Radiation-Induced Metabolomic Changes in Sterile Male Monochamus alternatus (Coleoptera: Cerambycidae)

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RESEARCH

Radiation-Induced Metabolomic Changes in Sterile Male *Monochamus alternatus* (Coleoptera: Cerambycidae)

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ABSTRACT. Radiation-induced sterile insect technique is a biologically based, environment-friendly method for the suppression or eradication of a number of insect pests. Although the basic mechanisms underlying the technology have been well studied, little is known about the cell responses in organisms. Characterization of the metabolic shift associated with radiation exposure in sterile insects would be helpful for understanding the detailed mechanism underlying this technique and promote its practical application. In this article, a metabolomic study was performed to characterize the global metabolic changes induced by radiation using untreated and 40 Gy ^{60}Coγ-irradiated testes of Japanese pine sawyer, *Monochamus alternatus* Hope. Differential metabolites were detected and tentatively identified. Many key metabolites in glycolysis and the tricarboxylic acid cycle, as well as most fatty and amino acids, were elevated in irradiated male *M. alternatus*, presumably resulting from depression of glycolysis and the tricarboxylic acid cycle, each of which are important pathways for energy generation Adenosine Triphosphate (ATP) in insect spermatozoa. The findings in this article will contribute to our knowledge of the characteristic metabolic changes associated with irradiation sterility and understand the molecular mechanisms underlying radiation-induced sterile insect technique.

Key Words: metabolite, vector insect, sterile insect technique, gas chromatography time-of-flight mass spectrometry

Radiation-induced sterile insect technique (RISIT) is an environment-friendly method of pest control that integrates well into area-wide integrated pest management programs. It has been used for the suppression or eradication of a number of insect pests (Dyck et al. 2005, Kumano et al. 2008). The basic mechanism of RISIT is that radiation damages genetic material, and the resulting chromosome breaks induce dominant lethal mutations in reproductive cells (Muller 1954, Tothová and Marec 2001, Dyck et al. 2005). It is well known that DNA, protein, and metabolite are fundamental elements of biology. DNA and proteins set the stage for what happens in the cell, but much of the actual activity is at the metabolite level. Metabolomics (or metabonomics), the analysis of the total population of metabolites in a given biological sample, is an important field of "-omics" study. It has been widely applied to uncover biomarkers and metabolic fingerprints in drug discovery and clinical toxicology, as well as to reveal active pathways and signaling metabolites (Bino et al. 2004, Griffin and Bollard 2004, Sreekumar et al. 2009). Metabolomic analysis can provide a "closer" glimpse into the phenotypic state of an organism because it is downstream of both transcriptomics and proteomics when compared with functional genomic analysis (Al-Rubeai and Fussenegger 2007). Therefore, metabolomic analysis is a very promising strategy for uncovering critical information about the mechanisms underlying RISIT.

Pine wilt disease, caused by *Bursaphelenchus xylophilus* Nickle, is one of the most destructive forest diseases in Japan, Korea, China, Laos, and Vietnam (Makihara 2004, Song et al. 2008). It has caused substantial ecological damage and enormous economic losses. Studies have indicated that *B. xylophilus* is transported between host trees almost exclusively by cerambycid beetles in the genus *Monochamus* (Linit 1988, Kishi 1995). In Asia, the Japanese pine sawyer, *Monochamus alternatus* Hope (Coleoptera: Cerambycidae), is the most important and efficient vector of *B. xylophilus* (Kobayashi et al. 1984, Akbulut and Stamps 2012). Controlling *M. alternatus* is an efficient

way to diminish the diffusion of *B. xylophilus* and reduce the damage caused by pine wilt disease (Togashi 1988). RISIT has been applied in the management of *M. alternatus* in China. Several studies have shown that this technique is effective and practical (Mou et al. 2005, 2009; Ma et al. 2008). In previous studies, the adults of *M. alternatus*, following emergence at days 3–5, were irradiated with 20–160 Gy ^{60}Coγ, and the optimal radiation dose for the beetles was investigated by evaluating the fertilization ability, longevity, mating propensity, oviposition, and egg hatching of the mated females. We found that the males were more sensitive to irradiation than the females, and 40 Gy was a suitable dose for full sterility of the male beetles. There was no significant difference in longevity, mating propensity, and egg quantity in mated females when comparing 40 Gy-irradiated beetles with untreated controls. However, the hatching rate in eggs fertilized by irradiated males was almost zero (Mou et al. 2005, 2009).

In this study, gas chromatography time-of-flight mass spectrometry (GC-TOF/MS) was used to determine the comprehensive metabolite profiles of untreated and 40 Gy-irradiated male *M. alternatus* and to further identify the metabolites and key pathways involved in radiation-induced sterile insects. The results showed that soluble sugars, amino acids, and lipid metabolites displayed the most significant differences between untreated and irradiated insects.

Materials and Methods

Insects and Irradiation. The fourth instar larvae of *M. alternatus* were collected from pine logs in Hangzhou, Zhejiang, China, in March 2001, and reared on an artificial diet (6.0% wheat bran, 3.9% soy protein, 1.2% Wesson salt mixture, 1.0% sucrose, 18.0% pine powder, 0.02% formaldehyde, 0.1% nipagine, 0.4% vitamin C, 0.0015% vitamin B$_{12}$, 1.4% agar, 1.8% yeast, 0.1785% choline chloride, 66% distilled water) until emergence under constant temperature ($25 \pm 1°C$) with 65% relative humidity (RH) and a photoperiod of 12:12 (L:D) h

cycle conditions in the laboratory. Newly emerged adults were maintained on the artificial diet. Individuals emerging after 4 d were manually separated according to their sex, and the males were selected for the experiment. These males were separated into two groups, one for irradiation and another for a nonirradiated control. Irradiation was performed at the Institute of Crops and Nuclear Technology Utilization, Zhejiang Academy of Agricultural Sciences of China, using ^{60}Coγ at a dose rate of 1.0 Gy/min. The total radiation dose was 40 Gy for the males. After radiation treatment, the treated and nonirradiated male beetles were reared with fresh pine branches in cages at room temperature. Two days after radiation, untreated and treated males were chosen for metabolites extraction, and were replicated seven times in the experiment.

Chemicals. Methanol (High-Performance Liquid (HPLC) grade; ≥99.9%; catalog no.: A454-4) and chloroform (HPLC grade; ≥99.9%; catalog no.: C607-4) were purchased from Fisher Scientific (Hampton, NH) (http://www.fishersci.com). Pyridine (GC grade; ≥99.8%; catalog no.: 82704-1L), *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA reagent) (catalog no.: M7891), the methoxy amination reagent, and the internal standard. reference compounds ribitol (≥99.0%; catalog no.: A5502-5G) and nonadecanoic acid (≥99.0%; catalog no.: N5252-5G) were purchased from Sigma-Aldrich (St. Louis, MO, USA) (http://www.sigmaaldrich.com) Distilled water was purified "in-house" using a Milli-Q system (Bedford, MA, USA) (http://www.millipore.com).

Metabolites Extraction and Derivatization. The extraction of metabolites was performed according to the protocol described as in the reference with some modification (Weckwerth et al. 2004). The testes of untreated and irradiated beetles were frozen with liquid nitrogen and ground to powder with a mortar and pestle. About 100 mg of powder was used for the extraction of the metabolites (untreated samples: 102.5, 103.0, 102.4, 105.2, 103.6, 99.5, 102.0 mg; irradiated samples: 96.8, 104.9, 101.9, 105.8, 103.4, 104.1, 101.0 mg). The extraction was carried out at room temperature. To an Eppendorf tube containing frozen powder, 1.5 ml of extraction buffer (methanol:chloroform:water 5:2:2), 10 µl of nonadecanoic acid (2.1 mg/ml), and 100 µl of ribitol (0.2 mg/ml) (internal quantitative standards) were added. The mixture was extracted using a supersonic instrument for 30 min and centrifuged at 11,000 rpm for 10 min. One milliliter of the supernatant was transferred to another tube, mixed with 300 µl of chloroform and 600 µl of dH$_2$O and centrifuged at 4,000 rpm for 5 min. The upper polar phase (100 µl) and the lower lipophilic phase (100 µl) were dried in centrifugal vacuum concentrators (ScanSpeed MaxiVac Beta, LaboGene ApS, Lynge, Denmark) rotating at 1,500 rpm at 4°C. The dried residues were oximated with 40 µl of methoxylamine hydrochloride (20 mg/ml) in anhydrous pyridine at 37°C for 2 h and then silylated at 37°C for 30 min with 70 µl of MSTFA. The derivatized samples were transferred into 250-µl glass vials (Agilent) for GC-TOF/MS analysis.

Gas Chromatography Time-of-Flight Mass Spectrometry. The polar and lipophilic phases were analyzed using a LECO Pegasus IV GC-TOF/MS system (http://www.leco.com). The GC-TOF/MS system composed of an Agilent autosampler, a 6980 gas chromatograph (Agilent, San Jose, CA, USA), and a LECO Pegasus IV time-of-flight mass spectrometer (Leco, St. Joseph, MI, USA). One microliter of each derivatized sample was injected by autosampler into the gas chromatograph, which was equipped with a 30 m by 0.25 mm internal diameter-fused silica capillary column with a chemically bonded 0.25-µm DB-5 MS stationary phase (J&W Scientific, Folsom, CA, USA). The injector temperature was 280°C. The helium gas flow rate through the column was 1.5 ml/min. The column temperature was held at 80°C for 4 min, then raised 5°C/min to 330°C and held for 5 min. The column effluent was introduced into the ion source of the mass spectrometer. The transfer line and the ion source temperatures were 250 and 200°C, respectively. Ions were generated by a 70 eV electron beam at an ionization current of 2.0 mA, and 20 spectra were recorded in the mass range from 80 to 500 m/z. The acceleration voltage was turned on after a solvent delay of 300 s. The detector voltage was 1,700 V.

Data Processing. The acquired MS files from the GC-TOF/MS analysis were exported in NetCDF format by ChromaTOF software (v3.3, Leco, St. Joseph, MI, USA). NetCDF files were processed by custom scripts in MATLAB (The Math Works, Natick, MA, USA) to carry out baseline correction, denoising, smoothing, alignment, time-window splitting, and multivariate curve resolution (Jonsson et al. 2004, 2005; Ni et al. 2008). The resulting three-dimensional matrix (referred to as the metabolic matrix hereafter) includes sample information, peak retention time, and peak intensities. Internal standards and any known artificial peaks, such as peaks caused by noise, column bleed, and the derivatization procedure, were removed from the matrix. The weight is normalized to the maximum for each sample to minimize the discrepancy resulting from different sample weight. Then, min–max normalization is used to transform the data into the range [0,1]. Missing values were assumed to result from areas falling below limits of detection. For each metabolite, missing values were imputed with its observed minimum after the normalization step.

Univariate and Multivariate Statistical Analysis. The metabolic matrix was analyzed further by both univariate and multivariate statistical techniques in an attempt to evaluate the predictive power of each metabolite and to select potential differential metabolites. Nonparametric Mann–Whitney tests (SPSS17.0, IBM Corporation, Chicago, IL, USA) were the univariate methods selected for differential metabolite evaluation and selection. A smaller *P* value for the Mann–Whitney test indicated a statistic support for difference between the control and the irradiated group. The critical *P* value was set at 0.01. False discovery rates were calculated using the *q* value conversion algorithm to correct for Mann–Whitney tests. In parallel, mean centering, unit variance scaling, and multivariate statistical analysis were carried out by the SIMCA-P 12.0 software package (Umetrics, Umeå, Sweden). Unsupervised principle component analysis (PCA), partial least squares discriminant analysis (PLS-DA), and sophisticated supervised orthogonal projections to latent structures discriminant analysis (OPLS-DA) were applied. OPLS-DA is a modification of the PLS-DA method (Trygg and Wold 2002). It separates the systematic variation in *X* (process variables) into two parts, one that is linearly related to *Y* (response variables) and one that is orthogonal to *Y*. This may lead to better class resolution in a discriminant problem, and give a more straight forward and realistic model (Kuhl et al. 2008, Wiklund et al. 2008, Graca et al. 2009, Wu et al. 2012). In OPLS-DA model, R^2X(cum) is the percentage of cumulative variation of the data explained by the mode and represents the goodness of the fit to the model. R^2Y(cum) is the percentage of all response variables explained by the mode. Q^2(cum) is the cumulative predicted variation in *Y* according to cross-validation. Values of R^2Y(cum) and Q^2(cum) close to 1 indicate an excellent model. S-plot from the OPLS-DA model was constructed. The S-plot shows the covariance *p* against the correlation *p*(corr) variables of the discriminating component. Cutoff values for the covariance of $p \geq |0.05|$ and for the correlation of p(corr) $\geq |0.05|$ were used (Sieber et al. 2009). The variables selected in this range represent the metabolites responsible for differentiation in OPLS-DA score plots. Based on the variable importance values (with a threshold of 1.0) from the OPLS-DA model, a number of metabolites responsible for the differentiation of the metabolic profiles were screened. Compound identification was performed by comparing the mass fragments with NIST 05 standard mass spectral databases and the Golm Metabolome Database in NIST MS Search 2.0 (NIST, Gaithersburg, MD) software with a similarity of more than 80%.

Results

Metabolomic Profiling of Untreated and Irradiated Beetles. GC-TOF/MS analyses were performed to determine the relative metabolite levels across untreated and irradiated samples. In total, 816 peaks were quantified, with 555 and 261 peaks identified in the lipophilic and polar phases, respectively. In total, 22 compounds were found in both phases, including glycine, L-asparagine, and L-homoserine. PCA, PLS-DA, and

OPLS-DA in SIMCA-P software were applied to spectral data to visualize metabolic differentiation between untreated and irradiated testes. The parameters R^2X(cum), R^2Y(cum), and Q^2(cum) were used to evaluate the models and determine the number of components to use. It was shown that the application of OPLS-DA resulted in a more discernible discrimination between the two group samples. The metabolic profiles of the untreatment group and irradiated group are significantly different with high values of explained variation and predictive ability (Fig. 1). In lipophilic profiling, OPLS-DA gave one predictive and two orthogonal (1 + 2) components with the cross-validated predictive ability Q^2(cum) = 89.4% and the total explained variance R^2X(cum) = 58.2%. In polar profiling, OPLS-DA gave one predictive and two orthogonal (1 + 2) components with the cross-validated predictive ability Q^2(cum) = 86.1% and the total explained variance R^2X(cum) = 66.0% (Fig. 1A and B).

Metabolomic Variations Associated *With* Radiation. To further understand the underlying variables contributing to the differentiation, S-plot from the OPLS-DA model was constructed. In S-plot, statistically significant metabolites that contributed to group separation were screened. Relying on two criteria, the variable importance value of the OPLS-DA model > 1.0 and the P value of the Mann–Whitney test < 0.01, a total of 94 differential metabolites (including 70 metabolites in lipophilic profiles and 24 metabolites in polar profiles) were detected between the untreated and irradiated testes. Among these perturbed metabolites, 72 were upregulated in irradiated samples, whereas 22 were downregulated. The top 29 differential metabolites, including a number of amino acids, sugars, fatty acids, organic acids, and some unidentified compounds, were displayed in a control-based z-score plot (Fig. 2). In this synthetic presentation, metabolites located on the right side were generally more abundant in irradiated testes, while those located on the left side were less abundant. It was shown that proline and the unidentified metabolite o234 had the highest and lowest z-score values, respectively, meaning that they exhibited the biggest difference between the untreated and irradiated testes. Five amino acids (proline, glycine, tryptophan, histidine, and aspartic acid), six fatty acids (oleic acid, eicosanoic acid, stearic acid, behenic acid, eicosatrienoic acid, and linoleic acid), two sugars (glucose and galactose), two organic acids (2-butenedioic acid and benzoic acid), and five unidentified compounds (o349, o348, o470, p1, and o234) contributed to the clear separation of irradiated samples from the untreated controls. These metabolites could serve as biomarkers for radiation-induced sterility.

In total, 58 differential metabolites were tentatively identified, including 30 compounds in the lipophilic phase and 28 compounds in the polar phase. These tentatively identified differential compounds were assigned to common metabolic pathways according to the Kyoto Encyclopedia of Genes and Genomes public pathway-related database. The amounts of metabolite in regular and irradiated samples were compared (Fig. 3). The results showed that the metabolome of *M. alternatus* was highly disturbed by radiation. Six sugars were detected, of which three displayed significant differences between untreated and irradiated testes. Irradiated testes had significantly higher relative levels of glucose (the primary substrate for glycolysis) and galactose, whereas untreated testes had higher levels of xylose. However, the level of fructose, sucrose, and mannose in testes was not significantly altered by radiation treatment. Furthermore, concentrations of the tricarboxylic acid (TCA) metabolites malic acid and fumaric acid were also significantly higher in irradiated testes. Several saturated fatty acids (palmitic acid, stearic acid, eicosanoic acid, and behenic acid) and monounsaturated fatty acids (palmitelaidic acid and oleic acid) accumulated in higher concentrations in irradiated samples, but the polyunsaturated fatty acids linoleic acid and eicosatrienoic acid were significantly reduced. The present investigation also revealed the general disturbance of the amino acid pool in irradiated testes. Among the 16 detected
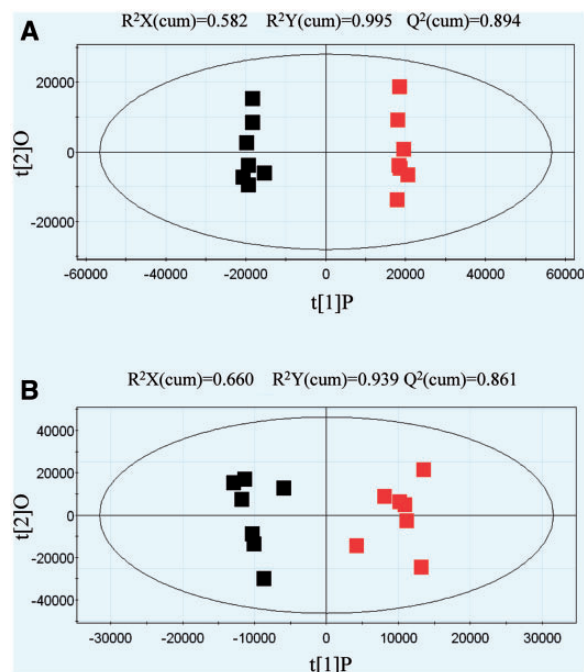

Fig. 1. The score plots for the OPLS-DA model of lipophilic (A) and polar (B) profiles for untreated and irradiated samples. The ellipses represent the Hotelling T^2 with 95% confidence. The black dots represent untreated samples, and the red dots represent irradiated samples.
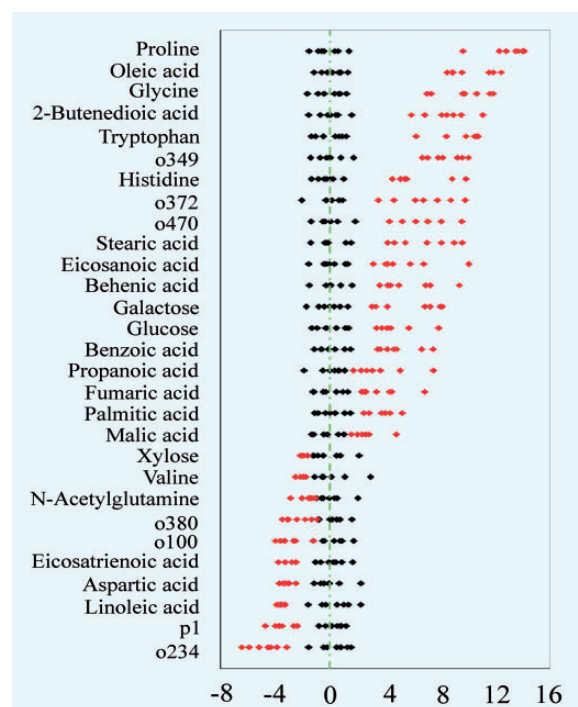

Fig. 2. z-score plot of the top 19 differential metabolites that have higher relative levels in irradiated samples (upper) and 10 differential metabolites that have higher relative levels in untreated samples (lower). Each point represents one metabolite in one sample, colored by phase type (black = untreated samples; red = irradiated samples). o349 (retension time [RT] = 1,114.6 s), o348 (RT = 111.5 s), o470 (RT = 1,814 s), o380 (RT = 1,244.7 s), o100 (RT = 518.3 s), p1 (RT = 319.6 s), and o234 (RT = 778.4 s) were unidentified compounds. The starting letters of the index "o" and "p" signify the unknown metabolites from the organic (lipophilic) and polar phase, respectively.
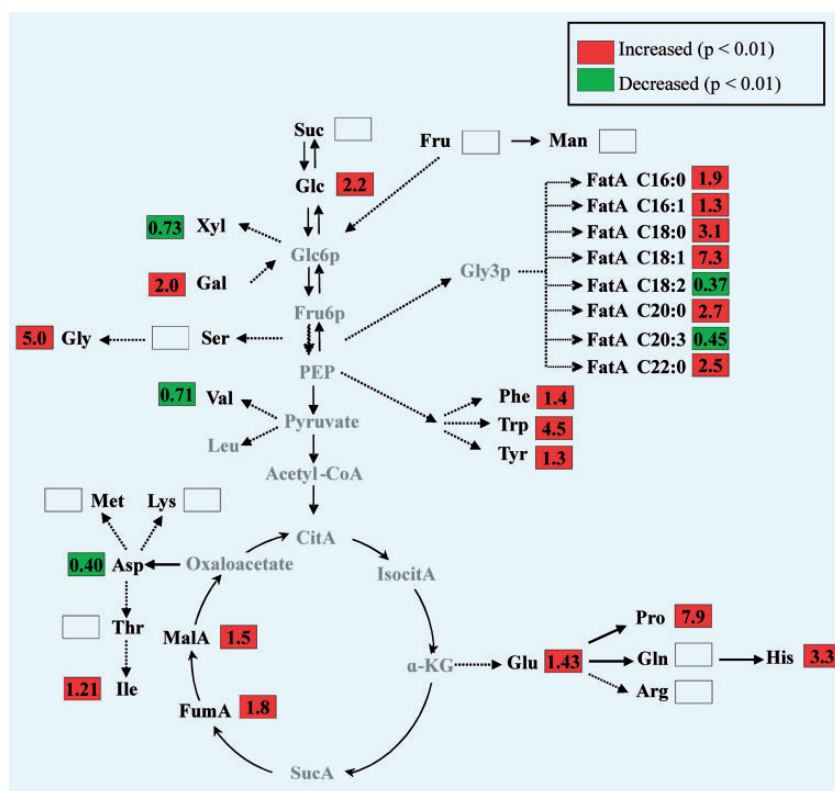
Fig. 3. Levels of metabolites in the main metabolic maps. The comparisons of metabolite contents were calculated by dividing the metabolite levels in irradiated testes by those in untreated testes. Metabolites with peak intensity ratios that were significantly different ($P < 0.01$) between the two groups were labeled either red or green. Extended metabolites were colored gray. α-KG, alpha-ketoglutarate; Arg, arginine; Asp, aspartic acid; Ala, alanine; CitA, citrate; FatA C16:0, palmitic acid; FatA C16:1, palmitoleic acid; FatA C18:0, stearic acid; FatA C18:1, oleic acid; FatA C18:2, linoleic acid; FatA C20:0, eicosanoic acid; FatA C20:3, eicosatrienoic acid; FatA C22:0, behenic acid; Fru, fructose; Fru6P, fructose 6-phosphate; FumA, fumaric acid; Gal, galactose; Glc, glucose; Glc6P, glucose-6-phosphate; Gln, glutamine; Glu, glutamate; Gly, glycine; Gly3P, glycerol-3-phosphate; His, histidine; Ile, isoleucine; IsocitA, isocitric acid; Leu, leucine; Lys, lysine; MalA, malic acid; Man, mannose; Met, methionine; PEP, phosyphoenol-pyruvate; Phe, phenylalanine; Pro, proline; Ser, serine; Suc, sucrose; SucA, succinate; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; Val, valine; and Xyl, xylose.

amino acids, 10 amino acids showed a significant difference. Most of the differential amino acids were more abundant in irradiated samples, with the exception of valine and aspartic acid, which were reduced (Fig. 3).

Discussion

Sterilized male insects are often used to control pest populations through RISIT. It is well known that the basic mechanism underlying radiation-induced sterility is dominant lethal DNA damage in reproductive cells (Robinson 2002, Dyck et al. 2005, Kumano et al. 2009). In this study, a metabolomic analysis was carried out across untreated and 40-Gy-treated male *M. alternatus* to get more information about the mechanism of RISIT. The results showed that the primary metabolic changes include metabolites involved in glycolysis and the TCA cycle, free fatty acids, and amino acids. This most likely indicates a lower glycolytic rate.

In this study, it was found that the glycolyzable substrates (glucose and galactose) and some TCA cycle intermediates (malic acid and fumaric acid) were significantly elevated in the irradiated samples compared with untreated controls. Generally, an increase in metabolites could translate into upregulation of the pathway, but it could also result from reduced utilization of these compounds. Ongoing research of Qu et al. has shown that the expression of glyceraldehyde-3-phosphate dehydrogenase, a key enzyme in glycolysis, and malate dehydrogenase, citrate synthase, and succinate–ubiquinone reductase, key enzymes in TCA cycle were clearly lower in irradiated males, probably resulting

from the radiation-induced damages. Furthermore, a large group of differentially expressed genes involved in embryo and postembryonic development were consistently expressed lower in treated males. Many of these genes were also involved in glycolysis and TCA cycle (L.J.Q. et al., unpublished data). This most likely indicates a lower glycolytic rate in treated males. Glycolysis and TCA cycle are main pathways for ATP production in insect and play an important role in embryo development (Chen 1966, Werner et al. 1999, Fernie et al. 2004, Nazaret et al. 2009). Based on the aforementioned information, it was speculated that radiation-damaged genes regulated glycolysis and TCA cycle. This damage might severely restrain hatch of fertilized eggs produced by irradiated males and regular females, although it did not have a significant influence on longevity and mating propensity of the former.

In this study, fatty acid and amino acid disorders were observed in irradiated males. In insect, the catabolism of sugars, fats, and proteins is converged on the TCA cycle because glucose, fatty acids, and most amino acids are metabolized to acetyl-CoA or intermediates of the cycle (Chino and Gilbert 1965, Osanai et al. 1987). TCA cycle also has a central role in gluconeogenesis, lipogenesis, and interconversion of amino acids (Mayes 1996). It was possible that the disturbance in fatty and amino acids was a result of TCA cycle disruption. Fatty acids, also as an energy source, are an integral part of cell membranes (Canavoso et al. 2001). Cell membrane fluidity, which is dependent on the lipid and fatty acid composition, is vital for normal cell functions (Werner and Simmons 2008). Amino acids play central roles both as building blocks of proteins and as intermediates in metabolism in insect. They

also perform critical biological roles including neurotransmitters, transport, and in synthesis. Disorders in these two groups of metabolites might contribute to the abort of fertilized eggs formed by irradiated males and regular females.

In summary, the metabolomic approach was able to identify characteristic changes in the metabolic signatures of irradiated male *M. alternates*. The present findings will contribute to our knowledge of the characteristic metabolic changes associated with irradiation sterility and understand the molecular mechanisms underlying RISIT.

Acknowledgments

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