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The use of general and specific combining abilities in a context of gene expression relevant to plant breeding

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Abstract Many common traits are believed to be a composite reflection of multiple genetic and environmental factors. Recent advances suggest that subtle variations in the regulation of gene expression may contribute to quantitative traits. The nature of sequence variation affecting the regulation of gene expression either in *cis* (that is, affecting the expression of only one of the two alleles in a heterozygous diploid) or in *trans* (that is, affecting the expression of both alleles in a heterozygous diploid) is a key and usually unknown feature for the breeders. If the change in expression acts entirely in *cis*, then the structural gene can be treated as a candidate gene and a potential target for marker-assisted selection. Therefore, gene surveys for *cis*-regulatory variation are a first step in identifying potential targets for marker-assisted breeding. Here, we discuss in detail the ''genome-wide analysis of allele-specific expression differences'' (GASED) approach. The GASED approach was developed to screen for *cis*-regulatory variation on a genome-wide scale. In GASED, mRNA abundance is treated as if it were a quantitative phenotypic response variable, whose genetic between-F_1 hybrid variance is partitioned into additive and non-additive components. In plant breeding, this partitioning of the genetic variance is well known in the context of estimation of general and specific combining abilities for diallel crossing schemes. We demonstrate the GASED method using *Arabidopsis thaliana* data. The method can be used to screen for *cis*-regulatory variation in any crop species for which diallel crossing schemes are appropriate and genomic tools are available.

Keywords General combining ability · Specific combining ability · Allele-specific expression · *Cis*-regulatory variation · Diallel

Abbreviations

ASE	Allele-specific expression
eQTL	Expression quantitative trait loci
FDR	False discovery rate
FNR	False non-discovery rate
GASED	Genome-wide analysis of ASE differences
GCA	General combining ability
LD	Linkage disequilibrium
MAS	Marker-assisted selection
SCA	Specific combining ability

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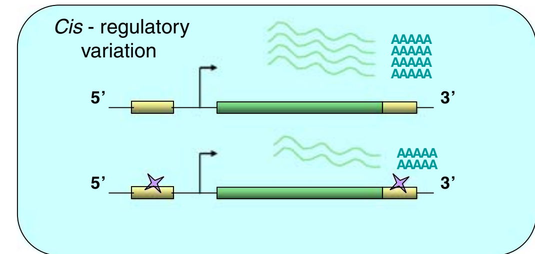
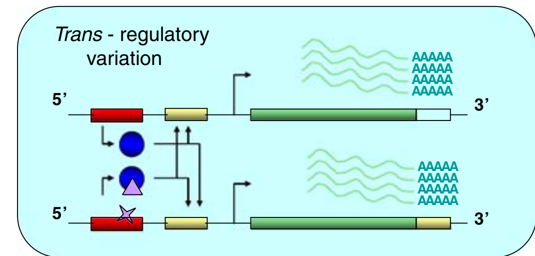
cis- versus *trans*-acting sequence polymorphisms

Sequence polymorphisms underlie phenotypic variation by affecting biological processes at the molecular level, such as protein structure, transcription, alternative splicing, etc.. There are a number of examples in which nucleotide polymorphisms in regulatory regions causing expression changes in the corresponding genes, have been found to be associated with phenotypic variation (Clark et al. 2006; Clop et al. 2006; Frary et al. 2000). The nature of the regulatory polymorphism that is causing expression changes associated with phenotypic variation is a key and usually unknown feature for the researchers or breeders. *Cis*-regulatory polymorphisms are expected to be in close proximity to the gene being regulated and directly affect the gene expression levels in an allele-specific manner. Such polymorphisms act in *cis*, i.e. in an allele-specific manner, either by altering classic 5’ upstream *cis*-acting regulatory elements in the promoter, or by modifying target sites for messenger RNA processing and stability, e.g. 3’-untranscribed regions (3’-UTR) (Fig. 1a). *Trans*-acting polymorphisms are not expected to be in close proximity to the gene regulated and modify either the expression level or activity of a factor (e.g. transcription factor) that interacts with *cis*-regulatory sequences of both alleles (Fig. 1b).

If the sequence polymorphism is entirely *cis*-acting, then one can treat the structural gene as a candidate gene and a potential target for marker-assisted selection (MAS) (Walsh and Henderson 2004). If the change in expression is due to one (or more) *trans*-acting polymorphisms, although correlations between target expression and phenotype may be very high, the only way for a breeder to exploit the existing variation is to perform a QTL mapping experiment to find markers for MAS on the *trans*-acting factor. If the change in expression is partly due to a major *trans*-regulatory element, this might have potentially significant implications for the correlated response in other, perhaps unwanted, traits (Walsh and Henderson 2004). Hence, it is crucial to the breeder to focus on *cis*-regulatory variants and to have the tools to clearly distinguish between *cis*- and *trans*-controlled expression changes of target genes.

(a) Local regulatory variation





(b) Distant regulatory variation

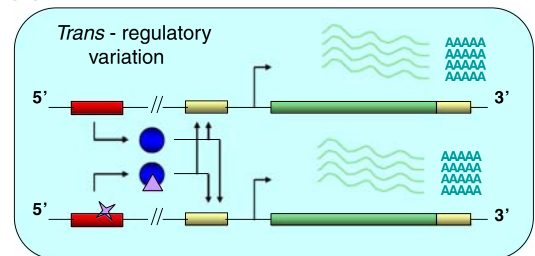


Fig. 1 Local and distant regulatory variation. (**a**) Local regulatory variation: local regulatory variation might be due *cis*-regulatory variation, that is a polymorphism (or polymorphisms) in the structural gene itself, either by altering classic 5’ upstream *cis*-acting regulatory elements in the promoter, or by modifying target sites for messenger RNA processing and stability, e.g. 3’-untranscribed regions (3’-UTR). Alternatively, local regulatory variation might be *trans*-regulatory variation due to a polymorphism in a nearby gene that regulates the expression of the structural gene by a protein (e.g. transcription factor). The star denotes the regulatory variant. The yellow and green rectangles denote the *cis*-regulatory elements and coding region, respectively, of the gene expressed. The red rectangle represents the *trans*-acting elements affecting the regulation of the gene expressed. The circle denotes the protein product (e.g. transcription factor) of the *trans*-acting gene, the triangle denotes the protein variant. The curved lines represent the transcripts or messenger RNA transcribed from the expressed gene. (**b**) Distant regulatory variation**:** Distant regulatory variation typically act in *trans* through the downstream effects of coding or *cis*-regulatory polymorphisms in different types of distantly located genes, with transcription factors being the most obvious example

Locating *cis*- and *trans*-acting loci using linkage analysis

Recently, geneticists have become interested in applying quantitative genetic methodologies to microarray expression data to estimate the genetic variance and heritability of gene expression (Jin et al. 2001; Gibson et al. 2004; Wayne et al. 2004; Gibson and Weir 2005), to estimate additive and dominance gene effects (Gibson et al. 2004; Vuylsteke et al. 2005), and to detect gene expression quantitative trait loci (eQTL) (Rockman and Kruglyak 2006). These eQTL are the statistically significant peaks in the profiles of test statistics versus genome positions in genome-wide scans for linkage between markers and transcript abundances (Jansen and Nap 2001). The basic experimental design of eQTL studies is identical to that of classical F_2 or recombinant inbred line linkage mapping for organismal quantitative traits, except that thousands of expression phenotypes (individual gene expression levels), modeled as quantitative traits, are analyzed simultaneously. The concept of mapping QTL underlying the observed expression variation was originally introduced by Damerval et al. (1994): spot intensities on two-dimensional gels were scored as measures of protein abundance in a F_2 progeny of a maize line cross, and QTL underlying the observed variation were mapped. In the recent years, however, the focus of such genomic approaches shifted towards gene expression data, and such eQTL studies have been reported to date for a number of organisms, such as yeast (Brem et al. 2002), C. elegans (Li et al. 2006) rodents (Schadt et al. 2003; Bystrykh et al. 2005; Chesler et al. 2005; Hubner et al. 2005; Petretto et al. 2006), human (Schadt et al. 2003; Monks et al. 2004; Morley et al. 2004), *Arabidopsis thaliana* (DeCook et al. 2005; Vuylsteke et al. 2006; West et al. 2007), eucalyptus (Kirst et al. 2004), and maize (Schadt et al. 2003). These recent ''genetics of gene expression'' studies all identified two types of correlations between markers and expression trait: those in which a transcript level maps near the genomic region containing the structural gene producing the transcript, classified as local eQTL (Fig. 1a); and those in which the expression level is associated with a distinct locus elsewhere in the genome, classified as distant eQTL (Fig. 1b). An interesting graphical way to display information from such an eQTL analysis is to plot the genomic location for the gene whose expression is being measured on one axis and genetic locations for any eQTL of this gene on the other axis. Points on and off the diagonal indicate local and distant eQTL, respectively (examplified in Bystrykh et al. 2005; Chesler et al. 2005; Li et al. 2006; Vuylsteke et al. 2006).

Distant eQTL typically act in *trans* through the downstream effects of coding or *cis*-regulatory polymorphisms in different types of distantly located genes, with transcription factors being the most obvious example (Fig. 1b). In contrast, local eQTL can arise as a result of several scenarios (Fig. 1a). First, the linkage might be due to a polymorphism in a nearby gene that regulates the expression of the structural gene by a protein (e.g. transcription factor) in *trans*. Second, and more typically, local eQTL might be due to a polymorphism (or polymorphisms) in the structural gene itself, acting at the level of DNA in *cis*. Hence, although expression changes with local eQTL are most likely caused by *cis*-regulatory variation in the corresponding gene, the high degrees of linkage disequilibrium (LD) between loci segregating in a mapping population complicate the distinction between *cis*-acting and local *trans*-acting eQTL.

Allele-specific gene expression

One approach to clearly differentiate between *cis*- and *trans*-control involves the quantification of allele-specific expression (ASE) in a heterozygous diploid individual, such as an F_1 hybrid (Cowles et al. 2002; Guo et al. 2003). Allele-specific expression differences in an F_1 individual are expected to be largely unaffected by *trans*-acting genetic variation and to be relatively robust against common environmental factors, because the allelic comparison is made within the heterozygous diploid individual (Fig. 2a, b and c). Therefore, ASE differences in a F_1 hybrid provide evidence for a model whereby only *cis*-acting sequence variation underlies the differential expression between the two alleles. Such *cis*-*trans* test, examining the differential expression of alleles in a F_1 hybrid, has been elegantly extended by including the parental expression ratio (Wittkopp et al. 2004). In this manner, different patterns of gene regulation could be distinguished: 1) genes with the same allelic ratios in the parents and hybrids were determined to

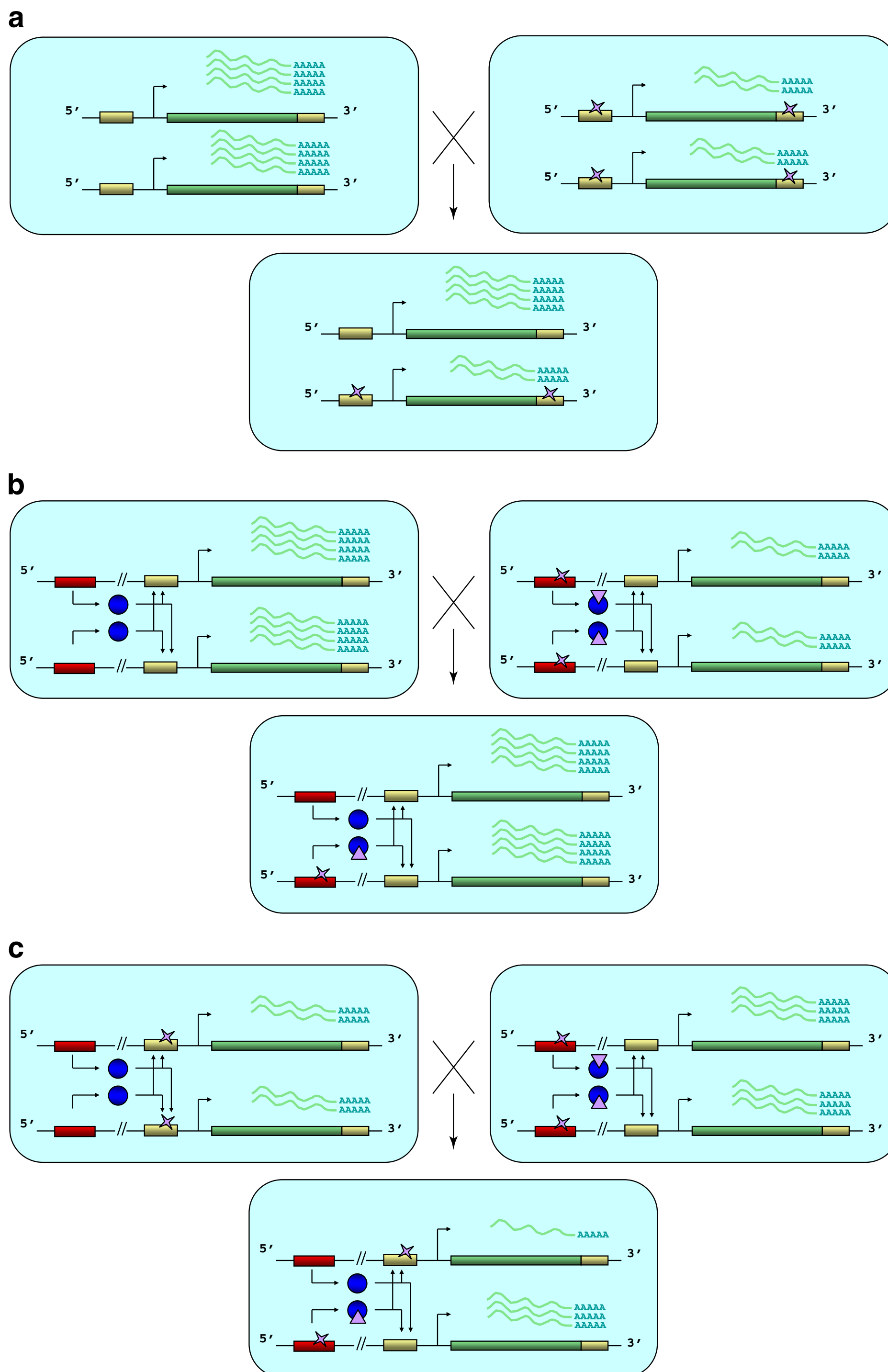


Fig. 2 Allele-specific expression differences in a heterozygous individual. (**a**) Genes with the same allelic ratios in the homozygous parents and hybrids are affected by *cis*-regulatory variants only. (**b**) Genes with allelic bias in the homozygous parents, but equal proportions in the hybrid, are strongly affected by *trans*-regulatory variants only. (**c**) Genes with hybrid allelic proportions that do not match either parental or equal proportions are regulated by a combination of *cis* and *trans* variants

be affected by *cis*-regulatory variants (Fig. 2a); 2) genes with allelic bias in the parents, but equal proportions in the hybrid, were determined to be strongly affected by *trans*-regulatory variants (Fig. 2b); and 3) genes with hybrid allelic proportions that do not match either parental or equal proportions were determined to be regulated by a combination of *cis* and *trans* variants (Fig. 2c).

The basic requirement to quantify ASE in a F_1 hybrid is a means of identifying the allelic source of the transcript. Single Nucleotide Polymorphisms (SNPs) in the transcripts lend themselves to easy quantify and differentiate the two allele-specific transcripts in the hybrid. Different assay techniques, such as allele-specific quantitative PCR (Cowles et al. 2002; Wittkopp et al. 2004; de Meaux et al. 2005; Doss et al. 2005), denaturing high-pressure liquid chromatography (Guo et al. 2003), and ASE arrays (Ronald et al. 2005; Pant et al. 2006) have been applied to compare the abundance of the allelic transcripts. Only the array-based method approaches a genome-wide scope. However, the need for allele-specific markers to distinguish between alleles limits the wider application of such ASE analyses, as many genes lack common exonic variants.

The GASED approach

To abrogate the requirement for transcribed or exonic sequence polymorphisms, Kiekens et al. (2006) proposed a genome-wide analysis of ASE differences, called GASED, based on partitioning between-F_1 hybrid genetic variance for mRNA abundance into additive and non-additive variance components. This partitioning allows the differentiation between strictly and non-strictly *cis*-regulatory changes and, hence, the identification of genes showing imbalances in allelic expression in a particular hybrid combination that arise primarily from *cis*-regulatory variants. We will discuss in more detail the rationale behind the GASED procedure and the obtained results.

Rationale of the GASED approach

Although transcript abundance, like any other quantitative trait, is potentially a complicated function of multiple loci, it has been found useful to consider transcript abundance as the summation of individual *cis* and *trans* effects. With the easiest case of a diploid individual with only two alleles at each *cis* and *trans* locus, the expression value of a gene in an F_1 hybrid resulting from the cross $i \times j$ can be modelled as:

$$y_{ijk} = \mu + c_i + ct_{ii} + c_j + ct_{jj} + ct_{ij} + ct_{ji} + \varepsilon_{ijk} \quad (1)$$

where y_{ijk} is the expression phenotype of the kth offspring from cross $i \times j$, μ is the mean of the expression values obtained in all crosses considered, c_i and c_j are the effects of the *cis* elements of the ith and jth gamete, respectively, ct_{ii} and ct_{jj} represent the *cis-trans* interaction at the ith and jth gamete, respectively, and ct_{ij} and ct_{ji} correspond to the interaction between *cis* and *trans* elements in one gamete with those of the other. Unless *trans*-acting factors bind with the *cis*-regulatory element directly or indirectly by forming complexes with other transcription factors, for instance, there will be no effect from the *trans*-acting factors per se. As LD is complete in heterozygous individuals coming from inbred parental lines, individual effects of *cis* and *cis-trans* interactions of the same gamete on the allelic expression cannot be distinguished (Fig. 2a, b and c), and equation (1) can be rewritten as follows

$$y_{ijk} = \mu + p_i + p_j + h_{ij} + \varepsilon_{ijk} \quad (2)$$

where the parental-specific p_i and p_j terms correspond to the $c_i + ct_{ii}$ and $c_j + ct_{jj}$ effects, respectively, and the hybrid-specific h_{ij} term to the $ct_{ij} + ct_{ji}$ effect.

In plant breeding programs, where the goal is to estimate the average effects of specific lines and to identify higher yielding hybrid combinations, diallel designs are often used. The model to be analyzed for a simple type of diallel analysis in which homozygous parents are included and reciprocal F_1 hybrids are pooled, is of the following form

$$y_{ijk} = \mu + g_i + g_j + s_{ij} + \varepsilon_{ijk} \quad (3)$$

where μ is the population mean effect, g_i and g_j are the general combining abilities (GCAs) of parents i and j, and s_{ij} is the specific combining ability (SCA) of $i \times j$ matings. In genetic terms, the GCAs represent the additive effects of the parental gametes and the

SCA the non-additive effect of putting gametes together in pairs to make the F_1 genotypes. From the comparison of equations (2) and (3), it is clear that the two models have an identical structure. As a consequence, in a context of gene expression, the GCAs may be regarded as the composite additive effects on the gene expression contributed by gametes i and j (i.e., the set of *cis* elements and *cis–trans* interactions in the gamete), respectively, and the SCA as the non-additive effect on the gene expression contributed by the interaction of the gametes (i.e., the interaction of the *cis* and *trans* elements in one gamete with those of the other).

As mentioned above, different patterns of gene regulation can be distinguished by comparing ASE ratios measured in hybrids and parents. The allelic expression ratio in the hybrid (ASE_H), representing the relative abundance of the allele-specific transcripts in a common hybrid genetic background $i \times j$, can be written as $ASE_H = \frac{c_i + ct_{ii} + ct_{ij}}{c_j + ct_{jj} + ct_{ji}}$, which simplifies to $ASE_H = \frac{c_i + ct_{ii}}{c_j + ct_{jj}} = \frac{g_i}{g_j}$ in a purely additive case, i.e. when $ct_{ij} = ct_{ji} = 0$ or the SCA equals zero. Writing the allelic expression ratio in the parents (ASE_p), which are homozygous for the expressed alleles, as function of *cis* and *trans*-acting elements, gives $ASE_P = \frac{2(c_i + ct_{ii})}{2(c_j + ct_{jj})}$. This equation can be simplified to$ASE_P = \frac{g_i}{g_j}$, which equals to ASE_H in the absence of non-additivity at the expression level. According to Wittkopp et al. (2004), this equality of expression ratios, $ASE_H = ASE_P$, implies that *cis*-regulatory divergence completely explains the expression difference between parents and that *trans*-regulatory variants are absent. From this, identification of genes with an ASE difference in a particular hybrid cross $i \times j$ arising primarily from *cis*-regulatory variants, implies the screening for imbalances in allelic expression, $g_i \neq g_j$, in the absence of the interaction of the *cis* and *trans* elements in one gamete with those of the other, i.e., $s_{ij} = 0$.

Empirical results

To apply the GASED approach, Kiekens et al. (2006) examined transcript levels in RNA samples collected from a diallel experiment in *Arabidopsis* with five parental lines and 10 F_1 hybrids. The hybrid samples consisted of a pooled progeny from reciprocal crosses. Gene expression from two independent samples for each genotype was analyzed, and a linear mixed model with the variance structure defined by the additive $\left(\sigma_g^2\right)$ and non-additive $\left(\sigma_s^2\right)$ variance components was fitted to the expression levels of 4,066 genes by restricted maximum likelihood. The 4,066 genes were identified to have a significant genetic variance component in their transcript abundance. To deal with the problem of multiple testing, estimated P-values were transformed into false discovery rates (FDRs; Storey and Tibshirani 2003), which are typically expressed in the form of Q-values, in analogy to P-values. A critical threshold for the Q-values can be understood as the proportion of significant features that is allowed to consist of false leads. Therefore, a Q-value threshold of 0.001, as applied in the identification of the genes having a significant genetic variance component in their transcript abundance, results in an FDR of 0.1% among the 4,066 genes called significant.

Because, according to our GASED procedure, screening for genes with an ASE difference caused by a *cis*-regulatory variant implies the screening for transcripts with $s_{ij} = 0$ and $g_i \neq g_j$ in a particular hybrid crossing $i \times j$, a first step is the selection of genes lacking evidence against the null hypothesis of no interaction, i.e. $s_{ij} = 0$. To correct for multiple testing, the selection of a subset of genes for which the null hypothesis of no interaction would hold, was performed under a modification of the FDR procedure, because the test statistic was a variance whose value should be non-negative (Genovese and Wasserman 2002; Taylor et al. 2005). In a subsequent step, the focus was on the difference between the estimated additive effects, i.e. g_i-g_j, for each gene with a non-significant non-additive effect in a particular hybrid. Rejecting H_0: $g_i = g_j$ resulted in a total of 1,574 genes displaying significant ASE differences across the 10 hybrids at a Q value of less than 0.001.

Kiekens and coworkers then sought to confirm these results by eQTL mapping directed at 41 genes. Linkage mapping of transcript abundance with 69 markers defining an equal number of genomic bins in a limited set of RILs was carried out (Kiekens et al. 2006). This eQTL analysis confirmed 31 cases (76%) identified to contain functional local regulatory variants, most probably affecting allelic expression levels in *cis*. All of the 31 local eQTL displayed higher expression of the allele predicted to be

preferentially expressed. For nine genes (22%), expression differences had the strongest linkage ($P < 0.05$) to genomic bins different from the genomic bin containing the gene in question, and accordingly, the *cis*-regulated ASE could not be confirmed by the eQTL mapping. For one gene, a significant linkage could not be detected neither to the target *cis* locus nor to *trans* loci.

Conclusions

The differential expression of alleles occurs commonly in plants (Guo et al. 2003, 2006; Kiekens et al. 2006; Stupar and Springer 2006) and is probably an important genetic factor underlying heritable differences in phenotypic and commercially interesting traits. Thus, identification of best performing alleles in terms of transcript abundance and the underlying *cis*-regulatory variant responsible for the superior allelic expression is an important challenge for the breeders.

Kiekens et al. (2006) have shown that in a context of gene expression, empirical estimates of GCA and SCA generated by a diallel design are valid parameters in large-scale detection of transcripts whose abundance is regulated by strong *cis*-acting variants. Compared to other ASE detection methods, GASED has major advantages. First, allelic variants in multiple genetic backgrounds can be examined in a large number of genes. Second, in contrast to the positional ASE detection methods, such as eQTL mapping, GASED is not affected by local *trans*-acting variants in LD with the *cis*-acting variants in question. Any effect of a *trans*-acting locus, irrespective its genomic location relative to the expressed gene, is captured as non-additive effect which is supposed to equal zero when ASE is strictly *cis*-regulated. Therefore, GASED leads to a more accurate identification of the truly *cis*-acting QTL. Third, the detection of *cis*-regulated ASE differences by GASED is not restricted to genes having allele-specific markers to distinguish between alleles. This feature is the major strength of the GASED approach and makes it a valuable prescreening method that accelerates systemic surveys of naturally occurring *cis*-regulatory variation among inbred strains. Although the underlying causal variants are not identified by the GASED approach (like any other ASE detection method), such gene survey for regulatory variation is a first step in identifying candidate genes that can be treated as potential targets for MAS.

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