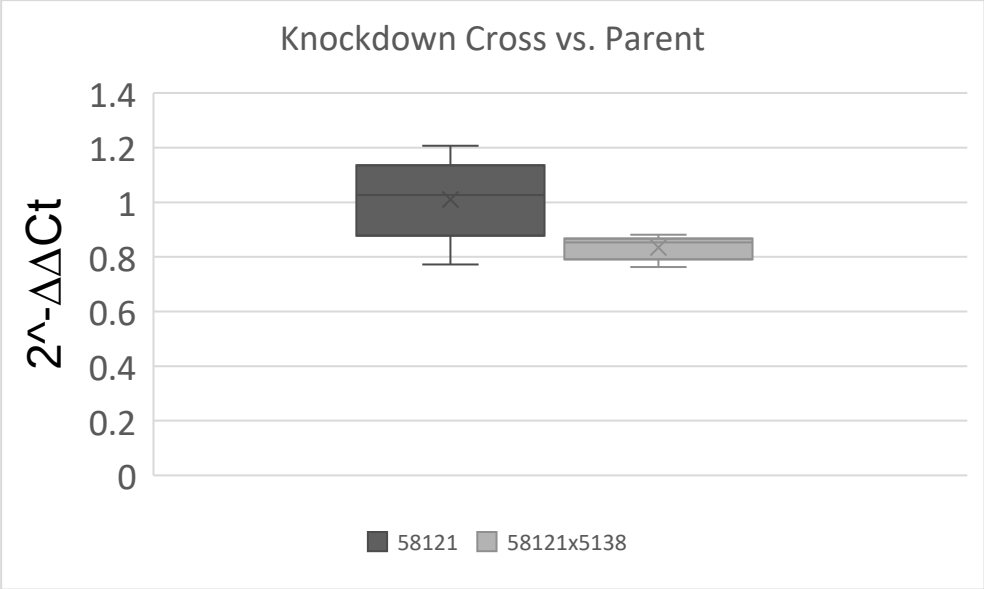
Supplementary Fig 7: Concentrations of total cytokinin [pmol/g fresh weight] isolated from *Drosophila melanogaster* newly emerged adults including parental lines (58121) transgenic RNAi line (Trip) and (5138) Gal4 driver line (Gal4) and RNA interference (RNAi) (58121x5138), (means ±SE, n=5 biological replicates)



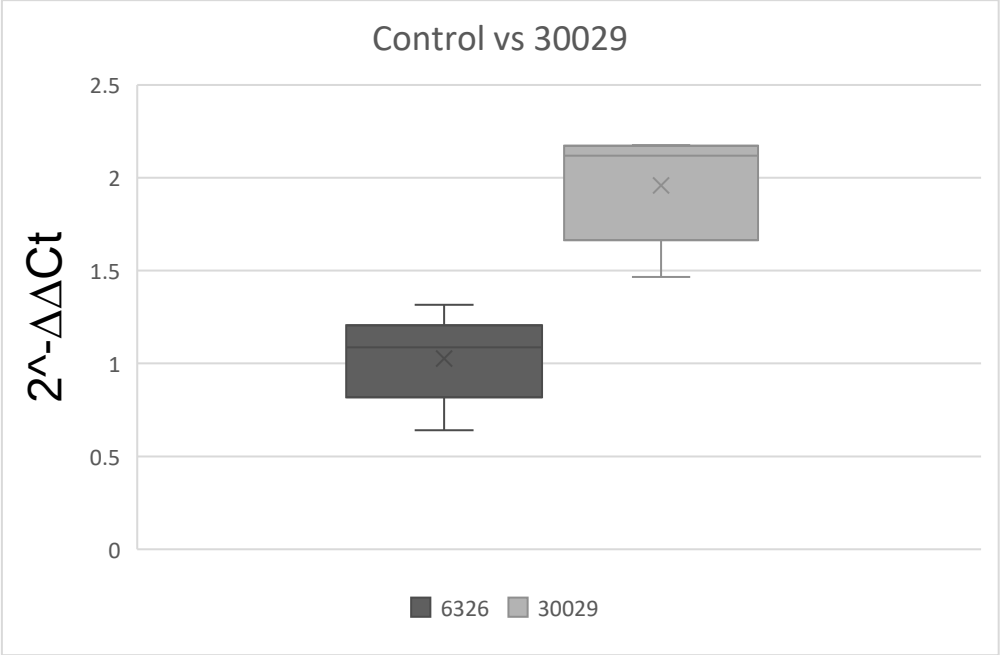
Supplementary Fig 8: Amplification plot showing qPCR signal for optimization of dilution series targeting CG31381/CG11089 using TaqMan Probe chemistry and CG31381/CG11089gene string construct synthesised from *Drosophila melanogaster* NCBI reference sequences.



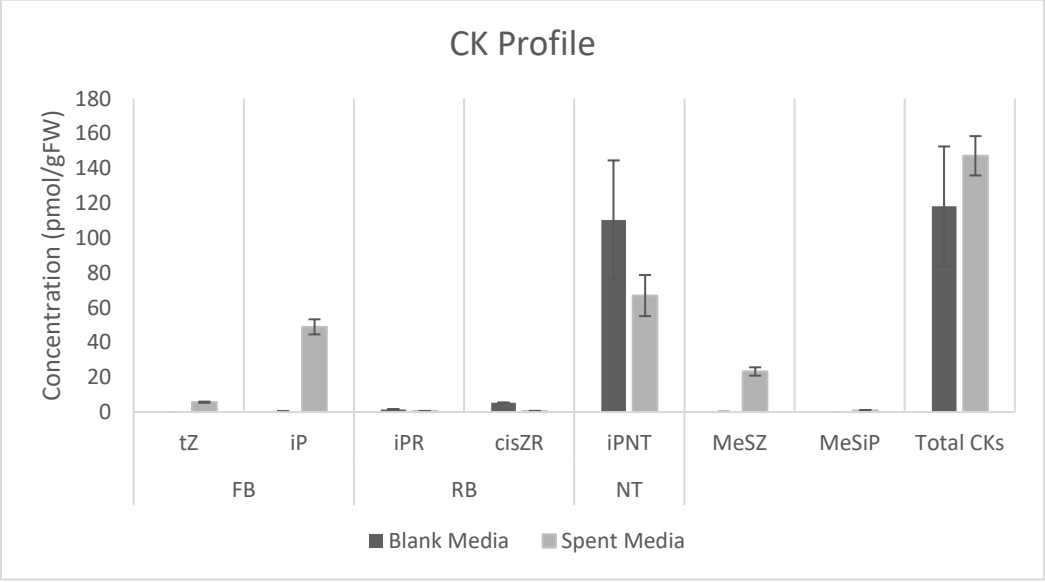
Supplementary Fig 9: Amplification plot showing qPCR signal for optimization targeting CG31381/CG11089 (iPT) and Rpl132 endogenous control gene using TaqMan Probe chemistry and reverse transcribed RNA - cDNA from *Drosophila melanogaster* parental control tissue tissues.



Supplementary Fig 10. Relative fold gene expression (2^-ΔΔ Ct) of *Drosophila melanogaster* newly emerged adults including parent lines and crosses: Including Parental control line (58121) and RNAi Knockdown IPT (58121x5138). Where means ±SE, n=5 biological and mean for n=3 technical replicates were used. No significance was noted.



Supplementary Fig 11. Relative fold gene expression (2^-ΔΔ Ct) of *Drosophila melanogaster* newly emerged adults including parent lines and crosses: Including A) balancer control line (6326) and B) parental line (30029). Where means ±SE, n=5 biological and mean for n=3 technical replicates were used. No significance was noted.



Supplementary Fig 12: Concentrations of cytokinin [pmol/g fresh weight] isolated from *Drosophila melanogaster* blank media (fresh media) and spent media (used media) (means ±SE, n=5 biological replicates).

Supplementary Background on Drosophila Genetic Toolbox:

Balancers are lines that prevent homozygous lethal or sterile mutations from being lost through meiotic cell division via recombination of specific regions of the genome and are used to cross with other lines to sustain a specific genotype. Balancer chromosomes are specialized chromosomes that help maintain the stability of experimental Drosophila stocks during genetic crosses. They carry specific genetic markers that allow researchers to easily identify and select flies carrying the balancer chromosome during breeding. The balancer chromosomes also contain inversions, which are rearrangements of chromosome segments. The inversions suppress recombination between homologous chromosomes, making them highly useful for preventing the loss of genetic modifications or other alleles of interest during breeding. This is because crossing over between homologous chromosomes can lead to the separation of linked genes and the loss of desired genetic elements (Miller et al. 2019).

Gal4 driver lines: The GAL4 (galactose –induced gene 4) protein was taken from yeast and does not have a specific target in Drosophila. Therefore, it serves as a transcriptional activator allowing specialized targeting in the system (Halpern et al. 2008). GAL4 is used in combination with the (UAS) upstream activation sequence and is specific to only the GAL4 information. This system initiates expression of a GOIs and facilitates downregulation mechanisms. The Gal4 driver lines are Drosophila strains that have been genetically engineered to express the Gal4 protein in specific cells or tissues of interest. These driver lines are created by placing the Gal4 gene under the control of a specific promoter. Promoters can be chosen based on their expression patterns, allowing

researchers to target Gal4 expression to specific cell types or developmental stages. For instance, tubulin is a commonly used because it expresses in all tissues and all developmental stages (Barwell et al. 2017).

UAS reporter lines: The UAS reporter lines carry a gene of interest (such as a fluorescent protein or a protein that can be used to ablate cells) downstream of a UAS sequence. The UAS sequence consists of repeated binding sites for the Gal4 protein. When Gal4 protein is present, it binds to the UAS sequence thus cascading the activation and expression of the downstream gene (Halpern et al. 2008).

Gal4 driver and UAS reporter lines: The Gal4 driver line is crossed with the UAS reporter line, resulting in progeny that carry both the Gal4 driver and the UAS reporter constructs. In the resulting flies, the Gal4 protein is expressed in the specific cells or tissues determined by the driver line, and the downstream gene carried by the UAS reporter line is activated only in those cells or tissues. Furthermore, the Gal4-UAS system allows genetic manipulation by crossing Gal4 driver lines with UAS lines carrying genes that interfere with normal gene function, such as genes that produce RNA interference (RNAi) or dominant-negative forms of proteins, thus blocking or altering gene activity in specific cells or tissues. Drosophila Gal4 genetics enables manipulation of gene expression in a precise and tissue-specific manner, facilitating the study of gene function, developmental processes, and disease models in Drosophila research

UAS-Cas9 lines: UAS-Cas9 lines are strains that carry the Cas9 gene under the control of UAS sequences. The Cas9 protein is an RNA-guided endonuclease but can be engineered to either target expression (GOF) using a so call dead Cas9 (dCas9-VPR) or cut specific DNA sequences using an active Cas9 system, as previously described.

Crossing Gal4 driver and UAS-dCas9-VPR lines: The Gal4 driver line can be crossed with the UAS-dCas9-VPR line, resulting in progeny that carry both the Gal4 driver construct and the UAS-dCas9-VPR construct. In these flies, Gal4 expression is restricted to specific cells or tissues determined by the Gal4 driver line (ie Tubulin -expressed in all cells), and dCas9-VPR expression is controlled by Gal4 through the UAS sequence. iiii) Gene activation: Once the Gal4-UAS-dCas9 flies are generated, researchers can introduce a third construct carrying a gene of interest downstream of a UAS sequence. This construct is referred to as the UAS-target gene construct. When the Gal4 protein binds to the UAS sequence in the UAS-target gene construct, it activates the expression of the target gene, leading to its increased transcription and subsequent phenotypic effects (Lin et al., 2015). Over expression stock lines expresses a specified guide RNA (gRNA) that can be used to direct a nuclease-dead Cas9 fused to a transcriptional tripartite activator (dCas9.VPR) to the GOI as long as a CG site is present allowing activation of transcription. Moreover, the gRNA ensures the specificity and location for dCas9 and further allowing the viral protein r (VPR) to assist by recruiting transcriptional cofactors to enhance expression at the UAS binding site (Upstream Activating System) when GAL4 is present. These genetic structures allow for a variety of lines containing different components to be crossed in an array of combinations to induce (OE) over expression (Ren et al. 2013, Lin et al. 2015, Kaufman 2017).

Supplementary Table 12: Raw data of expression levels IPT1/IPT2 using the delta-delta CT (2^-ΔΔ Ct) 'control vs. treated' relative fold gene expression method with endogenous control gene Rpl32 for normalization.

	Well	Well Positi	Omit	Sample Na	Target Nar	Task	Reporter	Quencher	Quantity	Quantity M	Quantity S	RQ	RQ Min	RQ Max	CT	Ct Mean	Ct SD	Delta Ct	Delta Ct M	Delta Ct SI	Delta Ct SI	Delta Delta Ct	2^-ΔΔCt
58121	7	A7	FALSE	11	Rpl32	UNKNOWI	VIC	NFQ-MGB							16.281	16.289	0.101						
	7	A7	FALSE	11	IPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	27.080	27.023	0.054	10.799					
	8	A8	FALSE	11	Rpl32	UNKNOWI	VIC	NFQ-MGB							16.193	16.289	0.101						
	8	A8	FALSE	11	IPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	26.971	27.023	0.054	10.778	10.734			-0.090	1.0642
	9	A9	FALSE	11	Rpl32	UNKNOWI	VIC	NFQ-MGB							16.393	16.289	0.101						
	9	A9	FALSE	11	IPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	27.018	27.023	0.054	10.624					
	19	B7	FALSE	12	Rpl32	UNKNOWI	VIC	NFQ-MGB							16.713	16.679	0.053						
	19	B7	FALSE	12	IPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	27.307	27.231	0.066	10.593					
	20	B8	FALSE	12	Rpl32	UNKNOWI	VIC	NFQ-MGB							16.618	16.679	0.053						
	20	B8	FALSE	12	IPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	27.183	27.231	0.066	10.565	10.552			-0.272	1.2073
	21	B9	FALSE	12	Rpl32	UNKNOWI	VIC	NFQ-MGB							16.707	16.679	0.053						
	21	B9	FALSE	12	IPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	27.204	27.231	0.066	10.497					
	31	C7	FALSE	13	Rpl32	UNKNOWI	VIC	NFQ-MGB							16.222	16.103	0.105						
	31	C7	FALSE	13	IPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	27.094	26.952	0.132	10.872					
	32	C8	FALSE	13	Rpl32	UNKNOWI	VIC	NFQ-MGB							16.064	16.103	0.105						
	32	C8	FALSE	13	IPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	26.928	26.952	0.132	10.864	10.849			0.025	0.9826	1.010432411798210
	33	C9	FALSE	13	Rpl32	UNKNOWI	VIC	NFQ-MGB							16.022	16.103	0.105						
	33	C9	FALSE	13	IPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	26.833	26.952	0.132	10.811					
	43	D7	FALSE	14	Rpl32	UNKNOWI	VIC	NFQ-MGB							17.054	16.894	0.215						
	43	D7	FALSE	14	IPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	28.225	28.091	0.293	11.170					
	44	D8	FALSE	14	Rpl32	UNKNOWI	VIC	NFQ-MGB							16.977	16.894	0.215						
	44	D8	FALSE	14	IPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	28.293	28.091	0.293	11.316	11.197			0.373	0.7719
	45	D9	FALSE	14	Rpl32	UNKNOWI	VIC	NFQ-MGB							16.650	16.894	0.215						
	45	D9	FALSE	14	IPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	27.754	28.091	0.293	11.105					
	55	E7	FALSE	15	Rpl32	UNKNOWI	VIC	NFQ-MGB							16.746	16.707	0.098						
	55	E7	FALSE	15	IPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	27.499	27.493	0.094	10.752					
	56	E8	FALSE	15	Rpl32	UNKNOWI	VIC	NFQ-MGB							16.779	16.707	0.098						
	56	E8	FALSE	15	IPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	27.584	27.493	0.094	10.805	10.786			-0.037	1.0262
	57	E9	FALSE	15	Rpl32	UNKNOWI	VIC	NFQ-MGB							16.595	16.707	0.098						
	57	E9	FALSE	15	IPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	27.397	27.493	0.094	10.802					
																	Avg. Delta	10.824					
	Well	Well Positi	Omit	Sample Na	Target Nar	Task	Reporter	Quencher	Quantity	Quantity M	Quantity S	RQ	RQ Min	RQ Max	CT	Ct Mean	Ct SD	Delta Ct	Delta Ct M	Delta Ct SI	Delta Ct SI	Delta Delta Ct	
"Treated" 58121 x 51	61	F1	FALSE	41	Rpl32	UNKNOWI	VIC	NFQ-MGB							16.248	16.232	0.040						
	61	F1	FALSE	41	IPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	27.449	27.393	0.055	11.202					
	62	F2	FALSE	42	Rpl32	UNKNOWI	VIC	NFQ-MGB							16.300	16.264	0.115						
	62	F2	FALSE	42	IPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	27.202	27.074	0.142	10.901	11.052			0.228	0.8537
	63	F3	FALSE	43	Rpl32	UNKNOWI	VIC	NFQ-MGB							15.868	15.704	0.166						
	63	F3	FALSE	43	IPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	26.920	26.828	0.134	11.052					
	64	F4	FALSE	44	Rpl32	UNKNOWI	VIC	NFQ-MGB							16.868	16.740	0.115						
	64	F4	FALSE	44	IPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	28.036	27.891	0.128	11.167					
	65	F5	FALSE	45	Rpl32	UNKNOWI	VIC	NFQ-MGB							16.686	16.619	0.061						
	65	F5	FALSE	45	IPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	28.060	27.985	0.093	11.374	11.231			0.408	0.7539
	73	G1	FALSE	41	Rpl32	UNKNOWI	VIC	NFQ-MGB							16.187	16.232	0.040						
	73	G1	FALSE	41	IPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	27.338	27.393	0.055	11.152					
	74	G2	FALSE	42	Rpl32	UNKNOWI	VIC	NFQ-MGB							16.356	16.264	0.115						
	74	G2	FALSE	42	IPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	27.099	27.074	0.142	10.743					
	75	G3	FALSE	43	Rpl32	UNKNOWI	VIC	NFQ-MGB							15.536	15.704	0.166						
	75	G3	FALSE	43	IPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	26.673	26.828	0.134	11.137	11.007			0.183	0.8808	0.814261551404010
	76	G4	FALSE	44	Rpl32	UNKNOWI	VIC	NFQ-MGB							16.706	16.740	0.115						
	76	G4	FALSE	44	IPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	27.845	27.891	0.128	11.139					
	77	G5	FALSE	45	Rpl32	UNKNOWI	VIC	NFQ-MGB							16.604	16.619	0.061						
	77	G5	FALSE	45	IPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	28.015	27.985	0.093	11.411					
	85	H1	FALSE	41	Rpl32	UNKNOWI	VIC	NFQ-MGB							16.261	16.232	0.040						
	85	H1	FALSE	41	IPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	27.392	27.393	0.055	11.131	11.109			0.286	0.8203
	86	H2	FALSE	42	Rpl32	UNKNOWI	VIC	NFQ-MGB							16.135	16.264	0.115						
	86	H2	FALSE	42	IPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	26.921	27.074	0.142	10.787					
	87	H3	FALSE	43	Rpl32	UNKNOWI	VIC	NFQ-MGB							15.708	15.704	0.166						
	87	H3	FALSE	43	IPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	26.890	26.828	0.134	11.182					
	88	H4	FALSE	44	Rpl32	UNKNOWI	VIC	NFQ-MGB							16.645	16.740	0.115						
	88	H4	FALSE	44	IPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	27.791	27.891	0.128	11.146	11.214			0.391	0.7626
	89	H5	FALSE	45	Rpl32	UNKNOWI	VIC	NFQ-MGB							16.566	16.619	0.061						
	89	H5	FALSE	45	IPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	27.882	27.985	0.093	11.315					
																	Avg	11.123					

		Well	Well Positi	Omit	Sample Na	Target Nai	Task	Reporter	Quencher	Quantity	Quantity M	Quantity S	RQ	RQ Min	RQ Max	CT	Ct Mean	Ct SD	Delta Ct	Delta Ct M	Delta Ct SD	Delta Ct SE	Delta Delta Ct	2^-ΔΔCt	
"Control"	24344	1	A1	FALSE	21	RpL32	UNKNOWN	VIC	NFQ-MGB							17.688	17.643	0.039							
		1	A1	FALSE	21	IPT	UNKNOWN	FAM	NFQ-MGB							29.665	29.468	0.194	11.977						
		2	A2	FALSE	21	RpL32	UNKNOWN	VIC	NFQ-MGB							17.624	17.643	0.039							
		2	A2	FALSE	21	IPT	UNKNOWN	FAM	NFQ-MGB							29.461	29.468	0.194	11.837	11.825			0.296	0.814711737177353	
		3	A3	FALSE	21	RpL32	UNKNOWN	VIC	NFQ-MGB							17.616	17.643	0.039							
		3	A3	FALSE	21	IPT	UNKNOWN	FAM	NFQ-MGB							29.278	29.468	0.194	11.662						
		13	B1	FALSE	22	RpL32	UNKNOWN	VIC	NFQ-MGB							17.057	16.962	0.098							
		13	B1	FALSE	22	IPT	UNKNOWN	FAM	NFQ-MGB							28.895	28.643	0.232	11.838						
		14	B2	FALSE	22	RpL32	UNKNOWN	VIC	NFQ-MGB							16.967	16.962	0.098							
		14	B2	FALSE	22	IPT	UNKNOWN	FAM	NFQ-MGB							28.594	28.643	0.232	11.627	11.681			0.151	0.900372581929174	
		15	B3	FALSE	22	RpL32	UNKNOWN	VIC	NFQ-MGB							16.861	16.962	0.098							
		15	B3	FALSE	22	IPT	UNKNOWN	FAM	NFQ-MGB							28.440	28.643	0.232	11.579						
		25	C1	FALSE	23	RpL32	UNKNOWN	VIC	NFQ-MGB							16.447	16.421	0.057							
		25	C1	FALSE	23	IPT	UNKNOWN	FAM	NFQ-MGB							27.951	27.890	0.075	11.505						
		26	C2	FALSE	23	RpL32	UNKNOWN	VIC	NFQ-MGB							16.356	16.421	0.057							
		26	C2	FALSE	23	IPT	UNKNOWN	FAM	NFQ-MGB							27.806	27.890	0.075	11.450	11.469			-0.061	1.04296256991989	1.059885925461600
		27	C3	FALSE	23	RpL32	UNKNOWN	VIC	NFQ-MGB							16.461	16.421	0.057							
		27	C3	FALSE	23	IPT	UNKNOWN	FAM	NFQ-MGB							27.914	27.890	0.075	11.452						
		25	D1	FALSE	23	RpL32	UNKNOWN	VIC	NFQ-MGB							16.447	16.421	0.057							
		25	D1	FALSE	23	IPT	UNKNOWN	FAM	NFQ-MGB							27.951	27.890	0.075	11.505						
		26	D2	FALSE	23	RpL32	UNKNOWN	VIC	NFQ-MGB							16.356	16.421	0.057							
		26	D2	FALSE	23	IPT	UNKNOWN	FAM	NFQ-MGB							27.806	27.890	0.075	11.450	11.469			-0.061	1.04296256991989	
		27	D3	FALSE	23	RpL32	UNKNOWN	VIC	NFQ-MGB							16.461	16.421	0.057							
		27	D3	FALSE	23	IPT	UNKNOWN	FAM	NFQ-MGB							27.914	27.890	0.075	11.452						
		49	E1	FALSE	25	RpL32	UNKNOWN	VIC	NFQ-MGB							17.525	17.363	0.170							
		49	E1	FALSE	25	IPT	UNKNOWN	FAM	NFQ-MGB							28.801	28.567	0.224	11.277						
		50	E2	FALSE	25	RpL32	UNKNOWN	VIC	NFQ-MGB							17.186	17.363	0.170							
		50	E2	FALSE	25	IPT	UNKNOWN	FAM	NFQ-MGB							28.356	28.567	0.224	11.170	11.204			-0.326	1.25324598007745	
		51	E3	FALSE	25	RpL32	UNKNOWN	VIC	NFQ-MGB							17.378	17.363	0.170							
		51	E3	FALSE	25	IPT	UNKNOWN	FAM	NFQ-MGB							28.544	28.567	0.224	11.166						
																		Avg. Delta	11.530						

		Well	Well Positi	Omit	Sample Na	Target Nai	Task	Reporter	Quencher	Quantity	Quantity M	Quantity S	RQ	RQ Min	RQ Max	CT	Ct Mean	Ct SD	Delta Ct	Delta Ct M	Delta Ct SD	Delta Ct SE	Delta Delta Ct		
"Treated"	24344 x 63	10	A10	FALSE	36	RpL32	UNKNOWN	VIC	NFQ-MGB							17.849	17.690	0.141							
		10	A10	FALSE	36	IPT	UNKNOWN	FAM	NFQ-MGB							29.349	29.227	0.117	11.500						
		11	A11	FALSE	36	RpL32	UNKNOWN	VIC	NFQ-MGB							17.641	17.690	0.141							
		11	A11	FALSE	36	IPT	UNKNOWN	FAM	NFQ-MGB							29.114	29.227	0.117	11.473	11.537			0.007	0.99514088505336	
		12	A12	FALSE	36	RpL32	UNKNOWN	VIC	NFQ-MGB							17.581	17.690	0.141							
		12	A12	FALSE	36	IPT	UNKNOWN	FAM	NFQ-MGB							29.219	29.227	0.117	11.637						
		22	B10	FALSE	37	RpL32	UNKNOWN	VIC	NFQ-MGB							18.428	18.431	0.004							
		22	B10	FALSE	37	IPT	UNKNOWN	FAM	NFQ-MGB							29.994	29.971	0.033	11.566						
		23	B11	FALSE	37	RpL32	UNKNOWN	VIC	NFQ-MGB							18.434	18.431	0.004							
		23	B11	FALSE	37	IPT	UNKNOWN	FAM	NFQ-MGB							29.947	29.971	0.033	11.513	11.539			0.010	0.99340224027817	
		24	B12	FALSE	37	RpL32	UNKNOWN	VIC	NFQ-MGB							Undetermi	18.431	0.004							
		24	B12	FALSE	37	IPT	UNKNOWN	FAM	NFQ-MGB							Undetermi	29.971	0.033							
		34	C10	FALSE	38	RpL32	UNKNOWN	VIC	NFQ-MGB							17.863	17.814	0.070							
		34	C10	FALSE	38	IPT	UNKNOWN	FAM	NFQ-MGB							29.691	29.669	0.062	11.828						
		35	C11	FALSE	38	RpL32	UNKNOWN	VIC	NFQ-MGB							17.734	17.814	0.070							
		35	C11	FALSE	38	IPT	UNKNOWN	FAM	NFQ-MGB							29.599	29.669	0.062	11.865	11.855			0.325	0.79833947800473	0.861856472267
		36	C12	FALSE	38	RpL32	UNKNOWN	VIC	NFQ-MGB							17.844	17.814	0.070							
		36	C12	FALSE	38	IPT	UNKNOWN	FAM	NFQ-MGB							29.716	29.669	0.062	11.872						
		46	D10	FALSE	39	RpL32	UNKNOWN	VIC	NFQ-MGB							16.962	16.968	0.061							
		46	D10	FALSE	39	IPT	UNKNOWN	FAM	NFQ-MGB							28.779	28.742	0.060	11.818						
		47	D11	FALSE	39	RpL32	UNKNOWN	VIC	NFQ-MGB							16.910	16.968	0.061							
		47	D11	FALSE	39	IPT	UNKNOWN	FAM	NFQ-MGB							28.672	28.742	0.060	11.762	11.774			0.244	0.84435765414474	
		48	D12	FALSE	39	RpL32	UNKNOWN	VIC	NFQ-MGB							17.032	16.968	0.061							
		48	D12	FALSE	39	IPT	UNKNOWN	FAM	NFQ-MGB							28.773	28.742	0.060	11.742						
		58	E10	FALSE	40	RpL32	UNKNOWN	VIC	NFQ-MGB							17.943	17.956	0.131							
		58	E10	FALSE	40	IPT	UNKNOWN	FAM	NFQ-MGB							30.098	30.046	0.129	12.155						
		59	E11	FALSE	40	RpL32	UNKNOWN	VIC	NFQ-MGB							17.832	17.956	0.131							
		59	E11	FALSE	40	IPT	UNKNOWN	FAM	NFQ-MGB							29.899	30.046	0.129	12.067	12.090			0.561	0.67804210385481	
		60	E12	FALSE	40	RpL32	UNKNOWN	VIC	NFQ-MGB							18.093	17.956	0.131							
		60	E12	FALSE	40	IPT	UNKNOWN	FAM	NFQ-MGB							30.142	30.046	0.129	12.049						
																		Avg	11.759						

		Well	Well Positi	Omit	Sample Na	Target Nai	Task	Reporter	Quencher	Quantity	Quantity M	Quantity S	RQ	RQ Min	RQ Max	CT	Ct Mean	Ct SD	Delta Ct	Delta Ct M	Delta Ct SD	Delta Ct SE	Delta Delta Ct		
"Control"	6326	4	A4	FALSE	26	RpL32	UNKNOWN	VIC	NFQ-MGB							17.880	17.699	0.174							
		4	A4	FALSE	26	IPT	UNKNOWN	FAM	NFQ-MGB							29.256	29.119	0.148	11.376						
		5	A5	FALSE	26	RpL32	UNKNOWN	VIC	NFQ-MGB							17.532	17.699	0.174							
		5	A5	FALSE	26	IPT	UNKNOWN	FAM	NFQ-MGB							28.962	29.119	0.148	11.429	11.420			0.642	0.6407176983422	
		6	A6	FALSE	26	RpL32	UNKNOWN	VIC	NFQ-MGB							17.685	17.699	0.174							
		6	A6	FALSE	26	IPT	UNKNOWN	FAM	NFQ-MGB							29.140	29.119	0.148	11.455						
		16	B4	FALSE	27	RpL32	UNKNOWN	VIC	NFQ-MGB							17.425	17.343	0.072							
		16	B4	FALSE	27	IPT	UNKNOWN	FAM	NFQ-MGB							28.051	27.988	0.056	10.625						
		17	B5	FALSE	27	RpL32	UNKNOWN	VIC	NFQ-MGB							17.292	17.343	0.072							
		17	B5	FALSE	27	IPT	UNKNOWN	FAM	NFQ-MGB							27.943	27.988	0.056	10.651	10.645			-0.133	1.09623195942679	
		18	B6	FALSE	27	RpL32	UNKNOWN	VIC	NFQ-MGB							17.311	17.343	0.072							
		18	B6	FALSE	27	IPT	UNKNOWN	FAM	NFQ-MGB							27.971	27.988	0.056	10.660						
		28	C4	FALSE	28	RpL32	UNKNOWN	VIC	NFQ-MGB							17.547	17.521	0.030							
		28	C4	FALSE	28	IPT	UNKNOWN	FAM	NFQ-MGB							28.344	28.305	0.063	10.797						
		29	C5	FALSE	28	RpL32	UNKNOWN	VIC	NFQ-MGB							17.527	17.521	0.030							
		29	C5	FALSE	28	IPT	UNKNOWN	FAM	NFQ-MGB							28.338	28.305	0.063	10.811	10.784			0.006	0.99562867187442	1.027042903665710
		30	C6	FALSE	28	RpL32	UNKNOWN	VIC	NFQ-MGB							17.488	17.521	0.030							
		30	C6	FALSE	28	IPT	UNKNOWN	FAM	NFQ-MGB							28.233	28.305	0.063	10.745						
		40	D4	FALSE	29	RpL32	UNKNOWN	VIC	NFQ-MGB							18.562	18.542	0.017							
		40	D4	FALSE	29	IPT	UNKNOWN	FAM	NFQ-MGB							28.977	28.924	0.061	10.415						
		41	D5	FALSE	29	RpL32	UNKNOWN	VIC	NFQ-MGB							18.534	18.542	0.017							
		41	D5	FALSE	29	IPT	UNKNOWN	FAM	NFQ-MGB							28.858	28.924	0.061	10.324	10.381			-0.396	1.31609511595451	
		42	D6	FALSE	29	RpL32	UNKNOWN	VIC	NFQ-MGB							18.532	18.542	0.017							
		42	D6	FALSE	29	IPT	UNKNOWN	FAM	NFQ-MGB							28.937	28.924	0.061	10.405						
		52	E4	FALSE	30	RpL32	UNKNOWN	VIC	NFQ-MGB							18.088	18.182	0.082							
		52	E4	FALSE	30	IPT	UNKNOWN	FAM	NFQ-MGB							28.669	28.840	0.149	10.581						
		53	E5	FALSE	30	RpL32	UNKNOWN	VIC	NFQ-MGB							18.242	18.182	0.082							
		53	E5	FALSE	30	IPT	UNKNOWN	FAM	NFQ-MGB							28.941	28.840	0.149	10.699	10.658			-0.120	1.08654107273062	
		54	E6	FALSE	30	RpL32	UNKNOWN	VIC	NFQ-MGB							18.215	18.182	0.082							
		54	E6	FALSE	30	IPT	UNKNOWN	FAM	NFQ-MGB							28.909	28.840	0.149	10.694						
																		Avg. Delta	10.778						

		Well	Well Positi	Omit	Sample Na	Target Nar	Task	Reporter	Quencher	Quantity	Quantity M	Quantity S	RQ	RQ Min	RQ Max	CT	Ct Mean	Ct SD	Delta Ct	Delta Ct M	Delta Ct SE	Delta Ct SE	Delta Delta Ct	2^-ΔΔCt	
"Control"	67048	4	A4	FALSE	6	Rpl32	UNKNOWI	VIC	NFQ-MGB							15.069	15.030	0.034							
		4	A4	FALSE	6	IPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	26.537	26.459	0.067	11.469						
		5	A5	FALSE	6	Rpl32	UNKNOWI	VIC	NFQ-MGB							15.018	15.030	0.034							
		5	A5	FALSE	6	IPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	26.423	26.459	0.067	11.405	11.429			-0.010	1.006997911929 71	
		6	A6	FALSE	6	Rpl32	UNKNOWI	VIC	NFQ-MGB							15.004	15.030	0.034							
		6	A6	FALSE	6	IPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	26.418	26.459	0.067	11.415						
		16	B4	FALSE	7	Rpl32	UNKNOWI	VIC	NFQ-MGB							15.236	15.239	0.015							
		16	B4	FALSE	7	IPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	26.537	26.446	0.079	11.301						
		17	B5	FALSE	7	Rpl32	UNKNOWI	VIC	NFQ-MGB							15.255	15.239	0.015							
		17	B5	FALSE	7	IPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	26.398	26.446	0.079	11.143	11.207			-0.232	1.174486634954070	
		18	B6	FALSE	7	Rpl32	UNKNOWI	VIC	NFQ-MGB							15.226	15.239	0.015							
		18	B6	FALSE	7	IPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	26.404	26.446	0.079	11.178						
		28	C4	FALSE	8	Rpl32	UNKNOWI	VIC	NFQ-MGB							15.990	15.895	0.112							
		28	C4	FALSE	8	IPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	26.928	26.964	0.058	10.938						
		29	C5	FALSE	8	Rpl32	UNKNOWI	VIC	NFQ-MGB							15.924	15.895	0.112							
		29	C5	FALSE	8	IPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	27.031	26.964	0.058	11.107	11.069			-0.371	1.292822161679 20	1.018446701307880
		30	C6	FALSE	8	Rpl32	UNKNOWI	VIC	NFQ-MGB							15.771	15.895	0.112							
		30	C6	FALSE	8	IPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	26.932	26.964	0.058	11.161						
		40	D4	FALSE	9	Rpl32	UNKNOWI	VIC	NFQ-MGB							15.414	15.525	0.129							
		40	D4	FALSE	9	IPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	27.162	27.307	0.167	11.748						
		41	D5	FALSE	9	Rpl32	UNKNOWI	VIC	NFQ-MGB							15.667	15.525	0.129							
		41	D5	FALSE	9	IPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	27.489	27.307	0.167	11.822	11.782			0.342	0.788677464006 59	
		42	D6	FALSE	9	Rpl32	UNKNOWI	VIC	NFQ-MGB							15.495	15.525	0.129							
		42	D6	FALSE	9	IPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	27.270	27.307	0.167	11.776						
		52	E4	FALSE	10	Rpl32	UNKNOWI	VIC	NFQ-MGB							15.207	15.238	0.046							
		52	E4	FALSE	10	IPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	26.950	26.947	0.100	11.743						
		53	E5	FALSE	10	Rpl32	UNKNOWI	VIC	NFQ-MGB							15.290	15.238	0.046							
		53	E5	FALSE	10	IPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	27.046	26.947	0.100	11.756	11.710			0.270	0.829249333969 82	
		54	E6	FALSE	10	Rpl32	UNKNOWI	VIC	NFQ-MGB							15.216	15.238	0.046							
		54	E6	FALSE	10	IPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	26.846	26.947	0.100	11.630						
																		Avg. Delta	11.439						
		Well	Well Positi	Omit	Sample Na	Target Nar	Task	Reporter	Quencher	Quantity	Quantity M	Quantity S	RQ	RQ Min	RQ Max	CT	Ct Mean	Ct SD	Delta Ct	Delta Ct M	Delta Ct SE	Delta Ct SE	Delta Delta Ct		
"Treated"	79824 x 67	7	A7	FALSE	31	Rpl32	UNKNOWI	VIC	NFQ-MGB							17.135	17.092	0.053							
		7	A7	FALSE	31	IPT	UNKNOWI	FAM	NFQ-MGB							27.913	27.891	0.081	10.778						
		8	A8	FALSE	31	Rpl32	UNKNOWI	VIC	NFQ-MGB							17.032	17.092	0.053							
		8	A8	FALSE	31	IPT	UNKNOWI	FAM	NFQ-MGB							27.801	27.891	0.081	10.769	10.799			-0.641	1.558990728214 16	
		9	A9	FALSE	31	Rpl32	UNKNOWI	VIC	NFQ-MGB							17.109	17.092	0.053							
		9	A9	FALSE	31	IPT	UNKNOWI	FAM	NFQ-MGB							27.958	27.891	0.081	10.850						
		19	B7	FALSE	32	Rpl32	UNKNOWI	VIC	NFQ-MGB							17.868	17.692	0.155							
		19	B7	FALSE	32	IPT	UNKNOWI	FAM	NFQ-MGB							28.019	27.877	0.123	10.151						
		20	B8	FALSE	32	Rpl32	UNKNOWI	VIC	NFQ-MGB							17.636	17.692	0.155							
		20	B8	FALSE	32	IPT	UNKNOWI	FAM	NFQ-MGB							27.809	27.877	0.123	10.173	10.185			-1.255	2.385860466338 03	
		21	B9	FALSE	32	Rpl32	UNKNOWI	VIC	NFQ-MGB							17.574	17.692	0.155							
		21	B9	FALSE	32	IPT	UNKNOWI	FAM	NFQ-MGB							27.804	27.877	0.123	10.230						
		31	C7	FALSE	33	Rpl32	UNKNOWI	VIC	NFQ-MGB							17.343	17.302	0.038							
		31	C7	FALSE	33	IPT	UNKNOWI	FAM	NFQ-MGB							27.515	27.490	0.032	10.172						
		32	C8	FALSE	33	Rpl32	UNKNOWI	VIC	NFQ-MGB							17.292	17.302	0.038							
		32	C8	FALSE	33	IPT	UNKNOWI	FAM	NFQ-MGB							27.454	27.490	0.032	10.162	10.188			-1.251	2.380062591274 68	2.351853618175 23
		33	C9	FALSE	33	Rpl32	UNKNOWI	VIC	NFQ-MGB							17.270	17.302	0.038							
		33	C9	FALSE	33	IPT	UNKNOWI	FAM	NFQ-MGB							27.501	27.490	0.032	10.231						
		43	D7	FALSE	34	Rpl32	UNKNOWI	VIC	NFQ-MGB							19.053	18.860	0.293							
		43	D7	FALSE	34	IPT	UNKNOWI	FAM	NFQ-MGB							29.073	28.985	0.248	10.020						
		44	D8	FALSE	34	Rpl32	UNKNOWI	VIC	NFQ-MGB							18.522	18.860	0.293							
		44	D8	FALSE	34	IPT	UNKNOWI	FAM	NFQ-MGB							28.704	28.985	0.248	10.182	10.125			-1.315	2.487387381238 15	
		45	D9	FALSE	34	Rpl32	UNKNOWI	VIC	NFQ-MGB							19.004	18.860	0.293							
		45	D9	FALSE	34	IPT	UNKNOWI	FAM	NFQ-MGB							29.177	28.985	0.248	10.173						
		55	E7	FALSE	35	Rpl32	UNKNOWI	VIC	NFQ-MGB							17.831	17.854	0.026							
		55	E7	FALSE	35	IPT	UNKNOWI	FAM	NFQ-MGB							27.687	27.734	0.041	9.856						
		56	E8	FALSE	35	Rpl32	UNKNOWI	VIC	NFQ-MGB							17.883	17.854	0.026							
		56	E8	FALSE	35	IPT	UNKNOWI	FAM	NFQ-MGB							27.760	27.734	0.041	9.878	9.880			-1.559	2.946966923811 12	
		57	E9	FALSE	35	Rpl32	UNKNOWI	VIC	NFQ-MGB							17.849	17.854	0.026							
		57	E9	FALSE	35	IPT	UNKNOWI	FAM	NFQ-MGB							27.756	27.734	0.041	9.907						
																		Avg	10.235						

		Well	Well Positi	Omit	Sample Na	Target Nar	Task	Reporter	Quencher	Quantity	Quantity M	Quantity S	RQ	RQ Min	RQ Max	CT	Ct Mean	Ct SD	Delta Ct	Delta Ct M	Delta Ct SE	Delta Ct SI	Delta Delta Ct	2^-ΔΔCt	
"Control"	79824	1	A1	FALSE	1	Rpl32	UNKNOWI	VIC	NFQ-MGB							33.579	33.769	0.170							
		1	A1	FALSE	1	iPT	UNKNOWI	FAM	NFQ-MGB				1.000	0.735	1.360	32.427	32.764	0.337	-1.153	-1.005					
		2	A2	FALSE	1	Rpl32	UNKNOWI	VIC	NFQ-MGB							33.820	33.769	0.170							
		2	A2	FALSE	1	iPT	UNKNOWI	FAM	NFQ-MGB				1.000	0.735	1.360	32.764	32.764	0.337	-1.056	-1.005					
		3	A3	FALSE	1	Rpl32	UNKNOWI	VIC	NFQ-MGB							33.907	33.769	0.170							
		3	A3	FALSE	1	iPT	UNKNOWI	FAM	NFQ-MGB				1.000	0.735	1.360	33.100	32.764	0.337	-0.807	-1.005					
		13	B1	FALSE	2	Rpl32	UNKNOWI	VIC	NFQ-MGB							16.162	16.233	0.069							
		13	B1	FALSE	2	iPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	27.552	27.571	0.042	11.390						
		14	B2	FALSE	2	Rpl32	UNKNOWI	VIC	NFQ-MGB							16.237	16.233	0.069							
		14	B2	FALSE	2	iPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	27.541	27.571	0.042	11.304	11.337			0.286	0.820150675168899	
		15	B3	FALSE	2	Rpl32	UNKNOWI	VIC	NFQ-MGB							16.300	16.233	0.069							
		15	B3	FALSE	2	iPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	27.619	27.571	0.042	11.318						
		25	C1	FALSE	3	Rpl32	UNKNOWI	VIC	NFQ-MGB							16.475	16.503	0.025							
		25	C1	FALSE	3	iPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	27.462	27.502	0.065	10.987						
		26	C2	FALSE	3	Rpl32	UNKNOWI	VIC	NFQ-MGB							16.516	16.503	0.025							
		26	C2	FALSE	3	iPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	27.468	27.502	0.065	10.952	10.999			-0.052	1.036964492900812	1.008147140699900
		27	C3	FALSE	3	Rpl32	UNKNOWI	VIC	NFQ-MGB							16.520	16.503	0.025							
		27	C3	FALSE	3	iPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	27.578	27.502	0.065	11.058						
		37	D1	FALSE	4	Rpl32	UNKNOWI	VIC	NFQ-MGB							16.175	16.060	0.105							
		37	D1	FALSE	4	iPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	27.137	27.109	0.047	10.961						
		38	D2	FALSE	4	Rpl32	UNKNOWI	VIC	NFQ-MGB							15.970	16.060	0.105							
		38	D2	FALSE	4	iPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	27.054	27.109	0.047	11.085	11.048			-0.003	1.00202366021852	
		39	D3	FALSE	4	Rpl32	UNKNOWI	VIC	NFQ-MGB							16.036	16.060	0.105							
		39	D3	FALSE	4	iPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	27.135	27.109	0.047	11.099						
		49	E1	FALSE	5	Rpl32	UNKNOWI	VIC	NFQ-MGB							16.097	16.014	0.074							
		49	E1	FALSE	5	iPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	26.895	26.835	0.060	10.799						
		50	E2	FALSE	5	Rpl32	UNKNOWI	VIC	NFQ-MGB							15.953	16.014	0.074							
		50	E2	FALSE	5	iPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	26.775	26.835	0.060	10.822	10.821			-0.231	1.17344973450406	
		51	E3	FALSE	5	Rpl32	UNKNOWI	VIC	NFQ-MGB							15.993	16.014	0.074							
		51	E3	FALSE	5	iPT	UNKNOWI	FAM	NFQ-MGB				0.000	0.000	0.000	26.834	26.835	0.060	10.841						
																		Avg. Delta	11.051						
		Well	Well Positi	Omit	Sample Na	Target Nar	Task	Reporter	Quencher	Quantity	Quantity M	Quantity S	RQ	RQ Min	RQ Max	CT	Ct Mean	Ct SD	Delta Ct	Delta Ct M	Delta Ct SE	Delta Ct SI	Delta Delta Ct		
"Treated"	79824 x 67	7	A7	FALSE	31	Rpl32	UNKNOWI	VIC	NFQ-MGB							17.135	17.092	0.053							
		7	A7	FALSE	31	iPT	UNKNOWI	FAM	NFQ-MGB							27.913	27.891	0.081	10.778						
		8	A8	FALSE	31	Rpl32	UNKNOWI	VIC	NFQ-MGB							17.032	17.092	0.053							
		8	A8	FALSE	31	iPT	UNKNOWI	FAM	NFQ-MGB							27.801	27.891	0.081	10.769	10.799			-0.253	1.19137134164317	
		9	A9	FALSE	31	Rpl32	UNKNOWI	VIC	NFQ-MGB							17.109	17.092	0.053							
		9	A9	FALSE	31	iPT	UNKNOWI	FAM	NFQ-MGB							27.958	27.891	0.081	10.850						
		19	B7	FALSE	32	Rpl32	UNKNOWI	VIC	NFQ-MGB							17.868	17.692	0.155							
		19	B7	FALSE	32	iPT	UNKNOWI	FAM	NFQ-MGB							28.019	27.877	0.123	10.151						
		20	B8	FALSE	32	Rpl32	UNKNOWI	VIC	NFQ-MGB							17.636	17.692	0.155							
		20	B8	FALSE	32	iPT	UNKNOWI	FAM	NFQ-MGB							27.809	27.877	0.123	10.173	10.185			-0.867	1.82326022426738	
		21	B9	FALSE	32	Rpl32	UNKNOWI	VIC	NFQ-MGB							17.574	17.692	0.155							
		21	B9	FALSE	32	iPT	UNKNOWI	FAM	NFQ-MGB							27.804	27.877	0.123	10.230						
		31	C7	FALSE	33	Rpl32	UNKNOWI	VIC	NFQ-MGB							17.343	17.302	0.038							
		31	C7	FALSE	33	iPT	UNKNOWI	FAM	NFQ-MGB							27.515	27.490	0.032	10.172						
		32	C8	FALSE	33	Rpl32	UNKNOWI	VIC	NFQ-MGB							17.292	17.302	0.038							
		32	C8	FALSE	33	iPT	UNKNOWI	FAM	NFQ-MGB							27.454	27.490	0.032	10.162	10.188			-0.863	1.81882952300157	1.79727239535503
		33	C9	FALSE	33	Rpl32	UNKNOWI	VIC	NFQ-MGB							17.270	17.302	0.038							
		33	C9	FALSE	33	iPT	UNKNOWI	FAM	NFQ-MGB							27.501	27.490	0.032	10.231						
		43	D7	FALSE	34	Rpl32	UNKNOWI	VIC	NFQ-MGB							19.053	18.860	0.293							
		43	D7	FALSE	34	iPT	UNKNOWI	FAM	NFQ-MGB							29.073	28.985	0.248	10.020						
		44	D8	FALSE	34	Rpl32	UNKNOWI	VIC	NFQ-MGB							18.522	18.860	0.293							
		44	D8	FALSE	34	iPT	UNKNOWI	FAM	NFQ-MGB							28.704	28.985	0.248	10.182	10.125			-0.927	1.90084648224084	
		45	D9	FALSE	34	Rpl32	UNKNOWI	VIC	NFQ-MGB							19.004	18.860	0.293							
		45	D9	FALSE	34	iPT	UNKNOWI	FAM	NFQ-MGB							29.177	28.985	0.248	10.173						
		55	E7	FALSE	35	Rpl32	UNKNOWI	VIC	NFQ-MGB							17.831	17.854	0.026							
		55	E7	FALSE	35	iPT	UNKNOWI	FAM	NFQ-MGB							27.687	27.734	0.041	9.856						
		56	E8	FALSE	35	Rpl32	UNKNOWI	VIC	NFQ-MGB							17.883	17.854	0.026							
		56	E8	FALSE	35	iPT	UNKNOWI	FAM	NFQ-MGB							27.760	27.734	0.041	9.878	9.880			-1.171	2.25205440562221	
		57	E9	FALSE	35	Rpl32	UNKNOWI	VIC	NFQ-MGB							17.849	17.854	0.026							
		57	E9	FALSE	35	iPT	UNKNOWI	FAM	NFQ-MGB							27.756	27.734	0.041	9.907						
																		Avg	10.235						