# Single-Embryo Metabolomics and Systematic Prediction of Developmental Stage in Zebrafish[§]

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Metabolites, the end products of gene expression in living organisms, are tightly correlated with an organism's development and growth. Thus, metabolic profiling is a potentially important tool for understanding the events that have occurred in cells, tissues, and individual organisms. Here, we present a method for predicting the developmental stage of zebrafish embryos using novel metabolomic non-target fingerprints of "single-embryos". With this method, we observed the rate of development at different temperatures. Our results suggest that this method allows us to analyse the condition, or distinguish the genotype, of single-embryos before expression of their ultimate phenotype.

*Key words:* Single-Embryo, Embryogenesis, Metabolomics

## Introduction

Early development is a dynamic process in higher organisms. A single fertilized egg gives rise to complex, standardized tissues, organs, and body plan. The genome sequence of several organisms has been determined, and the functions of individual genes associated with embryogenesis have been demonstrated in many studies (Stanford *et al*., 2001). The systematic analyses of the transcriptome and the proteome have been instrumental in understanding various developmental processes in zebrafish embryos (Mathavan *et al*., 2005; Link *et al*., 2006). However the mechanisms of early development are not yet completely understood.

Metabolomics can be understood as a comprehensive, simultaneous, and systematic determination of metabolite levels in cells, tissues, and/or organisms (Oliver *et al*., 1998; Nicholson *et al*., 1999; Lindon *et al*., 2007). The metabolome represents the final downstream product of gene expression (Dunn *et al*., 2005; Maharjan and Ferenci, 2005; Kell, 2006), thus the change in the metabolome could be regarded as the ultimate response of biological systems (Fiehn, 2002). Transcriptomics and proteomics are both considered to be intermediates in the flow of genetic information. In contrast, metabolomics should be thought of as being more proximate to the phenotype (Raamsdonk *et al*., 2001; Fukusaki and Kobayashi, 2005) and expressed at various stages after transcriptional control of mRNA, posttranslational modification of proteins, localization of gene products, and so on. Metabolomics might be the sole technology capable of detecting complex, biologically essential changes.

Zebrafish (*Danio rerio*) is a common and powerful model organism used to understand vertebrate embryogenesis and organ development (Hsu *et al*., 2007). Metabolites are known to play an important role in embryogenesis, and they enhance our understanding of this dynamic

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process. For example, analyses of the glucose and lipid metabolism revealed that alteration of these metabolites affect the embryonic development and/or cell fate determination (Miyazaki *et al*., 2005; Maden, 2007; Barceló-Fimbres and Seidel Jr., 2007; Dumollard *et al*., 2007). In our previous study, the metabolome of fertilized eggs of zebrafish was profiled using gas chromatography/time-of-flight mass spectrometry (GC/TOF-MS) (Hayashi *et al*., 2009). The metabolic profiles of other model organisms, including medaka fish (Viant, 2003) and *Caenorhabditis elegans* (Blaise *et al*., 2009), have been analysed using nuclear magnetic resonance (NMR). While a metabolomics approach has been applied to the analysis of early development, the established methods require many embryos, and the metabolome data from heterogeneous samples of embryos are likely to be ambiguous and have serious limitations. For example, embryonic lethal mutations are maintained in the heterozygote state because homozygous mutants are not viable; therefore, investigations of homozygous embryos depend on mating between heterozygous parents. Only 25% of the embryos are recessive lethal mutants, and hence each embryo must be separated to confirm the genotype and analyse its effect on the phenotype. Consequently, the metabolomic methods that require multiple embryos are difficult to use especially in the analysis of embryonic lethal mutants. In the present study, we developed a new method for "single-embryo" metabolomics.

For this purpose, we present a novel system of microanalysis and non-target profiling developed using ultra-performance liquid chromatography/TOF-MS (UPLC/TOF-MS) analysis of single zebrafish embryos. The data matrices, with the peak index as the independent variable and relative intensity as the dependent variable, were analysed using partial least square by means of projection to latent structure (PLS) regression to predict the developmental stage (Ammann *et al*., 2006) in order to compare our data with previous results of Hayashi *et al.* (2009) and to validate our metabolome data (Fig. 1). The developmental stage could be expressed as a regression value when this model was applied to fertilized eggs reared at different temperatures affecting the growth rate. Furthermore, application of this methodology could reveal a turning point, around 4–5 hours post fertilization (hpf), in metabolism correlated with maternal effects. This novel "single-embryo metabolomics system" represents a powerful tool for the elucidation of mechanisms important to early vertebrate development.

## Material and Methods

### *Maintenance of zebrafish*

*Danio rerio* adults were obtained from a local pet shop and maintained at (28.0 ± 0.5) °C with a 14-h/10-h light/dark cycle. They were fed a commercially available artificial diet (TetraMin™ flakes; Tetra, Melle, Germany) twice daily.

### *Embryo collection*

Fertilized eggs were collected immediately after spawning, and the embryos were washed several times in system water, maintained at 28.5 °C in the same water, and staged according to the standard morphological criteria. Embryos were collected at 21 time points (2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 10.5, 11, 11.5, and 12 hpf), immediately frozen using liquid nitrogen, and stored at −80 °C. The 21 time points were divided into the following developmental stages: cleavage period (2 hpf), blastula period (2.5–5 hpf), gastrula period (5.5–10 hpf), and segmentation period (10.5–12 hpf). To investigate growth rates at different rearing temperatures, embryos maintained at 25, 28.5, and 33 °C were collected at 7 time points (1, 2, 3, 4, 5, 6, and 7 hpf) and analysed using single-embryo UPLC/TOF-MS. The sample number of each point was 4–5.

### *Sample preparation*

The individual embryos stored at −80 °C were homogenized (20 Hz, 1 min) with MM 301 mixer mills (Retsch, Haan, Germany). Then, two prepared solutions, 64 $\mu$l of 80% aqueous methanol solution and 16 $\mu$l of 50% aqueous methanol solution containing 50 $\mu$mol of naringin as an internal standard, were added to the embryos, and the mixtures were homogenized under the same conditions. The homogenate was shaken for 30 min at 37 °C and centrifuged at 16,000 × *g* for 3 min at 4 °C. All supernatants were filtered by a 0.2-$\mu$m PTFE filter to generate samples for analysis.

### *UPLC/TOF-MS analysis*

Liquid chromatographic separations were performed on a 50 × 2.1 mm ACQUITY™ 1.7 $\mu$m C18 column (Waters, Milford, USA) using an

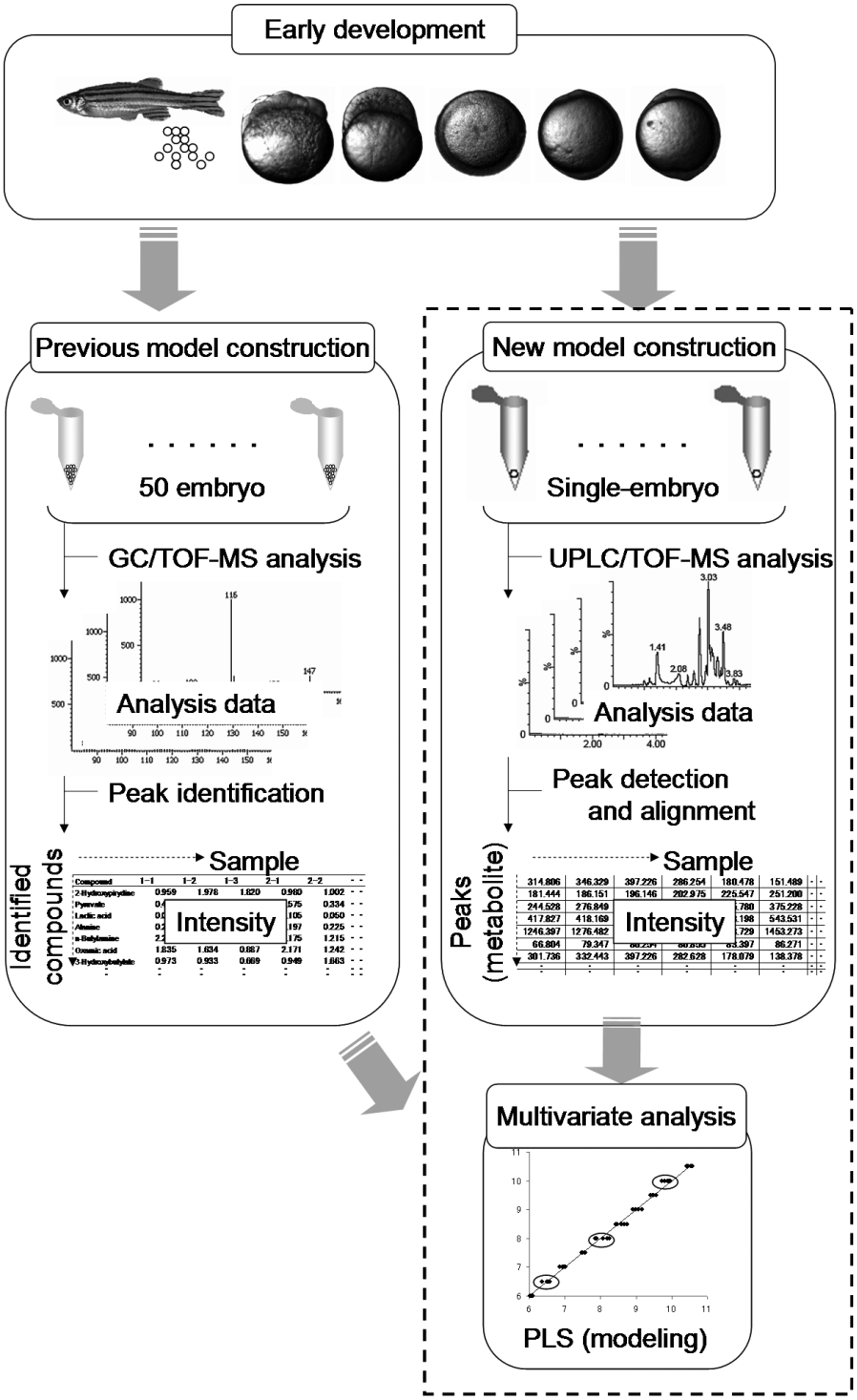


Fig. 1. Schematic diagram of our method developed to predict the developmental stages of zebrafish using single embryos. Here, we profiled the non-target metabolites for each developmental stage using UPLC/TOF-MS, as described in the text.

ACQUITY™ ultra-performance liquid chromatography (UPLC) system (Waters) coupled with a Micromass LCT Premier™ (Waters) orthogonal acceleration time-of-flight mass spectrometer operating in V mode and both positive and negative ion mode. The column was maintained at 40 °C and eluted under gradient conditions at a flow rate of 0.3 ml $min^{-1}$; mobile phase component A consisted of 0.1% aqueous formic acid and B consisted of 0.1% formic acid in acetonitrile. The composition was linearly increased from 0 to 100% B over 7 min, then maintained at 100% B over the next 7 min, returned to 100% A over 0.1 min. The column was re-equilibrated over a final 7 min prior to injection of the next sample. The nebulization gas was set to 500 l $h^{-1}$ at a temperature of 300 °C, the cone gas was set to 50 l $h^{-1}$, and the source temperature was set to 120 °C. The capillary voltage and the cone voltage were set to 3000 and 50 V in the positive ion mode and 2700 and 35 V in the negative ion mode, respectively. All analyses were acquired using lock spray to ensure accuracy and reproducibility. The flow rate of leucine-enkephalin solution (2 $\mu$g $ml^{-1}$) for lock spray was 5 $\mu$l $min^{-1}$.

### *Data processing and multivariate analysis*

Raw chromatographic data were converted into ANDI files (analytical data interchange protocol, *.cdf), and these data were subject to peak detection and alignment, and filtered of gap using MZmine software (Katajamaa *et al.*, 2006; Katajamaa and Oresic, 2007). Concerning peak detection parameters in positive ion mode, the *m/z* bin size was set to 0.05, the chromatographic threshold level was set to 0, the noise level was set to 5, the minimum peak height was set to 150, the minimum peak duration was 4.0, the tolerance for *m/z* variation was set to 0.05, and the tolerance for intensity variation was set to 50%. All parameter settings for the negative ion mode were the same except that the minimum peak height was set to 100. For fast aligner, the balance between *m/z* and retention time ($t_R$) was set to 10.0, *m/z* tolerance size was set to 0.05, and the $t_R$ tolerance size (absolute) was set to 4. The data normalization selected total raw signals. Projections to latent structures by means of partial least square, PLS (SIMCA-P version 11.5, Umetrics, Umeå, Sweden), were then chosen to create a prediction model (Fig. 1).

## Results

### *Prediction of developmental stage based on single-embryo metabolomics*

Previous analyses of metabolites in early zebrafish embryos have used GC/MS and NMR spectroscopy (Hayashi *et al.*, 2009; Viant, 2003). In order to develop a novel single-embryo metabolomics methodology, we employed UPLC/TOF-MS because it is highly sensitive, extracts a large volume of information from an exact mass, and has a wide dynamic range. Individual zebrafish embryos from twenty one different developmental stages (2 to 12 hpf taken at intervals of 30 min) were analysed using electrospray ionization (ESI) in both positive and negative ion modes. The collected data were aligned and normalized using MZmine software (Katajamaa *et al.*, 2006). In order to confirm this non-target profiling data, the data were analysed by projections to latent structures by means of partial least square (PLS) regression, one of the most reliable methods for the regression of multivariate data (Figs. 2A, B). We could construct regression models with high linearity because the $R^2$ values were over 0.99 and the root mean square errors (RMSEE) were superior to those of our previous model (Hayashi *et al.*, 2009). For cross-validation, we used a test data set from 3-, 6-, and 9-hpf embryos. The PLS results for the training set revealed high linearity without the test set, and the test set fitted perfectly into the predicted regression line (Figs. 2C, D). Furthermore, there was no difference between RMSEE and the root mean square error of predictions (RMSEP) (Table I). There was only a small influence of ionization mode, and the model using the negative ion mode was slightly superior to the model using the positive ion mode (Table I). It was significant that the noise peaks were less frequent and lower in the negative ionization mode. The accuracy and reproducibility of the analysis data were checked and calibrated by lock mass spray, so that the variability was mainly biological reproducibility. These results showed that our models are accurate and sensitive enough to predict the developmental stage based on metabolomics using a single zebrafish embryo.

### *Application of prediction model under different growth rate conditions*

We developed a single-embryo metabolomics approach and validated it with the construction

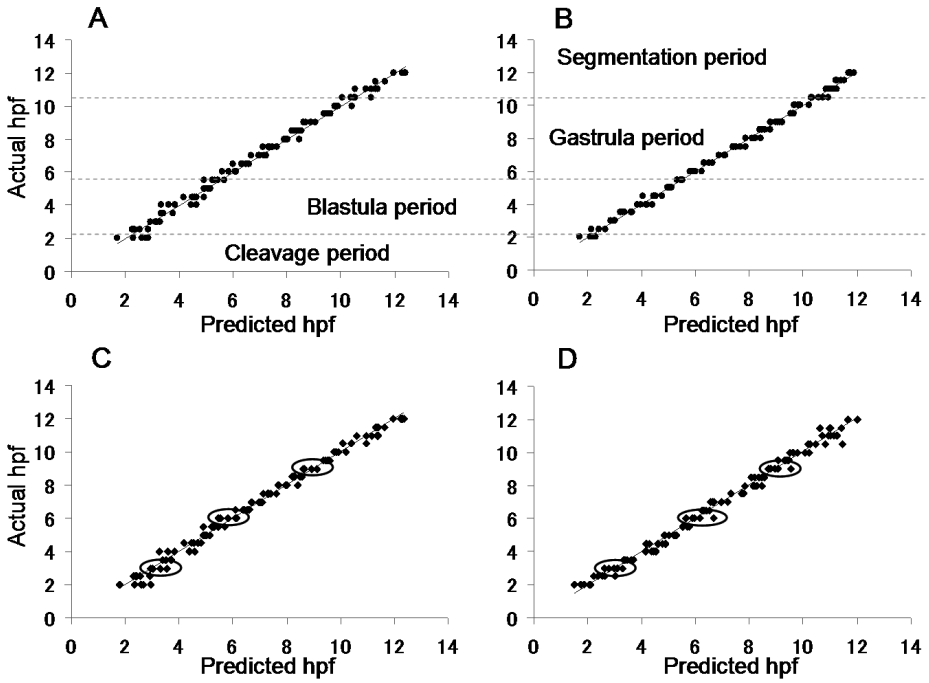


Fig. 2. A model constructed using partial least square (PLS) regression reveals a good correlation between the composition of the metabolome at a particular time and actual embryogenesis. Each dotted line (A and B) shows a distribution of developmental stages. (A) The model was constructed using the entire data set analysed in the positive ion mode and demonstrates an obvious correlation between the developmental stages and the metabolomes. (B) The model was constructed using the entire data set analysed in the negative ion mode and demonstrates an obvious correlation between the developmental stages and the metabolomes. (C) Cross-validation of the model using the data analysed by the positive ion mode. The test set (3-, 6-, and 9-hpf stages) was fitted onto the prediction model constructed using the training set. Circles correspond to the data for the 3-, 6-, and 9-hpf stages. (D) Cross-validation of the model using the data analysed by the negative ion mode. The test set (3-, 6-, and 9-hpf stages) was fitted onto the prediction model constructed using the training set. Circles correspond to the data for the 3-, 6-, and 9-hpf stages.

Table I. Validated prediction model using partial least square (PLS) regression.

| Matrix | Latent factors | $R^2$ | RMSEE | RMSEP | No. of peaks |
|---|---|---|---|---|---|
| *Positive ion mode* | | | | | 1875 |
| Entire data | 3 | 0.991 | 0.288 | - | |
| Cross-validation | 3 | 0.992 | 0.278 | 0.304 | |
| *Negative ion mode* | | | | | 1507 |
| Entire data | 3 | 0.997 | 0.184 | - | |
| Cross-validation | 3 | 0.991 | 0.283 | 0.342 | |
| Cultivated at 33 °C | 3 | 0.992 | 0.180 | - | 708 |

RMSEE and RMSEP are root mean square errors of the fit for observations using the training set and of the fit of the prediction for observations using the test set, respectively. Test sets were 3-, 6-, and 9-hpf samples, and the other samples were included in the training set.

of a model that predicts developmental stages under standard rearing conditions. Next, we analysed and incorporated single-embryo data from embryos maintained under different rearing temperatures that are known to change the rate of embryonic development (Kimmel *et al.*, 1995). Embryos were reared at 25, 28.5, and 33 °C; the developmental stages of 7-hpf embryos (Fig. 3) corresponded to 50% epiboly, 60% epiboly, and 90% epiboly, respectively, consistent with the

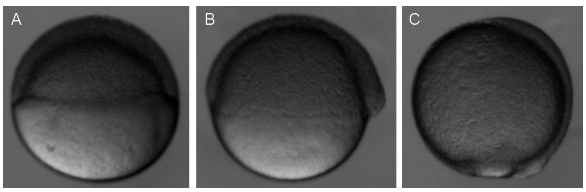


Fig. 3. Micrographs of 7-hpf embryos reared at (A) 25 °C, (B) 28.5 °C, and (C) 33 °C. The zygote is about 0.7 mm in diameter.

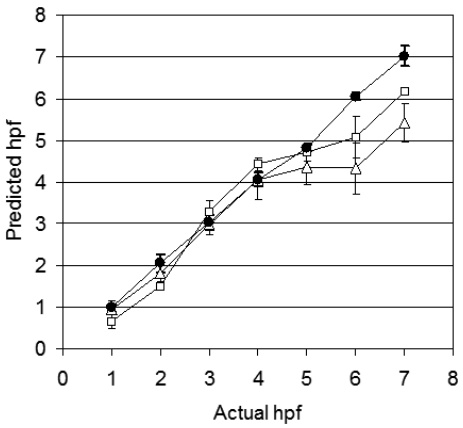


Fig. 4. The PLS regression of the embryogenesis of fish reared under different temperature conditions. The prediction model was constructed using data from embryos reared at 33 °C (black circles), and the data from embryos reared at 25 °C (open triangles) and 28.5 °C (open squares) were applied to this model.

previous report by Kimmel *et al*. (1995). In order to construct a prediction model, we used the single-embryo method and data from animals reared at 33 °C because the accelerated growth rate allowed the model to have the widest range of projection. The model using seven different developmental stages, from 1 to 7 hpf at intervals of 1 h, is summarized in Table I. The precision of this model was almost the same as that of the above-mentioned models. The data from embryos reared at 25 and 28.5 °C were applied to the model using the embryos reared at 33 °C, so that the projection values were approximately proportional to the developmental stages (Fig. 4). Interestingly, there were no significant differences in the metabolomes of embryos reared at 25 and 28.5 °C until 4 and 5 hpf, thereand after, the metabolome of early embryo was influenced by the rearing temperature. These results demonstrate that the prediction model using metabolic fingerprinting was suitable for evaluating developmental stages under various rearing conditions.

## Discussion

We developed a metabolomics methodology using single zebrafish embryos. This approach will benefit the research on embryogenesis, especially research using homozygous recessive lethal and sterile mutants that cannot grow and/or propagate. These mutations must be maintained in heterozygous animals, and when the homozygous recessive phenotypes are needed, the heterozygotes are mated. Consequently, embryos are produced in standard 3-to-1 Mendelian ratios, and homozygous recessive individuals must be identified and separated for analysis. Hence, metabolomics approaches analysing multiple embryos are difficult to adapt to these types of mutants. On the other hand, if we are to investigate the mechanism of action of the mutated genes in development, homozygous recessive mutant embryos should be investigated before, during, and/or after their phenotypic effects are evident. Conventionally, the related genes of mutation were monitored to elucidate the effects. In these cases, the single-embryo metabolomics methodology will be highly effective. Zebrafish embryos have been studied using not only mutations but also drugs and toxins; therefore, the single-embryo metabolomics approach might be useful in detecting individual errors and sensitivity. Moreover, understanding the differences between individual embryos may elucidate new mechanism of embryogenesis.

We were able to construct highly accurate PLS models of developmental stages using single-embryo metabolic non-target fingerprinting. The data matrices for the prediction models were obtained from UPLC/TOF-MS data processed by MZmine software, which had a large volume of information from an exact mass. The detected peak numbers, as shown in Table I, were actually lower by a few hundreds, because those contained the isotope peaks and the adduct ion formations etc.

Previously, we had detected 63 compounds when the 50 zebrafish embryos were profiled using GC/TOF-MS data (Hayashi *et al*., 2009). Therefore, the method in this report had a high sensitivity and provided a large amount of information.

The prediction model expressed the developmental stages under standard rearing conditions, and it was necessary to apply different rearing conditions to demonstrate the applicability and versatility of this single-embryo metabolomics approach. In the previous study, rates of zebrafish embryonic development changed when embryos were incubated at different temperatures (Kimmel *et al.*, 1995). Therefore, we attempted to apply the data from embryos reared at 25 and 28.5 °C to the model constructed using data from embryos reared at 33 °C. Regressions of the developmental stages were successful, demonstrating that the model was applicable to data from embryos reared under different conditions. Furthermore, the effect of the rearing temperatures on the metabolome clearly appeared around 4–5 hpf. Before 4 hpf, there was no obvious effect of the temperature on the metabolome even though the phenotype of developing embryos is clearly affected by the rearing temperature. The earliest stages of embryonic development in zebrafish rely predominately on maternal gene products generated during oogenesis and supplied to the egg. The mid-blastula transition (MBT) marks the onset of zygotic transcription. Accordingly, the finding that growth temperature begins to affect the metabolome only after MBT suggests two possibilities. First, the expression of maternal mRNA prior to the MBT period under different temperature conditions may barely affect the metabolome, contrary to a previous report (Kimmel *et al.*, 1995) that the growth temperatures affect the developmental stage proportionally. Alternatively, only zygotic gene expressions and/or translation are influenced by the growth temperature. There are no reports on transcriptomics and proteomics research around MBT under different temperature conditions. The metabolomics approach presented here used non-target profiling data constituted of compound information. Therefore, the metabolite target analysis, defined as quantitative analysis of one or several metabolites related to a specific metabolic reaction (Ellis *et al.*, 2007), is possible. If the $MS^n$ spectrum data were obtained by triple quadrupole (QqQ) and quadrupole time-of-flight (Q-TOF) mass spectrometry, specific compounds could be identified, and we might determine how growth temperature controls early embryogenesis.

The metabolomics approach will enable the identification of the developmental stage of embryos exhibiting no visible defects and clarification of novel aspects of known mutants, drug treatments, and other experimental manipulations. The single-embryo metabolomics approaches represent a powerful tool for elucidation of mechanisms during early development.

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