




# Single feature polymorphisms between two rice cultivars detected using a median polish method

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**Abstract** Expression levels measured in microarrays of probes and the targets had perfectly matched sequences. It has been shown that differences in minimum free energies of RNA secondary structure, may partly explain the SFPs polymorphisms (SFPs). Although there have been as detected. These SFPs may facilitate gene discovery in increasing interests in this approach, there is still need for future studies.

improving the algorithm in order to achieve high sensitivity and specificity especially with complex genome and large datasets, while maintaining optimal computational performance. We obtained microarray datasets for expression profiles of two rice cultivars and adapted a median polish method to detect SFPs. The analysis identified 6,655 SFPs between two the rice varieties representing 3,131 rice unique genes. We showed that the median polish method has the advantage of avoiding fitting complex linear models thus can be used to analyze complex transcriptome datasets like the ones in this study. The method is also superior in sensitivity, accuracy and computing time during molecular hybridization comparing to probes with requirement compared with two previously used methods. A comparison with data from a resequencing project indicated that 75.6% of the SFPs had SNP supports in the probe regions. Further comparison revealed that SNPs immediately flanking the probes also had contributions to the detection of SFPs in cases where

Short DNA oligonucleotides are commonly used as probes to interrogate nucleic acid targets in microarray analyses. These oligonucleotide probes, as short as 25 bases, are used in one of the most widely used microarray technology, Affymetrix GeneChip microarray. The short probes are generally more sensitive to mismatch bases to the targets than longer sequences. It is known that a mismatch base within the probe sequence could reduce the binding affinity between targets and probes (Borevitz et al. 2003, 2007; Ronald et al. 2005; Zhu and Salmeron 2007). The feature about the high sensitivity to sequence variations of the short oligonucleotide probes has two

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implications in the microarray analysis: (1) in order to accurately measure transcript abundance, perfect match is essential between probes and targets and between targets from different samples within the probe; (2) if target abundance is presumed to be at similar level in different samples, difference in hybridization signals measured by the same probe sequence could be used to infer sequence mismatch between targets from different samples. The latter has been developed into an approach to quickly identify genetic variations, referred to as single feature polymorphisms (SFPs), by genomic DNA hybridization

with microarrays in a number of species, including yeast probe sequences (Luo et al. 2007; Ronald et al. 2005). *Arabidopsis* and rice (Borevitz et al. 2003; Kumar et al. 2007; Winzeler et al. 1998). SFPs have also been detected in genomes with larger size and higher complexity from data from a single organism under study, the efficiency labeled RNA transcript derivatives (Cui et al. 2005; Das et al. 2008; Luo et al. 2007; Rostoks et al. 2005). Since oligonucleotide microarrays are composed of millions of probes representing most of the expressed unique sequences in the genome, SFPs can capture large number of sequence variations. Thus chip-based SFP detection can serve as high-throughput platforms for genetic analyses providing thousands of markers in a single experiment (Das et al. 2008; Ronald et al. 2005), which is much more efficient than other molecular markers such as RFLPs and SSRs that have been widely used in genetic analyses. SFP markers have been used for high-resolution genetic mapping (West et al. 2006), and for studying expression quantitative trait loci (eQTLs) (Luo et al. 2007; Potokina et al. 2008; West et al. 2006, 2007).

SFPs have been generally regarded as resulting from mismatched bases between the targets and probes. However, results from previous studies indicated that SFPs can also be repeatedly detected even though the targets and the probes have perfectly matched sequences (Borevitz et al. 2007; Luo et al. 2007; Zhu and Salmeron 2007). Such results have frequently been ignored in the analyses thus the cause has not been investigated. A possible cause for the SFPs resulting from perfectly matched probe-target sequences might be the influence of polymorphisms, mostly SNPs, between the targets in the sequences flanking the probes (probe flanking SNPs or flanking SNPs).

A highly efficient algorithm is critical for sensitivity and specificity of SFP detection, while maintaining optimal computational performance, especially with complex genome and large data sets. Several algorithms have been developed to detect SFPs from RNA derived microarray data (Cui et al. 2005; Luo et al. 2007; Ronald et al. 2005; Rostoks et al. 2005). These algorithms have to reduce the perturbation of hybridization signals from different transcript abundance rather than genetic variations to ensure sufficient detection power and accuracy. For example, Rostoks et al. provided a mean-based analysis of variance (ANOVA) method employing a complex linear model for the specific experimental designs, which successfully discriminated the differential hybridization signals induced by different tissues and genotypes (Rostoks et al. 2005). Ronald et al. inferred SFPs by comparing the observed hybridization signal with the expected using the position-dependent nearest-neighbour (PDNN) model which needs to estimate 82 energy parameters for specific RNA-DNA binding relying on information of Affymetrix standard protocols. Six replicates of labeled

RNA targets, representing three biological replicates and two technical replicates, were hybridized to the Rice GeneChip Genome Array (<http://www.affymetrix.com/products/arrays/speciPc/rice.affx>). This array contains 57,381 probe sets representing 51,279 transcripts from *japonica* and *indica*. Each probe set consists of 11 pairs of 25-mer perfect match (PM) and mismatch (MM) probes which differ only at the middle base.

#### Additional microarray data

In addition to the rice microarray data generated from this study, microarray data used for yeast allelic specific expression analysis (Ronald et al. 2005) and for barley SFP detection (Rostoks et al. 2005) were also used for the analysis. Yeast data generated by Affymetrix YGS98 GeneChip array were downloaded from NCBI Gene Expression Omnibus (GEO) (acc: GSE1975) (Barrett et al. 2007) and barley data generated by Affymetrix Barley1 GeneChip array can be downloaded from author's website (<http://naturalsystems.uchicago.edu/naturalvariation/barley/>).

#### Data processing and SFP detection

The CEL files containing raw intensity data for each probe were read into a R program (Ihaka and Gentleman 1996). Background correction and quantile normalization were performed using RMA methods in Bioconductor affy package (Gautier et al. 2004; Gentleman et al. 2004). The gene expression presence/absence detection (absolute calls) was calculated from the CEL files using the Microarray Analysis Suite (MAS 5.0, Affymetrix). Only probes from probe sets with "Present" value in the absolute calls at least four out of total six arrays of each sample were selected for further analysis. Matrices containing the background corrected and normalized data with "Present" calls by combining the information from the above processing procedures were subjected to median polish analysis to extract the residuals. The limma package (Smyth 2004) was then used to determine SFPs by a selected threshold based on Benjamini and Hochberg (BH) adjusted *P* values. The custom script and microarray data can be downloaded in our website (<http://www.ncpgr.cn/supplements/sfp/>).

The method of fitting linear model proposed by Rostoks et al. (2005) was also used for comparing the efficiency of SFP detection. Probes in a set were fit with the following linear model for rice and barley data:

$$\log(Y_{igrp}) = \mu + t + g + g \times t + p + \varepsilon$$

where *Y* is the background corrected normalized intensity of *t* (tissue), *g* (genotype), *r* (replicate), and *φ* (probe) in a

#### Rice probe sequence mapping

Sequences of 631,066 PM probes on the rice genome array downloaded from Affymetrix website were aligned against the pseudo-chromosomes of TIGR rice annotation version 5.0 using BLAT (Kent 2002) with parameters of minIdentity = 100, minMatch = 1 and stepSize = 5. Only probes with unique location to the rice genome were used to SFP confirmation and SNP analysis.

#### Rice and yeast SFP confirmation by SNP analysis in silico

Among 257,691 SNPs in OryzaSNP project (<http://www.oryzasnp.org/>), a total of 37,730 high-quality SNPs between Zhenshan 97 and Minhui63 were mapped to rice PM probes based on sequence identity and chromosomal location. The corresponding SNPs to SFP probes were identified as true detection positives. In addition, 100 Mb pseudo-sequences were downloaded from the same source and used to identify probes that are monomorphic between the two varieties.

To validate yeast SFPs, the whole genome shotgun sequences of *Saccharomyces cerevisiae* RM11-1a (RM) were downloaded from NCBI Nucleotide database by searching "txid285006[Organism:noexp]". Probe sequence alignments and polymorphisms were obtained by BLASTing (Altschul et al. 1990) the YG-S98 exemplar sequences from Affymetrix against the RM sequences using a custom perl script.

#### Calculation of minimum free energy of cRNA

The 75-bp reverse complementary sequences of BY and RM centered by each probe were extracted as cRNA sequences. UNAFold packages (Markham and Zuker 2005; Walter et al. 1994) were downloaded from author's website (<http://dinamelt.bioinfo.rpi.edu/>). The minimum RNA free energies of each extracted sequences were then calculated using "hybrid-ss-min" command in UNAFold packages with parameters "E -n RNA -t 45 -T 45" and the differences of free energies between the two genotypes were compared.

## Results

## SFP detection using median polish using rice data

## Transcriptome proPLing data of two indica rice cultivars

Zhenshan 97 and Minghui 63 were collected from panicles at three developmental stages using the rice GeneChip genome array. The median polish method, adapted from Tukey (1977), was used in SFP detection. Thirty-six CEL probe hybridizing to two different genotypes, three developmental stages, and six replicates (three biological replicates, each with two technical replicates) were subjected to the median polish analysis. Only probe sets producing detectable hybridization signal, as indicated by Present calls, were used for SFP detection.

MAS 5.0 (Affymetrix Inc 2001), in at least one developmental stage of both rice cultivars were selected to construct data matrices. A total of 20,220 matrices each representing a positive probe set were constructed using signal intensities of perfect match (PM) probes after background correction and quantile normalization. Probe sets with more or less than 11 probes were removed from the analysis. The residuals from applying median polish were grouped by genotypes, and significant differential residual groups were identified using limma package (Smyth 2004) in the Bioconductor (Gentleman et al. 2004). The custom R scripts and microarray data can be downloaded in our website (see Materials and methods).

Based on the assumption that the observed signal intensity of a PM probe can be decomposed to the corresponding transcript abundance level and a coefficient representing the binding affinity of the probe to the transcripts target with a random error, the following model, proposed previously with demonstrated utility (Cui et al. 2005; Li and Hung Wong 2001), was adopted for our data analysis:

$$S_{gij} = I_{gi} + A_{gij} + \varepsilon_{gij} \quad (1)$$

where  $S_{gij}$  is the log-scaled observed signal intensity of the  $j$ th PM probe in the  $i$ th probe set hybridized to RNA derived from the  $g$ th genotype.  $I_{gi}$  represents the expression index of the transcript,  $A_{gij}$  the binding affinity coefficient of the probe, and  $\varepsilon_{gij}$  the random error. Different samples from the same genotype are considered to have the same  $I_{gi}$  but may differ in  $A_{gij}$ , whereas samples from different genotypes especially ones having sequence polymorphisms within the probe sequence would have different  $A_{gij}$ , thus causing a SFP. For a  $N \times M$  matrix composed of  $N$  samples and  $M$  probes within the  $i$ th probe set, median polish (Tukey 1977) decomposes each probe signal in the matrix to the following components:

$$S_{gij} = T_i + R_{gi} + C_j + E_{gij} \quad (2)$$

where  $T_i$  represents the overall value of the matrix,  $R_{gi}$  the effect of each row,  $C_j$  the effect of each column and  $E_{gij}$  the

residuals. The relation of Eq. 1 and 2 can be expressed as the following:

$$\begin{cases} \hat{I}_{gi} = T_i + R_{gi} \\ \hat{A}_{gij} = C_j + E_{gij} \end{cases} \quad (3)$$

That means  $I_{gi}$  can be estimated by the overall value plus the row effect of median polished data.  $A_{gij}$  is directly proportional to the residual of median polished data. For a probe set representing two genotypes, three developmental stages, and six replicates (three biological replicates, each with two technical replicates) were subjected to the median polish analysis. Only probe sets producing detectable hybridization signal, as indicated by Present calls, were used for SFP detection.

$$\Delta A_{ij} = \hat{A}_{2ij} - \hat{A}_{1ij} = E_{2ij} - E_{1ij} \quad (4)$$

Based on the above assumptions, we can extract the residual effects resulting from the different hybridization affinities of the two genotypes, irrespective of the expression levels and tissues.

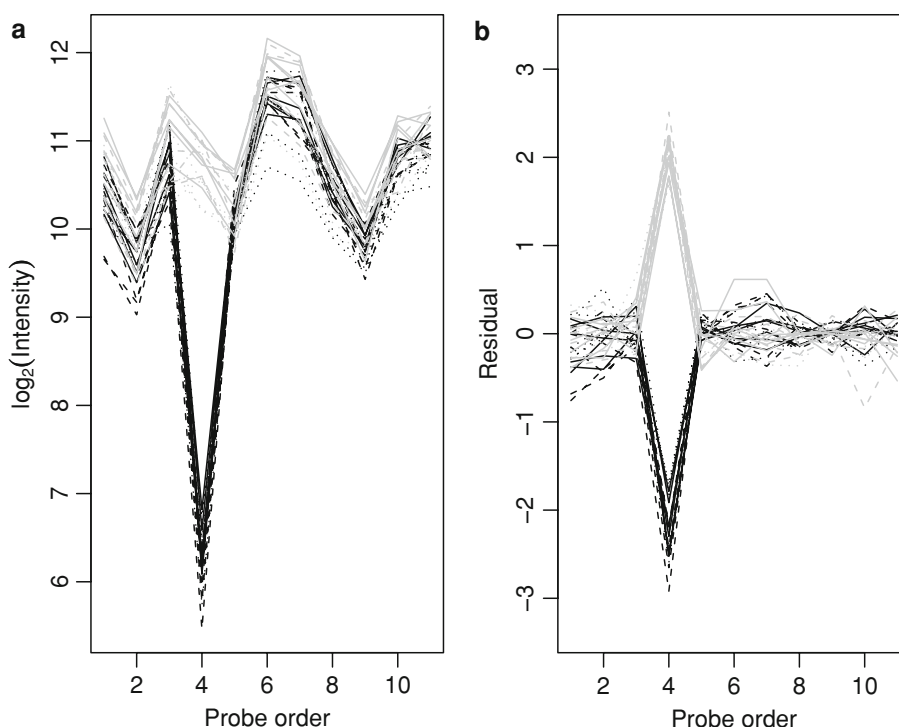
In calculating the residuals for a  $N \times M$  matrix composed of  $N$  samples (genotypes, tissues and replicates) and  $M$  probes, the normalized intensity values in each row were subtracted by the median of the respective row, which eliminates the effects of the expression levels. The residuals in each column of the resulting matrix were then subtracted by the median of the column resulting in a new matrix, which removes the noise from different affinity of the probes. Such calculation was iterated until the sum of the absolute residuals in the matrix becomes stabilized (the sums of two successive iterations differed by 1%). SFPs between the varieties were identified based on the residuals of median polish using the limma package (Smyth 2004), with BH adjusted  $P$  values. An example of the effect obtained by median polish on SFP detection between the two rice cultivars is demonstrated in Fig. 4 using the probe set Os.10369.1.S1\_a\_at.

Using a threshold of BH adjusted  $P \leq 0.001$ , 6,655 SFPs from 3,131 probe sets, representing 15.5% of the total probe sets with Present calls, were identified between the two rice cultivars. Among them, 231 (7.4%) probe sets contained six or more SFPs.

## SFP confirmation by SNP analysis

To evaluate the robustness of the method, detected SFPs were compared to the rice SNPs identified from oryzaSNP resequencing project (McNally et al. 2006). This project produced 100 Mb of unique and low copy rice genomic pseudo-sequences from 20 diverse rice lines, including Minghui 63 and Zhenshan 97, by microarray-based whole genome resequencing technology developed by Perlegen Sciences.

**Fig. 1** Effectiveness of the median polish method in SFP detection from transcript probe data. **a** Normalized probe level signals of a probe set, Os.10369.1.S1\_a\_at. The  $y$ -axis shows the background-corrected normalized  $\log_2$  intensity and the  $x$ -axis is the position of each probe of a probe set along the transcript. *Black lines* represent microarray results from Minghui 63 while *grey* are from Zhenshan 97. *Different line types* represent different tissues. **b** Same as **a** after median polish showing the effect on noise elimination of the probe signals and displaying the differential residuals of median polish at the SFP probe (the fourth probe) in the probe set



Based on the SNPs detected by OryzaSNP project, it was predicted SFPs using data from rice samples collected found that SNPs between these two varieties occurred at a single stage (single tissue), and compared the results to sequences corresponding to a total of 830 probe sets, 430 of which were resolved in our analysis, resulting in a false negative rate of 48.2% (Table 1). We checked the sequences number of 6,655 SFPs as in the previous analysis using for the false negative detections, and found that a SNP in the middle (the 13th base) of the 25-mer probe could be detected in all tissues were detected by at least one single tissue. However, as a SFP with a false negative rate of 26.9%, while a SNP at the edge (the 1st and 25th bases) of the probe could be detected with a false negative rate of 90.2% (see Supplementary data Fig. 1 for the effect of SNP position for SFP detection). Among the 400 false negative SNPs, 191 (47.8%) were located within 5 bases from the ends (1st to 5th and 21st to 25th) of the probe sequences, while only 116 (29.0%) false negative SNPs were located within the 9 (9th to 17th) bases in the middle. Such positional effect of false negative SFP identification is similar to the results from previous studies (Cupress et al. 2005; Ronald et al. 2005; Rostoks et al. 2005).

Sequences for 569 of the 6,655 SFP detecting probes were mapped to the oryzaSNP sequences, 430 (75.6%) of which mapped SFP probes have at least one SNP within the 25-mer probe region between Minghui 63 and Zhenshan 97. The remaining 139 (24.4%) SFPs did not show polymorphism between the two cultivars within the probe region (Table 1).

#### SFP detection in single tissue and multiple tissues

In order to assess the impact of multiple biological samples as replicates on SFP detection using median polish method, we compared the results with other methods. For comparison, we re-analyzed our transcriptome data for SFP identification using the method of fitting linear model proposed by Rostoks et al. (2005). The exact method described previously was used, except SAM which needed large permutation (Tusher et al. 2001) was replaced with the *t*-test (Smyth 2004) to reduce computational demands of SAM and SAM using rice data was found to be highly similar: only 0.8% of the SFPs detected was different. In morphism between the two cultivars within the probe region to make the results comparable, detection threshold was adjusted to obtain a consistent number of 6,655 SFPs for both methods. By doing so, the cutoff probability was slightly different from 0.001 in this analysis. Totally, 4,937 (74.2%) of the 6,655 SFPs were the same between the two methods. Under the selected threshold, the median polish method had lower false positive, false negative and false

Table 1 Comparison of the detection power of different methods

Organism	Method	SFP number	False negative rate (%)	False positive rate (%)	False discovery rate (%)
Rice	Median polish <sup>a</sup>	6,655	48.2	0.90	24.4
	Fitting linear model	6,655	56.7	1.21	34.2
	NONE	6,655	58.7	1.28	36.6
Yeast	Median polish <sup>a</sup>	3,387	78.9	0.25	11.1
	Fitting linear model	3,387	81.4	0.48	21.2
	Median polish <sup>b,c</sup>	1,049	53.5	0.09	2.5
	PDNN <sup>c</sup>	1,049	55.3	0.22	6.2
Barley	Median polish <sup>a</sup>	10,504	23.7	3.23	18.8
	Fitting linear model	10,504	26.4	7.04	34.4

Method: median polish, a median polish step was employed to remove the transcript abundance effects from PM intensities; fitting linear model, obtaining the genotype effect at the probe level to identify SFPs by fitting linear model, Rostoks et al. proposed (Rostoks et al. 2005); PDNN, estimating the binding affinity of the probe to the transcript target based on the PDNN model, Ronald et al. proposed (Ronald et al. 2005); NONE, using PM intensities to identify SFPs directly

<sup>a</sup> BH adjusted *P* values less than 0.001 were used in median polish method for claiming SFPs, and the threshold for the counterpart of the comparison was adjusted to obtain an equal number of the predicted SFPs

<sup>b</sup> The threshold was adjusted to yield the same number of the predicted SFPs as in the published result (Ronald et al. 2005) for comparison

<sup>c</sup> The yeast SFPs detected by the PDNN method (and the corresponding median polish method) used only three replicates from the diploid genomes of the strains and a subset of the probe sets (which the authors regarded as robustly expressed probe sets)

Table 2 Efficiency of SFP detection between two rice cultivars using single tissue relative to all three tissues

Tissue <sup>a</sup>	ALL (%)	SBP (%)	PSP (%)	PCF (%)
False negative rate	48.2	55.1	51.7	54.1
False positive rate	0.90	1.18	1.05	1.08
False discovery rate	24.4	32.9	28.9	30.6
Consistent SFP detection with ALL	100	67.6	76.8	71.3

<sup>a</sup> ALL, all the three rice panicle tissues; SBP, PSP, and PCF, samples from three different developing stages of panicle tissues (see Methods for details). Each tissue was sampled with three biological replicates each with two technical repeats

<sup>b</sup> False negative: the rate in which SFPs were not predicted for probe sequences with known SNPs

<sup>c</sup> False positive: the rate of predicted SFPs among the sequences known not to contain SNPs

<sup>d</sup> False discovery: the rate of predicted SFPs whose probe sequences did not contain SNPs to the total number of SFPs. Both false positive and false discovery include ones with flanking SNPs

<sup>e</sup> The rate of consistence of SFPs detected with single tissues relative to multiple tissues

discovery rates compared to the linear model fitting method, the median polish method has better detection sensitivity (Table 1). Moreover, even for the 139 SFPs called by the fitting linear model, the median polish method that did not have SNPs in the probes, We also directly used the original normalized PM intensities instead of residuals to identify SFPs. In doing so, all PM intensities of each probe were grouped by genotypes, and probes detecting significant difference between the two genotypes (SFPs) were identified using the same SNP data set from OryzaSNP probe set. When the detection threshold was adjusted to obtain 6,655 SFPs, this method obviously resulted in lower sensitivity and accuracy than both within the 25-base probe regions, which is lower than that of the Rostoks method and the median polish method (Table 1), indicating the positive gain of the median polish method.

The remaining 187 (34.2%) were not supported by the We also analyzed the barley microarray data composed of oryzaSNP resequencing data. These results suggested that six tissues including radicle, root, leaf, embryo,

coleoptile and seedling crown, using the median polish method, and compared the results with the original analysis. Interestingly, additional 546 SFPs were detected with method, and compared the results with the original analysis. Unexpectedly, lower residuals of median polish in BY than in using the method of fitting linear model (Rostoks et al. 2005). It was shown that, with the same threshold of 10,504 reference strain BY than that of RM. We examined 186 SFPs and based on the available data, the median polish method is superior in both sensitivity and accuracy (Table 1).

We further compared these methods using yeast datasets. However, the density distributions of the average absolute differences of residuals are similar between the detection between *S. cerevisiae* strains BY4716 (BY, an isogenic strain to the reference yeast) and RM11-1a (RM, a wild yeast strain) were downloaded from NCBI GEO (Barrett et al. 2007). The dataset included 12 microarrays, with three replicates from haploid and diploid genomes of each strain. Using the median polish method, 3,387 SFPs were detected between the two genotypes at 0.001 false discovery rates than the median polish method all array data. As expected, for most (2,841) of the SFPs, RM showed higher binding ability than RM as estimated by method was also made against the method based on the average of residuals of median polish (Zhang et al. 2003) as in the original analysis, also demonstrating that the median polish method has better detection sensitivity and accuracy (Table 1).

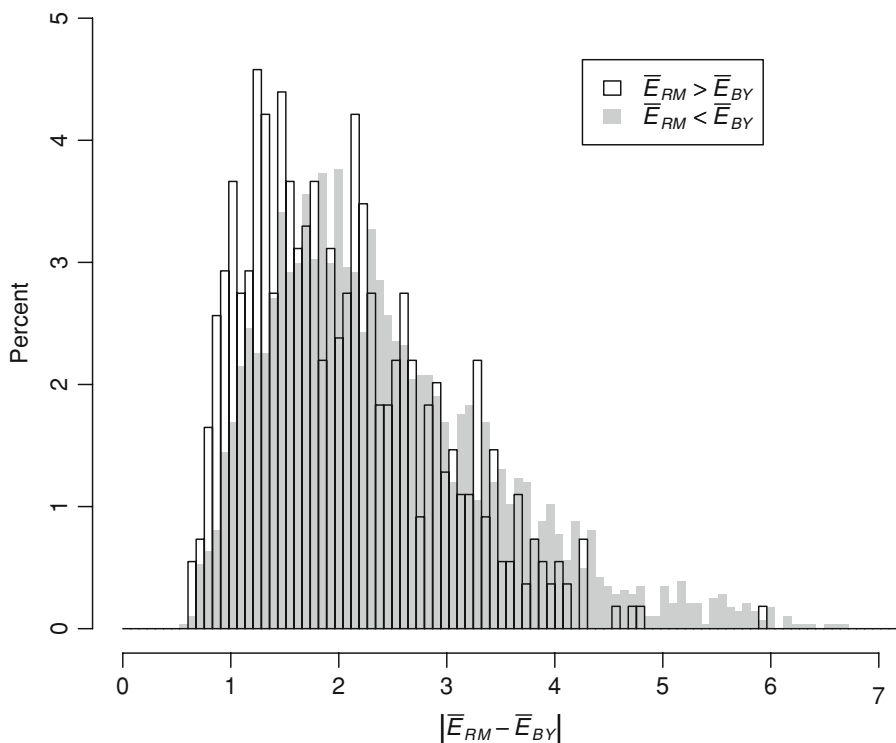


Fig. 2 The distribution of average absolute differences of residuals according to whether  $\bar{E}_{RM}$  is larger than  $\bar{E}_{BY}$  (white  $\bar{E}_{RM} > \bar{E}_{BY}$ ) and (grey  $\bar{E}_{RM} < \bar{E}_{BY}$ ) between two yeast strains BY and RM. Totally 3,387 SFPs between BY and RM were identified with BH adjusted  $p < 0.001$ . Their corresponding averages of residuals by median polish of (BY) and RM ( $\bar{E}_{RM}$ ) and their absolute differences ( $|\bar{E}_{RM} - \bar{E}_{BY}|$ ) were calculated. The x axis shows  $|\bar{E}_{RM} - \bar{E}_{BY}|$  and the y axis is the percentage of the distribution. The  $\bar{E}_{RM} - \bar{E}_{BY}$  are grouped as the affinity between transcript targets and the probe. The distribution of the average absolute differences  $\bar{E}_{RM} > \bar{E}_{BY}$  group are almost the same as  $\bar{E}_{RM} < \bar{E}_{BY}$  group, which means an equal chance to be detected as SFP although most SFPs in  $\bar{E}_{RM} > \bar{E}_{BY}$  group are non-polymorphic probes

SFPs and probe flanking polymorphisms

As presented above, 24.4% of the SFPs, that could be repeatedly detected using different tissues and methods, do not have SNPs in the probe regions according to the OryzaSNP data. It was recently speculated that variations between different targets in flanking sequences adjacent to the probes may affect SFP detection (Borevitz et al. 2007; Zhu and Salmeron 2007). To assess in what degree flanking SNPs affect the detection of SFPs, we analyzed flanking SNPs of 5,088 probes with available flanking sequences longer than 20 bases at each end and the sequences corresponding to all these probes are monomorphic between the two rice varieties according to the data by the oryzaSNP resequencing project (McNally et al. 2006). SNPs were found within flanking 20 bases between Zhenshan 97 and Minghui 63 for 217 probes. Among them, 77 probes have flanking SNPs in the immediate adjacent ten bases, and 140 probes have SNPs within the flanking 11th to 20th base region. Two probes with SNPs in both immediate adjacent 10 bases and 11th to 20th bases were removed from the analysis.

We also investigated the possible effect of the nucleotide compositions of the flanking SNPs on hybridization signals of the SFPs. We first categorized the yeast flanking SNPs into two groups according to their relative residuals of median flanking SNPs in the immediate adjacent ten bases, and 140 probes have SNPs within the flanking 11th to 20th base region. Two probes with SNPs in both immediate adjacent 10 bases and 11th to 20th bases were removed from the analysis.

It was found that the probe flanking SNPs were significantly associated with the SFP calls (Table 3a). Among the SFPs detected at  $P < 0.001$ , SNPs occurred with a frequency of 14.3% in the flanking ten bases, a 9.7-fold enrichment comparing to the background frequency (1.5%) resulting in  $P = 1.40 \times 10^{-4}$  by Fisher's exact test (FET). SFP calls were further enriched to 17-fold when the stringency increase to  $P < 0.00001$  (4/16, FET  $P = 6.94 \times 10^{-5}$ ). About a twofold enrichment (5.7%) of SNPs was also observed in the flanking 11th to 20th base regions among the SFP calls at  $P < 0.001$  compared with the background (2.8%).

We again used yeast microarray data (Ronald et al. 2005) to validate the above observation (Fig. 3b). We particularly focused on the erratic SFP probes with negative residuals ( $\bar{E}_{RM} > \bar{E}_{BY}$ ) detected by reference yeast strain BY. A total of 4,273 probes without polymorphisms within the 25 bases but with SNPs in the flanking 20 bases of the probes were selected for the analysis (Table 3). The concurrence of SFPs with probe flanking SNPs was found to be significantly higher than in the background. There are only 4.6% (2,127)

of the probes having SNPs in flanking ten bases among 46,595 monomorphic probes, compared to 15.1% (13) among the 86 significant SFPs detected at 0.001 (FET,  $df = 1.39 \times 10^{-4}$ ). However, the rate of SNPs was not any higher among the SFPs than the background in flanking 11th to 20th base regions (1/86, FET  $P = 0.98$ ). These results indicated that probe flanking polymorphisms were partially responsible for SFPs, including SFPs resulting from negative residuals.

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SFPs detection in each group (Fig. 3a). We found that adenines appear as predominant form of purines in the flanking SNPs under the same or more stringent values while guanines display opposite effects, suggesting possible influence of adenines and guanines in the flanking SNPs on the probe binding affinity. However, compared to the background, the observed differences are relatively small although they are statistically significant (when using subset flanking SNPs under SFPs detection  $P < 0.05$ ,  $\bar{E}_{RM} < \bar{E}_{BY}$ :  $\chi^2 = 7.00$ ,  $df = 3$ ,  $P = 0.072$ ;  $\bar{E}_{RM} > \bar{E}_{BY}$ :  $\chi^2 = 6.88$ ,  $df = 3$ ,  $P = 0.076$ ). Compared with purines, the curves of pyrimidines (thymines and cytosines) showed weaker correlation between the SNP nucleotide composition and the flanking SFP formation.

It was speculated that the secondary structure of RNA molecules in a solution may interfere with the binding to

Table 3 SFPs caused by flanking SNPs revealed with different thresholds

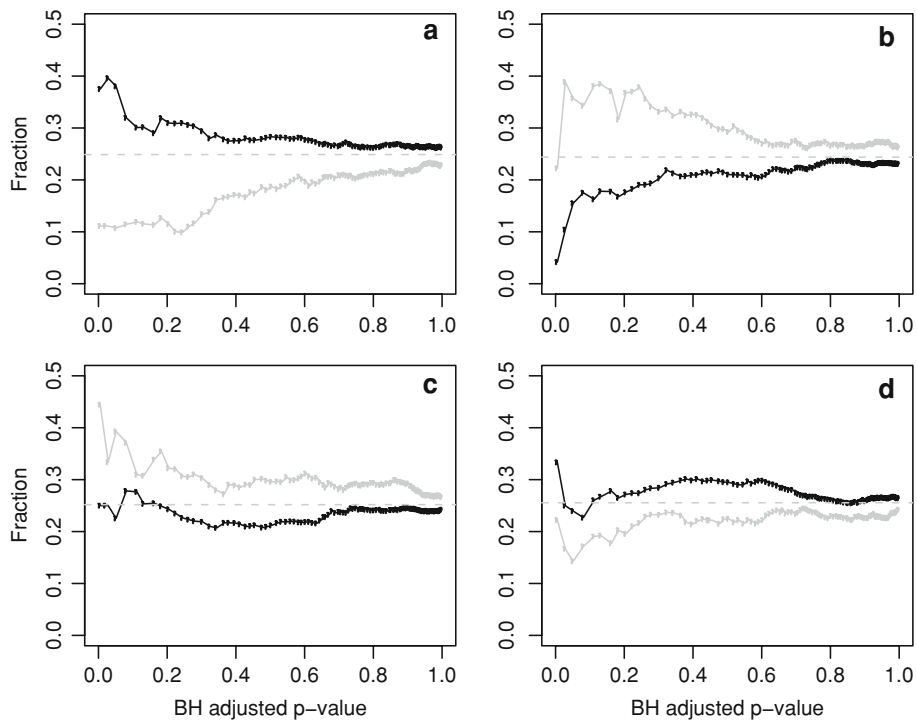
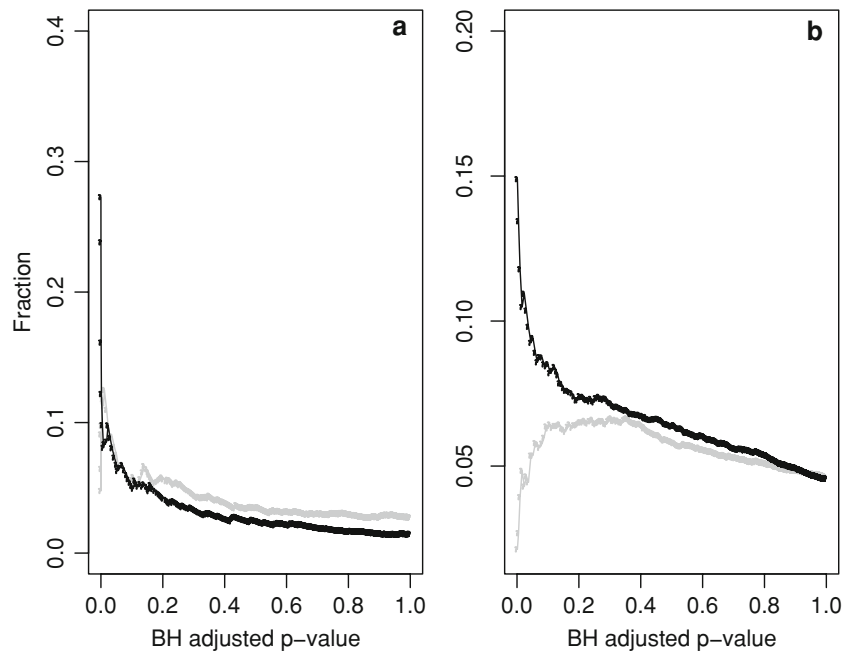
Perfect matched probe	Rice		Yeast			
		$P < 0.05^a$	$P < 0.001^b$	$P < 0.05^a$	$P < 0.001^b$	
Total	5,088 <sup>b</sup>	131	35	46,595	819	86
With SNPs in flanking 0–10 bp	75 (1.5%)	9 (6.9%)	5 (14.3%)	2,127 (4.6%)	74 (9.0%)	13 (15.1%)
With SNPs in flanking 11–20 bp	140 (2.8%)	10 (7.6%)	2 (5.7%)	2,146 (4.6%)	44 (5.4%)	1 (1.2%)

<sup>a</sup> Number of SFPs detected at the BH adjusted values of 0.05 and 0.001, respectively

<sup>b</sup> Number of probes that are monomorphic in the probe region with flanking sequences available in public database. For the yeast data, we only selected the probes with  $\bar{E}_{RM} > \bar{E}_{BY}$



**Fig. 3** The fraction distribution of non-polymorphic probes with banking SNPs resolved with different statistical thresholds in a rice and b yeast. The fractions of probes with banking SNPs were calculated using different thresholds of BH-adjusted  $P$  value generated from SFP detection. The x-axis shows the BH-adjusted  $P$  values and the y-axis is the fraction. The surveys in the banking 100 base region and 1100 base region are colored in black and grey, respectively



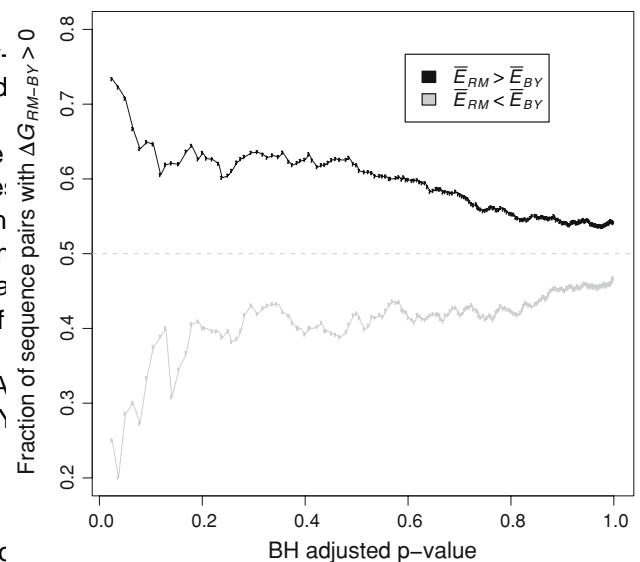
**Fig. 4** The fraction distribution of nucleotide content of SNPs is larger than  $\bar{E}_{BY}$  (black  $\bar{E}_{RM} > \bar{E}_{BY}$ ) and (grey  $\bar{E}_{RM} < \bar{E}_{BY}$ ). Banking non-polymorphic probes resolved with different statistical thresholds. Non-polymorphic probes with unique SNPs in the banking  $\bar{E}_{RM} > \bar{E}_{BY}$  group under more stringent BH-adjusted  $P$  values, while 1000 bases were selected and the nucleotide content of these SNPs in yeast RM11-1a genotype were then surveyed using different thresholds of BH-adjusted  $P$  values generated from SFP detection. The x-axis shows the BH adjusted  $P$  values and the y-axis is the fraction of certain nucleotide base. SNPs are grouped according to whether

probes (Southern et al. 1999). To examine the changes of cRNA secondary structure induced by banking polymorphisms, we used the UNAFold software (Markham and Zuker 2005; Walter et al. 1994) to calculate the minimum free energies ( $\Delta G$ ) of 75-bp cRNA sequences that bank the center of each probe extracted from BY and RM sequence. A total of 3,439 polymorphic banking sequence pairs with monomorphic probe region and polymorphic banking bases were obtained. We also divided these sequence pairs into two groups according to their relative residuals of median polish:  $\bar{E}_{RM} > \bar{E}_{BY}$  or  $\bar{E}_{RM} < \bar{E}_{BY}$ . We then calculated the proportion of sequence pairs in which the RNA free energy of RM sequences were greater than BY sequences (proportion of  $\Delta G_{RM-BY} > 0$ ) according to different SFP detection  $P$  values. The results showed that there was no change of  $\Delta G_{RM-BY}$  for 738 (21.5%) of the sequences. A threshold of 50% quantile (absolute value of  $\Delta G_{RM-BY} > 0.60$  kJ) was applied to filter sequence pairs with different minimum RNA free energy between BY and RM (see the Supplementary Fig. 2 for the distribution of  $\Delta G_{RM-BY}$ ). Although it is difficult to confirm all of the predicted changes in RNA secondary structures, as the accuracy of predictions of RNA secondary structures is only 74% (Walter et al. 1994), we found that the minimum free energy of RNA is correlated positively to the binding affinity (Fig. 5). Using a threshold of BH adjusted  $P$  values less than 0.05, the percentage of sequence pairs with  $\Delta G_{RM-BY} > 0$  (72.5%) in the  $\bar{E}_{RM} > \bar{E}_{BY}$  group is significantly higher than the background level (54.1%, FET,  $P = 0.012$ ). Reversely, only 28.6% of the sequence pairs with  $\Delta G_{RM-BY} > 0$  were found in the  $\bar{E}_{RM} < \bar{E}_{BY}$  group. However, the difference is statistically insignificant compared to the background level (46.5%, FET,  $P = 0.29$ ) due to the less frequent occurrence and subsequently smaller sample size ( $n = 7$ , at SFPs detection  $P < 0.05$ ). Unfortunately, we are unable to conduct similar analysis in rice as the banking sequences are not available presently.

## Discussion

### Median polish as a high performing method for SFP detection

Molecular interactions between probes and targets are the basis for microarray detection. Both binding affinity between targets and probes and target abundance contribute to the output signals measured by microarray probes. Ideally, one of the two factors ought to be fixed or ignored in order to measure the other. For example, in transcriptome analysis, only the abundance of the transcript targets is considered as the variable. In the sequence analysis, in contrast, the sequence differences between targets and



**Fig. 5** The fraction distribution of the free energy changes in non-polymorphic probes with banking SNPs resolved with different statistical thresholds. Non-polymorphic probes with SNPs in the banking 10 bases were selected and their 75-bp cRNA sequence pairs banking the center of these probes were extracted from yeast BY and RM sequences. The minimum RNA free energies of the sequence pair ( $\Delta G_{BY}$ ,  $\Delta G_{RM}$ ) are then calculated and the difference between  $\Delta G_{BY}$  and  $\Delta G_{RM}$  are calculated by subtracting  $\Delta G_{BY}$  from  $\Delta G_{RM}$  (denoted as  $\Delta G_{RM-BY}$ ). The fractions of  $\Delta G_{RM-BY} > 0$  were then surveyed under different thresholds of BH-adjusted  $P$  values generated from SFP detection. The x-axis shows the BH-adjusted  $P$  values and the y-axis is the fraction of  $\Delta G_{RM-BY} > 0$  with the statistical thresholds. Sequence pairs are divided into two groups according to whether  $\bar{E}_{RM}$  is larger than  $\bar{E}_{BY}$  (black  $\bar{E}_{RM} > \bar{E}_{BY}$ ) and (grey  $\bar{E}_{RM} < \bar{E}_{BY}$ ). There is a trend that the proportion of sequence pairs with  $\Delta G_{RM-BY} > 0$  increases in the group  $\bar{E}_{RM} > \bar{E}_{BY}$  while the proportion decreases in the group  $\bar{E}_{RM} < \bar{E}_{BY}$  with more stringent thresholds

probes are considered as the major variable while the abundance of the targets is assumed similar. However, such a highly simplified model is not applicable in a variety of microarray applications. SFPs are consequence of sequence difference between corresponding targets from two samples detected by the same probes, providing opportunity to detect sequence polymorphism, as well as challenge to ensure accuracy in transcript abundance measurement in a transcript analysis.

SFP detection from RNA derivative targets by microarray has the advantage of measuring both transcript abundance and sequence variation at the same time (Cui et al. 2005; Luo et al. 2007; Ronald et al. 2005; Rostoks et al. 2005; West et al. 2007). It makes the SFP detection more cost effective and possible in complex genomes by using expressed transcripts as genome complexity reduction method (Cui et al. 2005; Gore et al. 2007; Rostoks et al. 2005; Zhu and Salmeron 2007). However, the algorithms used for analyzing such data requires not only

identifying differential hybridization signals but also dissecting contributing factors to these differential signals to ensure sufficient detection power and accuracy.

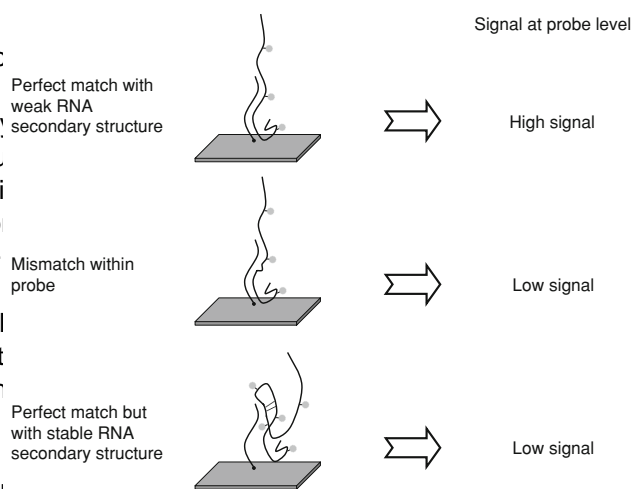
We adapted a median polish based method to identify SFPs using expression data from multiple tissues. Our study suggested that the median polish method has superior performance in SFP detection as evidenced by validation using data from multiple genomes with different composition, structure, and complexity (Table 1). An additional advantage of this method is that most methods for SFP prediction need specific design of microarray experiment (generally two groups under the same experimental conditions with several repeats in each group) (Cui et al. 2005; Das et al. 2008; Luo et al. 2007; West et al. 2006), our median polish based method provides some uniquely useful features for SFP detection. This method works for dissecting the expression data into and eliminate the effects of transcript abundance and variations of binding affinity, thus perturbation of transcript abundance resulting from different experimental conditions and data types had little interference with the sensitivity and accuracy of the SFP detection. This method can also significantly reduce the computing time required to perform the same SFP analysis

using the same data set, thus enable one to perform a SFP analysis using more complex data set. The computing time required for analyzing the rice data set in this study is less than 1 min by the median polish method and 21 min by the fitting linear model. The replacement of SAM with limma could further reduce the memory requirements and computing time by 50 times.

### Complexity of SFP formation and the contribution from probe flanking SNP

There are many factors influencing hybridization signal from a RNA target, including whether the RNA target was expressed in the sample, the abundance of the RNA target, the GC content of the binding region, and the presence of mismatch bases between the target and probe and their location (Rostoks et al. 2005; Zhu and Salmeron 2007). It was generally considered that mismatch bases within probe sequences lead to reduced hybridization intensities and the formation of SFPs in comparative studies (Fig. 1).

However, the interaction between short oligonucleotide probe and target is complex (Naef et al. 2002b) and affected by many variables in addition to the above. Our data clearly demonstrated that the probe described sequence specificity and abundance of target flanking SNPs could significantly contribute to SFP formation as over 15% of non-polymorphic SFPs are associated with flanking SNPs, of which the background contribution was less than 5%. To uncover the molecular basis of the SFP formation by flanking SNPs, we surveyed the nucleotide composition of the flanking SNPs and confirmed that adenines are over-



**Fig. 6** Probe-target interaction and contribution to hybridization signals. When target abundance is presumed to be at similar level, target that matches probe perfectly and with weak secondary structure produces a relative high signal, while target with mismatch within probe region produces a relative low signal. When the flanking polymorphisms can make more stable RNA internal secondary structure, the binding affinity between probes and targets may lead to reduced signal

than the PM probes (Naef et al. 2002a, b; Zhang et al. 2003). In this study, 32 yeast SFPs with confirmed mismatch bases between RM targets and probes was found to have greater residuals of median polish in RM and more than half of them (18/32) was found to have greater hybridization signal in RM. Second, probes could produce different hybridization signals from targets with identical perfect complementary sequences. A total of 266 probes detected statistically significant differential signals from targets of RM and BY strains even these targets have sequences perfectly matched the probes.

The interference of biotinylated fluorescent labels (Naef and Magnasco 2003) and stacking free energies of unlabeled nucleic acids in solution (Carlson et al. 2006) are among the hypothesized contributing factors to the first phenomenon. However, neither of the hypotheses was fully supported by experimental evidence (Wang et al. 2007).

The second phenomenon may relate to the influence of the mismatch bases in the flanking region (Fig. 6). Mismatch bases in the flanking region of a probe are generally considered to have less direct effect on SFP detection and thus less studied. However, our results from analyzing both yeast and yeast data clearly demonstrated that the probe flanking SNPs could significantly contribute to SFP formation as over 15% of non-polymorphic SFPs are associated with flanking SNPs, of which the background contribution was less than 5%.

To uncover the molecular basis of the SFP formation by flanking SNPs, we surveyed the nucleotide composition of the flanking SNPs and confirmed that adenines are over-

represented and guanines are underrepresented in the large array of traits and yield heterosis have been mapped flanking SNPs that lead to higher binding affinity. We also using populations derived from this cross. Recently, a gene calculated minimum free energies of 75-bp cRNA underlying a major QTL for pleiotropic effects on heading sequences flanking the center of each probe and compared date and yield potential was cloned (Xue et al. 2008). their difference between targets. We found that the Although this is probably the most intensively studied cross hybridization affinity was correlated positively to the in rice genetic mapping, several large gaps (20 cM) still minimum free energy of cRNA. The changes of predicted exist in the genetic map due to lack of polymorphic minimum free energy of cRNA are significant. These markers in these regions (Hua et al. 2002; Xing et al. observations suggest that the flanking SNPs could lead 2002), despite large efforts in adding more markers. Such the formation of SFPs by changing minimum free energies large gaps would have certainly affected the completeness of unlabeled RNA targets: a lower minimum free energy of the information for genetic mapping. The discovery of may lead to more stable RNA internal secondary structure. 6,655 SFPs in this study representing 3,131 rice unique thus reducing the hybridization affinity between probes and genes with a high validation rate supported by large scale targets. resequencing data, could generate an extra-high density

To further investigate possible causes for the SFPs, we genetic map, thus greatly facilitate gene discovery in this inspected 28 rice SFPs (Table 3) that were detected at population.  $P < 0.001$  but had no SNPs in either the probes or the In conclusion, median polish has superior performance flanking 20 bp regions, 24 of which were genes within SFP detection. This method avoids constructing complex EST/cDNA support in the TIGR database. Ten (41.7%) of linear models thus could be used to genotype recombinant the 24 genes have more than one gene model presumably hybrid lines or double haploid population which cannot be due to alternative splicing, much higher than the estimated into linear models easily. Furthermore, it could be used mated 21.2% alternative splicing level of the rice genome to remove the influence of SFPs in the expression analyses (Wang and Brende 2006), suggesting the likelihood that using GeneChip arrays to improve accuracy of measure-alternative splicing may also contribute to the SFPment. In this aspect, it would be especially powerful when detection. Moreover, 7 of the 28 SFP detecting probes the method is combined in the same automated workflows were the last ones (No. 11) in the respective probe sets with the robust multi-array average (RMA) (Irizarry et al. Recently, unexpected alternative termination of gene 2003), one of the most adapted algorithm for expression transcription was observed by RNA sequencing of mamamalysis using GeneChip microarrays, which uses the same mal and yeast genomes (Carninci et al. 2005) algorithm. Nagalakshmi et al. 2008). Considering that the microarray We confirmed that probe flanking SNPs could lead to we used was Affymetrix expression arrays, in which SFPs in yeast and rice genomes that significantly different the probes primarily target the 3' end of the genes, these in genome size, composition, and complexity level. This observations might also suggest possible differences in 3' binding has impact not only in SFP detection and its alternative termination between the two genotypes. Fur application in genetic analysis, but also in expression thermore, the widely reported copy number variations analysis using GeneChip and other short oligonucleotide (insertions/deletions) of small DNA segments exist not probe arrays. These probe flanking SNPs could affect the only in human and rat (Guryev et al. 2008; Stranger et al. binding affinity by changing the minimum free energy of 2007), but also in plants and other taxa (Clark et al. 2007; the unlabeled RNA target molecules. Our results also Gresham et al. 2006). These copy number variations indicated the complex compositions of SFP formation, for along with alternative splicing and/or alternative ter- a large of SFPs still cannot be explained by either probe mination may affect the probe signal intensities, thus SNPs or flanking SNPs. might have been detected as SFPs in this analysis while To the best of our knowledge, our study was the first not necessarily detected by sequencing, thus contributing application of detecting SFPs using expression data in rice. to the discrepancies between the sequence data and SFPs. The success of our study demonstrated the applicability of detection. this approach. The detected rice SFPs would greatly facilitate the effort of saturating the genome with molecular markers.

#### Usefulness of the detected rice SFPs

Rice is a staple cereal for a large segment of the world population. The two varieties used in this study, Zhenshan 97 and Minghui 63, are the parents of Shanyou 63, the most widely grown hybrid in China. Hundreds of QTLs for a Acknowledgments We thank Dr. James Ronald and Dr. Rachel B. Brem for help and suggestions in yeast data. This work was supported by grants from the National Special Key Project of China on Functional Genomics of Major Plants and Animals, and the National Natural Science Foundation of China.

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