An averaging model for analysis and interpretation of high-order genetic interactions

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ABSTRACT

While combinatorial genetic data collection from biological systems in which quantitative phenotypes are controlled by functional and non-functional alleles of multiple genes (multi-gene systems) is becoming common, a standard analysis method for such data has not been established. A common additive model of the non-functional allele effects contrasted against the functional alleles, based on ANOVA with interaction, has three issues. First, although it is a long tradition in genetics, modeling the effect of the non-functional allele (a null mutant allele) contrasted against that of the functional allele (the wild-type allele) is not suitable for mechanistic understanding of multi-gene systems. Second, an additive model fails in estimation of interactions among more than two genes when the phenotypic response is not linear. Third, interpretation of higher-order interactions defined by an additive model is not intuitive. I propose an averaging model, which is suitable for mechanistic understanding of multi-gene systems: the effect of the functional allele is contrasted against the effect of the non-functional allele for easier mechanistic interpretations; it is stable in estimation of higher-order interactions even when the phenotypic response is not linear; and the higher-order interactions it defines are highly intuitive. Yet, the averaging model is still a general linear model, so model fitting is easy and accurate using common statistical tools.

INTRODUCTION

Accumulation of genetic knowledge in many biological systems and technological advances that made combining multiple genetic loci easier have facilitated combinatorial genetic analysis among multiple genes, each of which has functional ("wild-type") and non-functional (null "mutant") allele states, involved in single quantitative traits, e.g., (1-4), which I here call multi-gene systems. However, conventional genetics is not well built for analysis and interpretation of high-order genetic interactions among multiple genes involved in a single quantitative trait. This is because conventional genetics is an extension of early objectives of analyzing functionally independent and/or qualitative genes. First, comparing multiple mutant phenotypes to the wild-type phenotype does not allow simple mechanistic interpretations. The phenotype of a particular genotype should be compared to the phenotype of the most disrupted mutant state (e.g., a quadruple null mutant in a 4-gene system) for simple mechanistic interpretations.

Second, a method to define and interpret interactions among multiple genes is not definitively integrated. The main topic of this paper concerns this second point. An additive model based on ANOVA with interaction is a simple implementation for analysis of high-order genetic interactions. However, such an additive model fails with a nonlinear system, such as a system with a saturating response, when it contains genetic interactions among more than two genes. This problem is caused by the fact that the additive model requires the conservation of the distributive law ((A + B):C = A:C + B:C, where ":" indicates the interaction defined in the additive model), whereas conservation of this law cannot be assumed in a nonlinear system. We previously proposed a network reconstitution (formerly called signaling allocation) general linear model (NR model), which does not assume conservation of the distributive law, to resolve this problem (2, 5). This was achieved by averaging the interaction terms at each order ("averaging principle"), instead of simply adding them (e.g., *ABC* = A + B + C + (A:B + B:C + C:A) / 3 +A:B:C in the NR model, while *ABC* = A + B + C + A:B + B:C + C:A + A:B:C in the additive model; the italicized upper-case letters denote the genotype carrying wild-type alleles *A*, *B*, and *C*; the intercept is omitted).

I recently realized that the interaction operator in the NR model is not consistent, e.g., in the above example of the *ABC* description, The ":" operators in A:B and A:B:C are not defined in a consistent manner. This was caused by the fact that the 2-gene interaction was defined in the same way in both the NR and additive models, *AB* = A + B + A:B. To resolve this issue, the averaging principle should be extended to the 1-gene effect terms as well: e.g., *ABC* = (A + B + C) / 3 + (A;B + B;C + C;A) / 3 +A;B;C. I call this an averaging model. It is a general linear model with the averaging principle applied consistently. I use a ";" to denote the interaction operator defined in the averaging model ("averaging interaction").

The averaging model does not assume distributivity. Consequently, it can estimate high-order genetic interactions in a stable manner with nonlinear multi-gene systems. This is because each averaging interaction can be defined using only observed values, e.g., A;B;C = *ABC* – (*AB* + *AC* + *BC*) / 3. Since all the interaction terms are defined simply and consistently, the interpretations of averaging interactions are intuitive and consistent: a genetic interaction is the average impact of adding the last gene; note that the above definition of A;B;C is the equivalent of A;B;C = { (*ABC* – *AB*) + (*ABC* – *AC*) + (*ABC* – *BC*) } / 3. I

further demonstrate that the additive and averaging models without interaction can be considered as two extreme approximations in a simple 2-gene system: there is no mechanistic reason to favor one of the models without additional mechanistic information. Since the averaging model does not fail in estimations of high-order genetic interactions, unlike the additive model, I propose the averaging model as a standard general linear model for descriptions of multi-gene system behaviors.

RESULTS AND DISCUSSION

Objective of the study

I define a multi-gene system as one in which multiple genes affect a single quantitative trait while each gene can have two states, functional and non-functional, arising from the wild-type and null mutant alleles, respectively. Such a system necessarily implies a gene network, in which the gene functions are not organized in a series (i.e., not in a single pathway). This is because a series of genes, each of which can only take a functional or non-functional state, can only generate an on-or-off, non-quantitative output. Instead, a gene network must have a converging node(s) to generate a single trait. Converging nodes are sources of complex system behaviors (6, 7). For a data set, I consider the measurement of the quantitative trait as the phenotype and measurements made with exhaustively combinatorial genotypes (i.e., for a *n*-gene system, the number of the exhaustively combinatorial genotypes is 2^n) as the data.

The objective of this study is to best describe the output of such a multi-gene system using the general linear model framework to facilitate mechanistic interpretations of the system behavior. The approach was limited to a general linear model framework due to assumed lack of mechanistic information for more complex (nonlinear) modeling. Fitting a general linear model is computationally easy and accurate. The general linear model could serve as a simple and versatile platform in many cases.

General notation rules

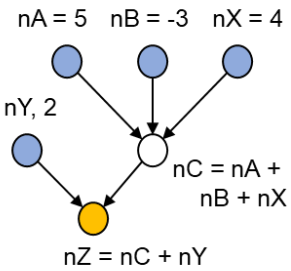
I assume that all the genes of interest are homozygous in diploid organisms. A single gene is denoted by a single alphabetical letter in italics, with upper-case and lower-case letters denoting the wild-type and null mutant alleles, respectively. For example, *ABc* represents the genotype with the wild-type alleles for genes *A* and *B* and the mutant allele for gene *C*. I also use the genotype notation omitting the mutant alleles, such as *AB* instead of *ABc*, for simplicity, clarity, and generalization. The notation of a genotype can represent its phenotype as well. The non-italic lower-case letters, such as a, b, and c, represent the mutant allele effects defined in comparison to the wild-type alleles. The wild-type allele effects, represented by non-italic upper-case letters, such as A, B, and C, are defined in comparison with the mutant allele. The additive effect of A and B is denoted A + B. The additive and averaging interactions between A and B are denoted A:B and A;B, respectively. In a mechanistic network model underlying the observation, the node corresponding to gene A and its output are denoted as nA. Although I often use a

3-gene system, *ABC*, with the intercept term omitted for simple examples, the points discussed subsequently can be generalized to a system consisting of an arbitrary number of genes.

Comparing to the most disrupted state instead of the intact state gives better interpretability

A convention in genetics is to compare a mutant phenotype to the wild-type phenotype. Here I argue that instead, comparing a phenotype of any genotype to the phenotype of the most disrupted state, e.g., comparing to the triple mutant state in a 3-gene system, leads to much better mechanistic interpretations. For a simple example, I use a system defined by an ANOVA-based, 3-gene additive model, i.e., assuming a linear phenotypic response (although I will later discuss a linear assumption issue).

a



b

Genotype	Z value
ABC	8
aBC	3
AbC	11
ABc	2
abC	6
aBc	2
Abc	2
abc	2

c

Effect or Interaction	ANOVA-lof
ABC (intercept)	8
a	-5
b	3
c	-6
a:b	0
a:c	5
b:c	-3
a:b:c	0

d

Effect or Interaction	ANOVA-GOF
abc (intercept)	2
A	0
B	0
C	4
A:B	0
A:C	5
B:C	-3
A:B:C	0

Fig. 1. A simple network behavior can be well described by the wild-type allele effects of a multi-gene system but not by the mutant allele effects. (a) A mechanistic model of a network containing 3 nodes that can be mutationally manipulated (a 3-gene system). The network consists of 6 nodes, among which nA, nB, and nC are mutationally manipulable and nX, nY, and nZ are not. The output of each node is given either as a value or an equation. The output of nZ is the quantitative phenotype of the system. (b) The phenotype values of all 8 combinatorial genotypes. (c) The values for the mutant allele effects and interactions. (d) The values for the wild-type allele effects and interactions.

Fig. 1a shows a mechanistic network underlying a system with 6 nodes, in which three nodes (nA, nB, and nC) can be manipulated by mutations and the other three (nX, nY, and nZ) cannot. Thus, for the purpose of genetic analysis, this is a 3-gene system. nA, nB, nX, and nY are input nodes, and their values are arbitrarily set at 5, -3, 4, and 2, respectively. nZ is the output node, and the output of nZ can be measured as the quantitative trait of the system. Simple additive rules at nodes nC and nZ are assumed, nC = nA + nB + nX and nZ = nC + nY, respectively. Fig. 1b shows the nZ output (i.e., phenotype) of 8 exhaustively combinatorial genotypes. Fig. 1c shows the effects and interactions of the mutant alleles that are calculated according to an ANOVA model with interaction. Fig. 1d shows the effects and interactions of the wild-type alleles that are calculated according to an ANOVA model with interaction. With Fig. 1d, it is easy to reconstitute the mechanistic network shown in Fig. 1a: there is a basal activity

of 2 in the absence of A, B, and C; A and B are not active by themselves, while C has its own activity of 4 regardless of A and B; the connection between A and C is positive with a value of 5, and the connection between B and C is negative with a value of -3; No A:B:C interaction means that additive effects up to two-gene interactions can explain the system behavior completely. In comparison, mechanistic interpretations based on Fig. 1c are not simple: e.g., it is not easy to decipher that B is completely dependent on C from *ABC* = 8, b = 3, c = -6, and b:c = -3. I conclude that the behavior of a multi-gene system should be interpreted using wild-type allele effects. I will subsequently model a system with wild-type allele effects and their interactions.

Derivation of the averaging model

See Text S1 for details of this section. The additive model requires the distributive law but this law is violated when the system behavior is nonlinear. A typical biological system has a limited phenotype range. When the sizes of the effects and interactions of the genes in a multi-gene system are substantial compared to the phenotype range, the system behavior is nonlinear, and the distributive law is violated - consequently the additive model fails. The NR model (2, 5) allows non-distributivity. However, the definitions of the 2-gene interaction and of the 3-or-more-genes interaction were not consistent in the NR model. The averaging model was derived by extending the definition of the 3-or-more-genes interaction in the NR model to the 2-gene interaction. Thus, the averaging model allows non-distributivity, and its interaction definition is consistent regardless of the number of genes involved. The three models and a 1-way ANOVA model for each genotype are all general linear models with different ways to decompose the fitted values. When the models are fit to data with replication as statistical models, their fitted values and residuals are identical. Therefore, subsequently I sometimes use only the mean estimates of the models for model comparisons (such as Fig. 2).

Unlike the additive model, the averaging model stably estimates high-order genetic interactions

The situation in which the additive model fails due to a limited phenotype range was simulated using a 7-gene system. A system with many genes was used because it shows the problem more clearly. With 7 genes, the number of combinatorial genotypes is $2^7 = 128$. A simulated mean phenotype value for each genotype was randomly sampled from a uniform distribution ranging from 1 to 10, and each model was solved using these randomly generated data values. This simulation procedure was repeated 10,000 times and the model estimate distributions, except for the model intercept (i.e., the septuple mutant value), were visualized as a box plot (Fig. 2). Fig. 2a shows that in the additive model, the higher the order of interactions is, the higher the representations of the interactions are. The length of the box (the difference between the 75th and 25th percentiles) of the 7-gene interaction is about 7.5 times larger than those of the 1-gene effects and about 5 times larger than the phenotype range. Therefore, if the additive model is used, the absolute values of higher order additive interactions are grossly overestimated in general. This problem was solved in the NR model, except for the 2-gene interactions, which were still additive interactions (Fig. 2b). Note the *y*-axis scale difference between Fig. 2a and Figs.

2b and 2c: the distributions of the 1-gene effects are essentially the same across the models. The distributions of estimates were very consistent across all the effects and averaging interactions in the averaging model (Fig. 2c). These results strongly suggest that the averaging interactions that do not assume distributivity can be stably estimated even when the system behavior is nonlinear.

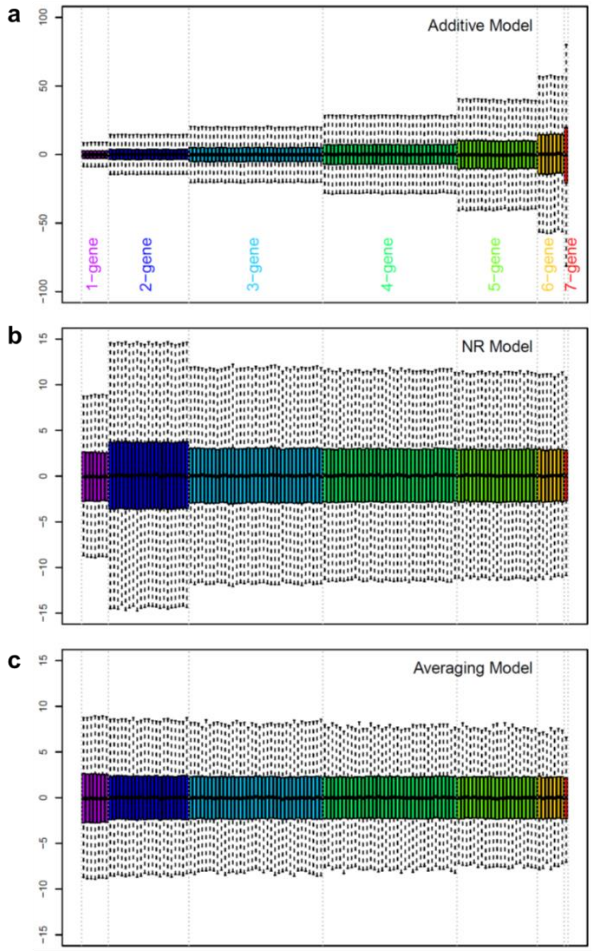


Fig. 2. Distributions of the gene effects and interaction values when the phenotype values were randomly sampled from a uniform distribution with (a) additive, (b) NR, or (c) averaging models. Each order of interactions is color-coded separately, and the color coding is shown at the bottom of (a). Note that the scales in the y-axes are much larger in (a) than in (b) or (c).

To investigate a situation with actual biological data, we reanalyzed our previously published data set with a 4-gene system (2) using the additive, NR, and averaging models (Fig. 3). Here we focus on the behaviors of the model estimates from the three models. Biological information about the data set is described later. Note that the data set had replicated observations for each combinatorial mutant, the models were fit to the data (instead of solving), and the confidence intervals of the model estimates were obtained. The 95% confidence intervals of the full additive models (which include up to the 4-gene interaction) were evidently wider with high-order interactions in the additive model (Fig. 3a, black bars). This problem of uneven confidence interval sizes was moderated in the NR model (Fig. 3b) and not noticeable in the averaging model (Fig. 3c). Furthermore, in the additive model, since the effect sizes of higher order interactions were overestimated, the estimates for the 1-gene effect and lower order interactions were substantially affected when higher-order interactions were omitted (model reduction; Fig. 3a, compare red to black and blue to red). In addition, the 95% confidence interval sizes of the same

terms were affected by model reduction in the additive model. These problems in model reduction were moderated in the NR model, and almost non-existent in the averaging model (Figs. 3b and 3c). In summary, the problems associated with non-distributivity in this biological data set are evident in the additive model while the averaging model appears free of these problems. These observations made with a biological data set directly corroborate with the simulation results in Fig. 2

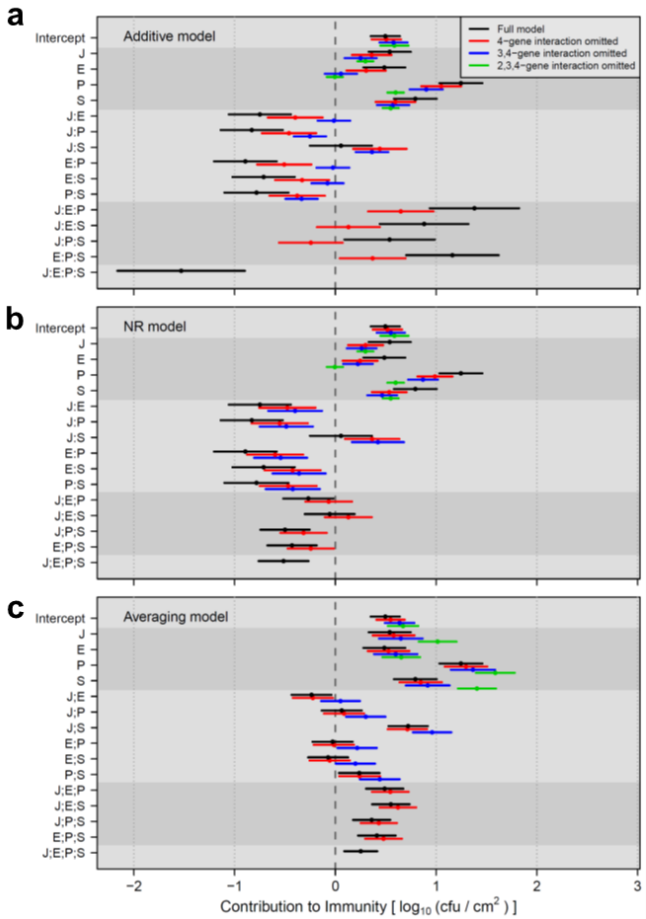


Fig. 3. Coefficient estimates for the contribution to immunity using the data from Tsuda et al. in (a) additive, (b) NR, and (c) averaging models. The 95% confidence interval is shown as a horizontal bar, with the mean as a point. Different levels of model reduction (omitting higher order interactions from the model) are color-coded according to the color code in (a). Different shades of gray background are used to show different orders of interactions. ":" and ";" indicate additive and averaging interactions, respectively.

Why does the averaging model describe a multi-gene system better than the additive model?

In the 2-gene additive interaction, A:B = $AB - (A + B)$, the 2-gene additive interaction A:B can be estimated using only observed values, i.e., estimation of A:B can be independent of other model estimates. Essentially, an error originated from the nonlinearity of data is absorbed by the 2-gene additive interaction A:B. However, estimation of a 3-gene additive interaction, A:B:C = $ABC - (A + B + C)$ - (A:B + A:C + B:C), is not independent of other model estimates, A:B, A:C, and B:C. In a nonlinear system, there is no guarantee that the value of A:B in the 3-gene model is the same as that of A:B estimated by the 2-gene model. Thus, on average, higher-order interactions accumulate errors from lower-order interactions in the additive model with a nonlinear system, which was demonstrated in simulation and analysis of an actual data set (Figs. 2a and 3a).

On the other hand, any order of averaging interactions can be estimated using only observed values, e.g., A;B;C = *ABC* – (*AB* + *AC* + *BC*) / 3, so their estimations can be independent of other model estimates. Thus, nonlinearity-originated errors are confined to individual averaging interactions and do not accumulate toward higher-order interactions (Figs. 2c and 3c). This is the reason the additive model including 3-or-more-gene interactions fails with nonlinear systems while the averaging model does not.

Interpretation of the averaging model outcome

It should be emphasized that the definitions of the additive and averaging interactions are different, and consequently their interpretations are different. The 2-gene additive interaction is understood as the difference from the addition of the 1-gene effects, A:B = *AB* – (A + B). When A, B, A:B > 0, A and B have a synergistic effect. When A, B > 0, A:B < 0, A and B have a compensating effect (Fig. 4a). However, synergistic or compensating interpretations of additive interactions become unclear when A and B have opposite signs (Fig. 4b) or the additive interactions are higher-order, e.g., A:B:C = *ABC* - (A + B + C + A:B + A:C + B:C) (Fig. 4c).

In contrast, the interpretation of averaging interactions is consistent and highly interpretable at all orders of the averaging interactions, i.e., the averaging model is highly scalable to the number of genes in the system. An averaging interaction is the deviation of the corresponding genotype from the average of all involved genotypes that have one gene fewer. For example, in a 2-gene system, A;B = *AB* – (*A* + *B*)/2 (Fig. 4d). Note that not just the values but also the signs of the interactions could be different between the additive and averaging interactions (compare *AB* (case 1) in Figs. 4a and 4d). The interpretations of averaging interactions are consistent even when A and B have opposite signs (Fig. 4e) or in the case of higher-order averaging interactions, e.g., A;B;C = *ABC* – (*AB* + *AC* + *BC*) / 3 (Fig. 4f).

A 3-gene averaging interaction, A;B;C = *ABC* – (*AB* + *AC* + *BC*) / 3, (Fig. 4d) could be a 3-gene interaction in a 7-gene system, A;B;C = *ABCdefg* – (*ABcdefg* + *AbCdefg* + a*BCdefg*) / 3. Thus, a genotype notation that omits the mutant alleles is a generalized notation for the averaging model.

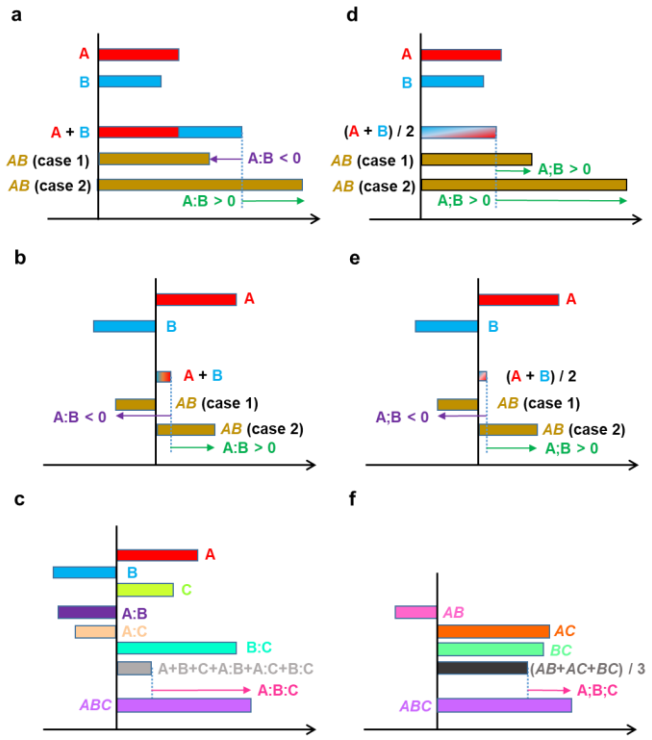


Fig. 4. Interpretations of interactions in (a-c) additive and (d-f) averaging models. (a, d) Two-gene interactions when both 1-gene effects A and B are positive. Two different cases (cases 1 and 2) of the *AB* phenotype values are used. (b, e) Two-gene interactions when the 1-gene effects have opposite signs. (c, f) Three-gene interactions. ":" and ";" indicate additive and averaging interactions, respectively.

An averaging model-based multi-gene analysis should only contain genes significantly involved in the phenotype.

Since the averaging interaction is the phenotypic deviation of the corresponding genotype from the average of all genotypes with one gene fewer, it is affected if the analysis includes unnecessary genes. Such unnecessary genes can be detected by comparing all the genotypes containing the gene in question to the corresponding genotypes without the gene. For example, in a 3-gene system with genes *A*, *B*, and *C*, the test for whether gene *C* should be included is whether any of *ABC* – *AB*, *AC* – *A*, *BC* – *B*, and *C* – *abc* have values significantly different from 0. If none of them are significant, gene *C* should be removed from the averaging model.

Reinterpretation of previous results using the averaging model

Using the averaging model, I reinterpreted results from my laboratory of exhaustively combinatorial genotype analysis in a 4-gene system, which were originally analyzed using the NR model shown in Fig. 4 of (2). The study consisted of four cases of inducible immunity in the model plant Arabidopsis against strains of the bacterial pathogen *Pseudomonas syringae*, which are designated as the AvrRpt2-ETI, AvrRpm1-ETI, flg22-PTI, and elf18-PTI cases. ETI is Effector-Triggered Immunity, and AvrRpt2 and AvrRpm1 are triggering effectors (8-12). PTI is Pattern-Triggered Immunity, and flg22 and elf18 are triggering molecular patterns (13-15). The inhibition of bacterial growth in the plant leaf, in

$\log_{10}$(cfu/cm^2), was the immunity phenotype measure. The hub genes of four major signaling sectors (subnetworks) in the plant immune signaling network were subjected to mutational analysis. The signaling sectors were the jasmonate, ethylene, PAD4, and salicylate sectors, which are indicated as J, E, P, and S, respectively. I also call their hub genes *J*, *E*, *P*, and *S*, in this context of analysis of the 4-gene system. Biological and experimental details are provided in (2).

Each of the AvrRpt2-ETI, AvrRpm1-ETI, flg22-PTI, and elf18-PTI cases was first tested to determine whether all four genes were significantly involved in the phenotype variation. Except for the elf18-PTI case, all four genes were significant, and the averaging model for the 4-gene system was used. In the elf18-PTI case, the phenotype was not significantly affected by the *J* gene in any genotype context, so the averaging model for the 3-gene system with the *E*, *P*, and *S* genes was used. The results of applications of the averaging model to these four immunity cases are shown in Fig. 5.

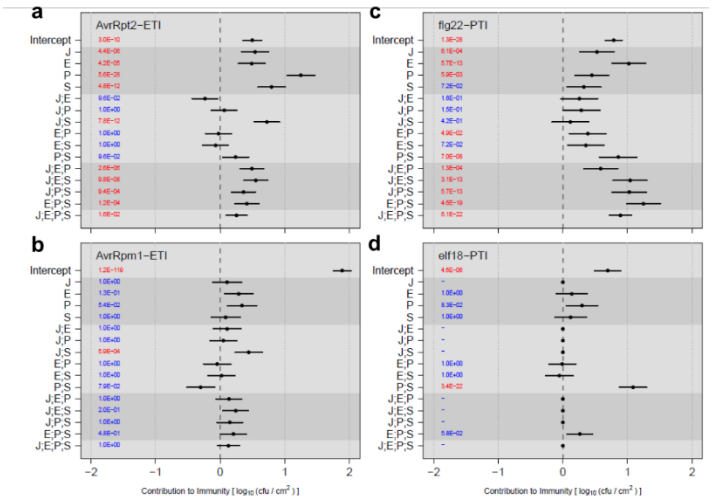


Fig. 5. Coefficient estimates for the contribution to immunity from averaging model analysis of the data in Tsuda et al. (a) AvrRpt2-ETI, (b) AvrRpm1-ETI, (c) flg22-PTI, and (d) elf18-PTI. The 95% confidence intervals are shown as horizontal bars, with the means as points. The Holm-corrected *p*-values are shown in the left part of each plot: red, $p < 0.05$; blue, $p \geq 0.05$. The dataset used for AvrRpt2-ETI in Fig. 5a is the same as that in used in Fig. 3, and Fig. 5a is the same as the full model (black lines) in Fig. 3c. ";" indicates the averaging interaction.

In AvrRpt2-ETI with the averaging model (Fig. 5a), the values for the 1-gene effects were all positive. P had the largest effect, which was close to the wild-type level. Among the 2-gene averaging interactions, only J;S was significantly positive: while the J and S effects are both positive, combining these two genes together (*JS* genotype) increases the immunity from the average of the *J* and *S* genotypes, which makes the *JS* phenotype similar to wild type.

In AvrRpm1-ETI with the averaging model (Fig. 5b), most immunity was explained by the intercept (i.e., the immunity level in the *jeps* genotype), showing that the quadruple mutant still maintains most of the immunity of wild-type plants. This observation can be explained by the fast kinetics of AvrRpm1-ETI signaling compared to AvrRpt2-ETI, in respect to the gating timing of the ETI-Mediated and PTI-Inhibited Sector (EMPIS) by PTI signaling (16). Although all the 1-gene effects and the averaging interactions had lower amplitudes, their general up and down trends were similar to those of AvrRpt2-ETI, suggesting that the 4-gene network apart from EMPIS behaves similarly in AvrRpm1-ETI and AvrRpt2 ETI. J;S was the only significant averaging interaction with a positive contribution to immunity. In addition, the positive P effect was marginally significant (Holm-corrected $p = 0.054$).

In flg22-PTI (Fig. 5c), all the 1-gene effects except S were significantly positive with E as the highest. The 2-gene averaging interactions were largely low and/or not significant, except P;S, which was significantly and strongly positive. The 3-gene and 4-gene averaging interactions were significantly and strongly positive, indicating that all the genes substantially increase the immunity level when added to the system as the 3rd or 4th genes.

In elf18-PTI (Fig. 5d), only P;S was significant among all the averaging model terms, except the intercept. The P;S averaging interaction was strongly positive, indicating that a single mutation in genes *P* or *S* almost completely abolishes elf18-PTI. The difference in the importance of the *E* gene clearly separated flg22-PTI and elf18-PTI. Another difference between flg22-PTI and elf18-PTI was the 3-gene and 4-gene averaging interactions. All were strongly positive in flg22-PTI, and none were significant in elf18-PTI.

It is noteworthy that the roles of J;S and P;S were very different in ETI and PTI. A strongly positive P;S averaging interaction was observed in PTI (Figs. 5c and 5d). Positive functional interactions between the *P* and *S* genes have been well documented in many aspects of plant immunity (17). In contrast, this averaging interaction was insignificant in ETI, except for their contributions through higher-order averaging interactions, J;P;S, E;P;S, and J;E;P;S. On the other hand, a strongly positive J;S averaging interaction was observed in ETI while it was insignificant in PTI (Fig. 5). Although negative functional interactions between the *J* and *S* genes are often described in plant immunity (17), these two genes interact positively in ETI (Figs. 5a and 5b). In addition, in flg22-PTI the 3-gene and 4-gene averaging interactions were strongly positive while they were moderately positive in AvrRpt2-ETI. A disadvantage of strongly positive 3-gene and 4-gene averaging interactions is that a mutation(s) in one or two genes results in large loss of immunity. Relatively weak 3-gene and 4-gene averaging interactions in ETI indicates that ETI is more resilient against damage to one or two of these major immune signaling sectors, which could be caused by pathogen effectors (7). In summary, the averaging model analysis highlighted that while the 4-gene system is important in both ETI and PTI (with flg22), the way they are used in ETI and PTI is quite different, and ETI is more resilient than PTI against perturbations to the signaling sectors. It also highlighted substantial differences, particularly in the roles of the *J* and *E* genes, in regulation between flg22-PTI and elf18-PTI.

Mechanistic basis of the additive and averaging models

To provide a concrete idea about what the additive and averaging models without interaction represent mechanistically, I use the following idealized 2-gene signaling network based on chemical reactions. Two genes *A* and *B* qualitatively (i.e., on or off) control two input nodes nA and nB of an output node nC (i.e., directed links from nA and nB converging on nC). The activity of each node is represented by the corresponding chemical, A, B, or C, and the chemical reactions involved are A + X → C and B + Y → C. We assume an equilibrium (or a steady state) for the reactions, and the concentration relationships, [X], [Y] >> [A], [B], [C], i.e., [X] and [Y] can be considered constant in the reactions. Under these conditions, we can describe the relationships among [A], [B], and [C] as: k_A[A] = [C] and k_B[B] = [C], where k_A and k_B are

positive constants. Then the additive and averaging models without interaction can well approximate the system behavior when k_A, k_B >> 1 (i.e., [C] >> [A],[B]) and when k_A, k_B << 1 (i.e., [C] << [A],[B]), respectively (Text S2). This discussion of an idealized system shows that the additive and averaging models without interaction could represent two opposing extreme approximations of the underlying mechanistic state. Therefore, one of the models cannot be favored over the other unless mechanistic information about the system is available in addition to the phenotypic values.

Limitation in mechanistic interpretations with a general linear model

The chemical reaction system discussion in the last section also shows that when the actual values of k_A and k_B are between the extreme values, both models handle the deviation with the interaction, A:B or A;B (with opposite signs). Note that the interaction is a consequence of shared [C] between two reactions: one reaction affects the other, i.e., it is truly a mechanistic interaction. However, the interaction value from a model cannot reveal the nature of the interaction – we can interpret it mechanistically in this example only because we know the underlying system mechanism. This example illustrates a limitation to describing a nonlinear system with a general linear model in terms of mechanistic interpretations based on the model. However, it should also be reemphasized that the genetic interpretations of higher-order interactions are always clear with the averaging model while this is not the case with the additive model.

Concluding Remarks

I have demonstrated that multi-gene systems subjected to exhaustively combinatorial mutation analysis typically violate the distributive law due to their nonlinearity and that therefore, the additive model that requires the conservation of the distributive law is not appropriate for analysis of such systems. In contrast, an averaging model allows non-distributivity and maintains consistency from the 1-gene effects to the highest order of averaging interactions. Furthermore, averaging model results are consistently and intuitively interpretable from the 1-gene effects to the highest order of averaging interactions. I propose the averaging model as a standard general linear model for combinatorial mutation analysis of multi-gene systems.

METHODS

Data sets

Biological data sets used in this study are the same data sets used in Figs. 4A and 4B in Tsuda et al. (2009). Each data set consists of bacterial counts ($\log_{10}$(colony forming units/cm^2)) for 16 exhaustively

398 combinatorial genotypes for a 4-gene system, with or without treatment, with replication. Since the raw
399 bacterial count data were not published previously, they are provided as Supplemental Dataset 1.
400
401
402 *Random simulation with three models*
403
404 The simulation was performed with a 7-gene system. The phenotype values for 2^7 = 128 genotypes were
405 randomly sampled from a uniform distribution ranging from 1 to 10. The 128 phenotype values were
406 solved for the coefficients (gene effects and interactions) in each of the additive, NR, and averaging
407 models. To solve the 128 equations per model, the 7-gene system matrix equivalent of the 3-gene
408 system matrix in Fig. TS1.1 (Text S1) was used (the matrices are provided in an R workspace file in
409 Supplemental Dataset 2). This procedure was repeated 10,000 times for each model, and the
410 distribution of each coefficient (except the intercept) across the repeats is shown by a box-and-whiskers
411 in Fig. 2.
412
413
414 *Fitting averaging models to the data*
415
416 A linear mixed-effect model (the lme function in the nlme R package (18)) was used. This was because (i)
417 each data set has factors regarding the experimental design, which were included as random effects in
418 the model and (ii) the numbers of replicates were not the same across the genotype x treatment
419 combinations. First, a linear mixed-effect model with the genotype x treatment interactions was fit to
420 each of the data sets for "AvrRpt2-ETI", "AvrRpm1-ETI", "flg22-PTI", and "elf18-PTI". The formula for the
421 fixed effects was "`~ genotype/treatment -1`". The random effects for the data sets were "~
422 `1|replicate/flat/pot`". The interaction coefficients of the linear mixed-effect model were used
423 to test whether each gene is significant. For example, to test the significance of the *J* gene, the estimate
424 differences, *JEPS – EPS*, *JEP – EP*, *JPS – PS*, *JES – ES*, *JE – E*, *JP – P*, *JS – S*, and *J – jeps* were subjected to *t*-
425 tests using the associated standard errors calculated from the variance/covariance matrix and the
426 residual degree of freedom. The *p*-values from all *t*-tests for a single data set were correct by the
427 Benjamini-Hochberg FDR. If none of the corrected *p*-values were smaller than 0.05 for the gene, the
428 gene was designated insignificant and omitted from the following averaging model analysis. Only the *J*
429 gene in "elf18-PTI" was insignificant. In this case, the data were bundled by ignoring the *J* gene. For
430 example, the *JEPS* data were considered as part of the *EPS* data.
431
432 Second, the averaging model using the significant genes was fit. The 4-gene system equivalent matrix of
433 the 3-gene system matrix in Fig. TS1.1c (Text S1) or the 3-gene system matrix was used (the matrices are
434 provided in an R workspace file in Supplemental Dataset 2). The rows were replicated according to the
435 genotypes of the observations (the design matrix for the averaging model coefficients, denoted as "m."),
436 Using the design matrix m., the fixed effects were, "`~ m. -1 + genotype`" and the random effects
437 were, "`~ 1|replicate/flat/pot`" in the lme function. The averaging model coefficient estimates,
438 their standard errors, and the *p*-values were extracted from the coefficient table of the lme model. The
439 estimates, the standard errors, and the residual degree of freedom of the lme model were used to

calculate the 95% confidence intervals. The *p*-values were subjected to the Holm multiple tests correction. The R script used to generate Fig. 5 from the raw bacterial count data sets is provided as Supplemental Dataset 3. Supplemental Datasets are available from https://github.com/fumikatagiri/Averaging_Model.

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FIGURE LEGENDS

Fig. 1. A simple network behavior can be well described by the wild-type allele effects of a multi-gene system but not by the mutant allele effects. (a) A mechanistic model of a network containing 3 nodes that can be mutationally manipulated (a 3-gene system). The network consists of 6 nodes, among which nA, nB, and nC are mutationally manipulable and nX, nY, and nZ are not. The output of each node is given either as a value or an equation. The output of nZ is the quantitative phenotype of the system. (b) The phenotype values of all 8 combinatorial genotypes. (c) The values for the mutant allele effects and interactions. (d) The values for the wild-type allele effects and interactions.

Fig. 2. Distributions of the gene effects and interaction values when the phenotype values were randomly sampled from a uniform distribution with (a) additive, (b) NR, or (c) averaging models. Each order of interactions is color-coded separately, and the color coding is shown at the bottom of (a). Note that the scales in the y-axes are much larger in (a) than in (b) or (c).

Fig. 3. Coefficient estimates for the contribution to immunity using the data from Tsuda et al. in (a) additive, (b) NR, and (c) averaging models. The 95% confidence interval is shown as a horizontal bar, with the mean as a point. Different levels of model reduction (omitting higher order interactions from the model) are color-coded according to the color code in (a). Different shades of gray background are used to show different orders of interactions. ":" and ";" indicate additive and averaging interactions, respectively.

Fig. 4. Interpretations of interactions in (a-c) additive and (d-f) averaging models. (a, d) Two-gene interactions when both 1-gene effects A and B are positive. Two different cases (cases 1 and 2) of the *AB* phenotype values are used. (b, e) Two-gene interactions when the 1-gene effects have opposite signs. (c, f) Three-gene interactions. ":" and ";" indicate additive and averaging interactions, respectively.

527 Fig. 5. Coefficient estimates for the contribution to immunity from averaging model analysis of the data
528 in Tsuda et al. (a) AvrRpt2-ETI, (b) AvrRpm1-ETI, (c) flg22-PTI, and (d) elf18-PTI. The 95% confidence
529 intervals are shown as horizontal bars, with the means as points. The Holm-corrected *p*-values are
530 shown in the left part of each plot: red, $p < 0.05$; blue, $p \geq 0.05$. The dataset used for AvrRpt2-ETI in Fig.
531 5a is the same as that in used in Fig. 3, and Fig. 5a is the same as the full model (black lines) in Fig. 3c. ";"
532 indicates the averaging interaction.
533
534
535 **SUPPLEMENTAL TEXTS**
536 Text S1. Derivation of the averaging model and non-distributivity of the data
537
538 Text S2. Averaging and additive models without interaction are two extreme approximations of the
539 behavior of a two-input chemical signaling system
540
541
542 **SUPPLEMENTAL DATASETS**
543 Supplemental Datasets are available from https://github.com/fumikatagiri/Averaging_Model.
544
545 Supplemental Dataset 1. A folder containing four bacterial count data files (tab-delimited text) for
546 "AvrRpt2_ETI", "AvrRpm1_ETI", "flg22_PTI", and "elf18_PTI". Each has columns of genotype, treatment,
547 replicate, flat, pot, and colony. The colony column has log10-transformed colony counts (colony forming
548 unit/cm2). Although the summarized data were originally reported in (2), these raw data were not
549 published.

550 Supplemental Dataset 2. A .RData file (R workspace file) containing a list object, "ave.model.mats",
551 which contains the matrices for the averaging model for 2- to 7-gene systems (equivalents of matrix in
552 Fig. TS1.1 in Text S1 for different number-gene systems).

553 Supplemental Dataset 3. An R script file (.r file), which was used to generate Fig. 5 from the data in
554 Supplemental Dataset 1 (the data should be in the subfolder "Supplemental.Dataset1" as in this folder).
555 In addition, an empty "outputs" subfolder is required for the outputs of this script (the "outputs"
556 subfolder in this folder is already populated with the actual outputs of the script). The outputs of the
557 script includes "reanalyzed.w.average.model....pdf", which is Fig. 5. The script includes algorithms for
558 selecting significant genes for the analysis and the averaging model. In the script, the object of a 3-gene
559 or 4-gene matrix included in Supplemental Dataset 2 is called "rec.mx", which is generated by a
560 function, "make.rec.mx".

561

Text S1. Derivation of the averaging model and non-distributivity of the data

Laws of algebra

An additive model of gene effects and interactions involves two operators: additive, "+", and interactive, ":". Different models can be derived if we assume different laws for these operations. Three types of laws define algebra involving two operators: commutative, associative, and distributive laws. The commutative laws are A + B = B + A and A:B = B:A. The associative laws are (A + B) + C = A + (B + C) and (A:B):C = A:(B:C). The distributive law is (A + B):C = A:C + B:C.

I assume the commutative laws for both "+" and ":" because a single quantitative phenotype cannot experimentally distinguish A + B from B + A or A:B from B:A. I also assume the associative law for "+" since without this assumption the general linear model framework cannot be used.

The associative law for ":" is also required for the general linear model framework (see below). However, as I show below, the impact of a violation of the associative law for ":" can be moderated using an averaging principle, in which the arithmetic mean of multiple different expressions for the same quantity is taken as the true value of the quantity. This moderation by the averaging principle is important in applications of the general linear model framework to multi-gene systems because we cannot generally assume that the associative law for ":" holds. For example, in the case of Fig. 1 in the main text, A:B = 0, and thus, (A:B):C = 0. However, B:C ≠ 0, so, A:(B:C) may not be 0 particularly when A:C ≠ 0. Thus, (A:B):C ≠ A:(B:C), i.e., a violation of the associative law, could happen.

In the following sections, I will show that the distributive law is required in the additive model. I will also show that a range-limiting nonlinearity of a system, such as a saturation response, would violate the distributive law. Such responses are common in biological systems. I will also show that there is a general linear model that allows violation of the distributive law under the assumption of the averaging principle. I call this model an averaging model.

Derivation of the averaging model

The part of the following discussion describing derivation of the NR model was modified from Text S1 in (1). In this section, for simplicity, the intercept value (i.e., the phenotype value for the most disrupted state) is subtracted from all the measured values so that the intercept value is 0.

According to the additive model, I assume *AB* = A + B + A:B … (1) as the starting point. In this case, the interaction is the deviation of the corresponding genotype from arithmetic addition of the 1-gene effects. Let's extend this to a system consisting of three genes *A*, *B*, and *C*. The phenotype *ABC* can be considered as being expressed in three different ways: adding C to the genetic background of *AB*; adding A to the genetic background of *BC*; or adding B to the genetic background of *CA*.
By adding C to *AB*, the genotype *ABC* is expressed as:

$ABC = AB + C + AB{:}C = (A + B + A{:}B) + C + AB{:}C = A + B + C + A{:}B + AB{:}C$ … (2)

If the distributive law is not assumed, (2) cannot be simplified.

If the distributive law is assumed, (2) can be simplified to:

$ABC = A + B + C + A{:}B + (A + B + A{:}B){:}C = A + B + C + A{:}B + B{:}C + C{:}A + (A{:}B){:}C$ … (3)

Similarly, if the distributive law is not assumed:

By adding A to *BC*, $ABC = A + B + C + B{:}C + BC{:}A$ … (4)

By adding B to *CA*, $ABC = A + B + C + C{:}A + CA{:}B$ … (5)

If the distributive law is assumed:

By adding A to *BC*, $ABC = A + B + C + A{:}B + B{:}C + C{:}A + A{:}(B{:}C)$ … (6)

By adding B to *CA*, $ABC = A + B + C + A{:}B + B{:}C + C{:}A + B{:}(C{:}A)$ … (7)

Although the expressions are not the same, (3), (6), and (7) must be the same in a model to explain *ABC*. Therefore, for this model framework to work exactly, the associative law for the interaction operator ":" is necessary,

$(A{:}B){:}C = A{:}(B{:}C) = B{:}(C{:}A) = A{:}B{:}C$ … (8)

If (8) is true, (3), (6), and (7) become the same expression:

$ABC = A + B + C + A{:}B + B{:}C + C{:}A + A{:}B{:}C$ … (9)

(9) is the additive model for three genes. This can be extended to a system consisting of more genes. In summary, the additive model is a good description of a multi-gene system if the associative law for ":" and the distributive law are conserved.

However, as discussed above, the associative law cannot be generally assumed for the ":" operator in a multi-gene system. This contradiction about associativity indicates a failure of the general linear model as a general description of a multi-gene system. A compromise to maintain the general linear model framework is to define *ABC* as the arithmetic mean of (3), (6), and (7):

$$ABC = [\{A + B + C + A{:}B + B{:}C + C{:}A + (A{:}B){:}C\} + \{A + B + C + A{:}B + B{:}C + C{:}A + A{:}(B{:}C)\} + \{A + B + C + A{:}B + B{:}C + C{:}A + B{:}(C{:}A)\}] / 3$$

$$= A + B + C + A{:}B + B{:}C + C{:}A + \{(A{:}B){:}C + A{:}(B{:}C) + B{:}(C{:}A)\} / 3 \quad \text{… (10)}$$

I call this practical approach to avoiding the contradiction in the general linear model by averaging all possible cases the averaging principle.

Since $\{(A{:}B){:}C + A{:}(B{:}C) + B{:}(C{:}A)\}$ cannot be expressed by the lower order terms, A, B, C, A:B, B:C, and C:A, it is reasonable to define $A{:}B{:}C = \{(A{:}B){:}C + A{:}(B{:}C) + B{:}(C{:}A)\} / 3$ … (11). Then,

$ABC = A + B + C + A{:}B + B{:}C + C{:}A + A{:}B{:}C$ … (9)

Thus, with the averaging principle, the additive model can conform to the assumption of no associativity in the interaction operator ":". This can be extended to a system consisting of more genes. In summary, the additive model should be a reasonable description of a multi-gene system if the distributive law holds.

If the distributive law cannot be assumed, (2), (4), and (5) must still be the same to express *ABC*. Here again we observe a failure of the general linear model as a general description of a multi-gene system. I apply the averaging principle to (2), (4), and (5) to express *ABC*:

$ABC = [\{A + B + C + A{:}B + AB{:}C\} + \{A + B + C + B{:}C + BC{:}A\} + \{A + B + C + C{:}A + CA{:}B\}] / 3$

= A + B + C + (A:B + B:C + C:A) / 3 + (*AB*:C + *BC*:A + *CA*:B) / 3 … (12)

Since (*AB*:C + *BC*:A + *CA*:B) cannot be expressed by the lower order terms, A, B, C, A:B, B:C, and C:A, it is reasonable to define A;B;C = (*AB*:C + *BC*:A + *CA*:B) / 3 … (13). I use the semicolon ";" to distinguish this different definition of interaction from that of the interaction in the additive model and call ";" the averaging interaction operator and ":" the additive interaction operator.

ABC = A + B + C + (A:B + B:C + C:A) / 3 + A;B;C … (14)

Therefore, if the distributive law is not assumed, the average of the 2-gene additive interactions should be used to express the all wild-type allele state of *ABC*. In general, the rule that the terms in each order of the interactions (2-gene additive interactions, 3-gene averaging interactions, 4-gene averaging interactions, …) must be averaged can be derived by extending this to a system with more genes. For example, with a system consisting of 4 genes, *A*, *B*, *C*, and *D*:

ABCD = A + B + C + D + (A:B + A:C + A:D + B:C + B:D + C:D) / 6 + (A;B;C + A;B;D + A;C;D + B;C;D) /4 + A;B;C;D … (15)

This is the NR model (2) (previously called the signaling allocation model (1)). Note that in the NR model, 2-gene interactions are additive interactions while 3- or higher-order interactions are averaging interactions.

The assumption of non-distributivity does not require any more changes in the model. However, the above derivation of the NR model began with an arbitrary definition of the 2-gene additive interaction, *AB* = A + B + A:B … (1), which is the reason the NR model is a mixture of additive and averaging interactions. The model would be more mathematically consistent if the averaging interaction definition were extended to 1-gene effect terms to make the 2-gene interactions averaging interactions as well, i.e., *AB* = (A + B) / 2 + A;B … (16). I demonstrate in Figs. 2 and 3 in the main text that (16) is indeed required for mathematical consistency of the model.

By applying (16):

ABC = (A + B + C) / 3 + (A;B + B;C + C;A) / 3 + A;B;C … (17)

ABCD = (A + B + C + D) / 4 + (A;B + A;C + A;D + B;C + B;D + C;D) / 6 + (A;B;C + A;B;D + A;C;D + B;C;D) /4 + A;B;C;D … (18)

Now the rule is that the terms in each order of the interactions, including 1-gene effects (the first order), must be averaged. (17) and (18) are equivalents of:

ABC = (*AB* + *BC* + *CA*) / 3 + A;B;C … (19)

A;B;C = *ABC* - (*AB* + *BC* + *CA*) / 3 … (19)' (variated from (19))

ABCD = (*ABC* + *ABD* + *ACD* + *BCD*) /4 + A;B;C;D … (20)

A;B;C;D = *ABCD* - (*ABC* + *ABD* + *ACD* + *BCD*) /4 …(20)' (variated from (20))

From (19)' and (20)', the highest order averaging interaction is defined as the deviation of the corresponding genotype from the average of all genotypes with one gene fewer. This definition of the averaging interaction is highly interpretable. I call this extended model with all averaging interactions an averaging model. With the definitions of the averaging interactions of different orders in (16), (19), and (20), it is clear that the averaging model does not require the distributive law because these definitions do not include any terms that could be affected by whether the distributive law holds or not.

Note that the mean estimates from the additive model, NR model, averaging model, and 1-way ANOVA for all genotypes are just different ways to linearly decompose the phenotype values (when the full model terms are kept). Thus, when the models are fit to actual data with replication, all these models yield the same fitted and residual values. The numbers of estimated values are the same, i.e., the models have the same residual degree of freedom. Therefore, I sometimes use only the mean estimates of the models for my arguments. The coefficient matrices to solve the linear equations for the means in the three models using the genotype mean values in a 3-gene system are shown in Fig. TS1.1.

a Additive model

	Intercept	A	B	C	A:B	A:C	B:C	A:B:C
triple.mut	1	0	0	0	0	0	0	0
A	1	1	0	0	0	0	0	0
B	1	0	1	0	0	0	0	0
C	1	0	0	1	0	0	0	0
AB	1	1	1	0	1	0	0	0
AC	1	1	0	1	0	1	0	0
BC	1	0	1	1	0	0	1	0
ABC	1	1	1	1	1	1	1	1

b NR model

	Intercept	A	B	C	A:B	A:C	B:C	A:B:C
triple.mut	1	0	0	0	0	0	0	0
A	1	1	0	0	0	0	0	0
B	1	0	1	0	0	0	0	0
C	1	0	0	1	0	0	0	0
AB	1	1	1	0	1	0	0	0
AC	1	1	0	1	0	1	0	0
BC	1	0	1	1	0	0	1	0
ABC	1	1	1	1	1/3	1/3	1/3	1

c Averaging model

	Intercept	A	B	C	A;B	A;C	B;C	A;B;C
triple.mut	1	0	0	0	0	0	0	0
A	1	1	0	0	0	0	0	0
B	1	0	1	0	0	0	0	0
C	1	0	0	1	0	0	0	0
AB	1	1/2	1/2	0	1	0	0	0
AC	1	1/2	0	1/2	0	1	0	0
BC	1	0	1/2	1/2	0	0	1	0
ABC	1	1/3	1/3	1/3	1/3	1/3	1/3	1

Fig. TS1.1. Matrices for the linear equations to obtain model coefficients from the genotype values in a 3-gene system for (a) additive, (b) NR, and (c) averaging models. The rows are genotypes and the columns are model variables. ":" and ";" indicate the additive and averaging interactions, respectively.

Violation of the distributive law is prevalent in multi-gene systems

The averaging model does not assume the distributive law. Do we really need to consider non-distributivity in a biological system? Let's consider a simple 3-gene system, in which nA and nB are input nodes and nC is the output node (Fig. TS1.2). Mechanistically, signals from nA and nB are first summed, and then modulated by a nonlinear function f_1 before the signal is output from nC. Thus,

(A + B):C = nC = f_1(nA + nB) … (21)

A:C + B:C = f_1 (nA) + f_1 (nB) … (22)

Therefore, if the distributive law holds,

f_1(nA + nB) = f_1 (nA) + f_1 (nB) … (23)

Let's make f_1 a Michaelis-Menten function for a saturating response (Fig. TS1.2b):

$f_1(x) = \frac{10}{1+\frac{7}{x}}$ … (24)

When nA = 5, nB = 2,

f_1(nA + nB) = $f_1(5+2)$ = 5

f_1 (nA) + f_1 (nB) = $f_1(5) + f_1(2)$ = 4.16… + 2.22… = 6.38…

Thus, f_1(nA + nB) ≠ f_1 (nA) + f_1 (nB) … (25)
and the distributive law is violated.
Generally, nonlinearity in a system leads to violation of the distributive law.

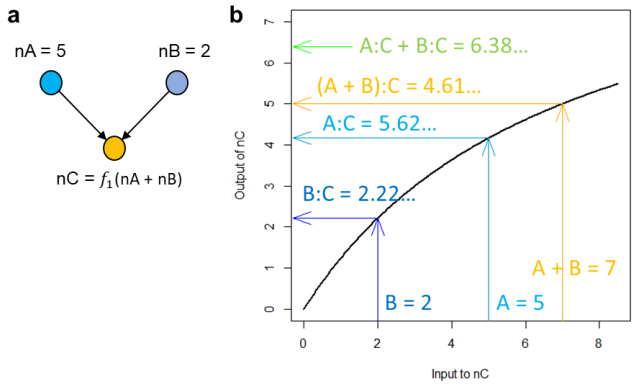

Fig. TS1.2. Non-linearity in a system violates the distributive law. (a) a 3-gene system, in which signals from nA and nB feed into nC. The output of nC is defined as f_1(nA + nB). (b) When $f_1(x) = \frac{10}{1+\frac{7}{x}}$, the input-output relationships at nC are shown. If the input is nA, the output is expressed as A:C in the additive model. This plot clearly shows that (A + B):C ≠ A:C + B:C (Y-axis values in orange and green, respectively), a violation of the distributive law.

A saturating response limits the output range. Without nonlinearity, the range of the system output is not limited, and this is the condition the additive model requires. Thus, the additive model generally cannot be used in a system consisting of multiple genes (when the model includes 3 -or-more-gene interactions, strictly speaking) when the phenotype value range is limited compared to the ranges of the gene effects and interactions.

References

1. K. Tsuda, M. Sato, T. Stoddard, J. Glazebrook, F. Katagiri, Network properties of robust immunity in plants. *PLoS Genet* **5**, e1000772 (2009).
2. F. Katagiri, Network Reconstitution for Quantitative Subnetwork Interaction Analysis. *Methods Mol Biol* **1578**, 223-231 (2017).

Text S2. Averaging and additive models without interaction are two extreme approximations of the behavior of a two-input chemical signaling system

We consider a hypothetical system with two converging input signals. One input is signal molecule A, the other input is signal molecule B, and the output is signal molecule C (Fig. S2.1).

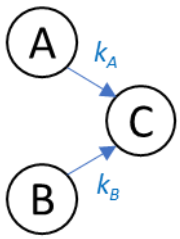

Fig. S2.1. The structure of a two-input system.

The system is modeled assuming an equilibrium among the three signal molecules. It can also be considered as a steady state of a dynamical system, which is more realistic for a signaling system. The system can be considered as a system of chemical reactions (e.g., A + X → C and B + Y → C), in which other molecules in the reactions (like X and Y) are not limiting (i.e., [X], [Y] >> [A], [B], [C]). We can only observe the final output of [C], the concentration of C, under the conditions of presence or absence of A and B. We cannot observe [A] and [B].

In the system, the sum of [A] and the fraction of [C] that derived from [A] is denoted as $[A]_0$, and the sum of [B] and the fraction of [C] that derived from [B] is denoted as $[B]_0$. When the system lacks B (genotype *Ab*), $[C]_A$, the concentration of C specific to this condition is expressed with $[A]_A$, the concentration of A specific to this condition, and the conversion constant k_A as $[C]_A = k_A[A]_A$. Since $[C]_A = [A]_0 - [A]_A$, by eliminating $[A]_A$,

$[C]_A = \{ k_A / (k_A + 1) \} [A]_0$ … (1)

Similarly, when the system lacks A (genotype *aB*), with the conversion constant k_B,

$[C]_B = \{ k_B / (k_B + 1) \} [B]_0$ … (2)

When the system has both A and B (genotype *AB*), with $[A]_{AB}$ and $[B]_{AB}$, the concentration of A and B specific to this condition, $[C]_{AB}$, the concentration of C specific to this condition, can be expressed as:

$[C]_{AB} = k_A[A]_{AB} = k_B[B]_{AB}$ … (3)

Also, $[C]_{AB} = ([A]_0 - [A]_{AB}) + ([B]_0 - [B]_{AB})$ … (4)

By eliminating $[A]_{AB}$ and $[B]_{AB}$ from (3) and (4),

$[C]_{AB} = \{ 1 / (1 / k_A + 1/ k_B + 1) \} ([A]_0 + [B]_0)$ … (5)

We now consider the situations with different k_A and k_B values.

I. When $0 < k_A, k_B << 1$,

(1), (2), and (5) can be approximated as:

$[C]_A \approx k_A[A]_0$ … (6)

$[C]_B \approx k_B[B]_0$ … (7)

$[C]_{AB} \approx \{ 1 / (1 / k_A + 1 / k_B) \} ([A]_0 + [B]_0)$ … (8)

k_A and $[A]_0$ cannot be distinguished as we only know $[C]_A$ as the phenotype, and similarly, k_B and $[B]_0$ cannot be distinguished as we only know $[C]_B$ as the phenotype. Thus, we assume $k \approx k_A \approx k_B$ to remove unnecessary complexity from the model. Then,

$[C]_A \approx k[A]_0$ … (6)'

$[C]_B \approx k[B]_0$ … (7)'

$[C]_{AB} \approx k ([A]_0 + [B]_0) / 2$ … (8)'

From (6)', (7)', and (8)',

$[C]_{AB} \approx ([C]_A + [C]_B) / 2$ … (9)

Therefore, under the conditions of $0 < k_A, k_B << 1$ (meaning [A],[B] >> [C]), the averaging model without interaction describes the system well.

II. When $k_A, k_B >> 1$,

(1), (2), and (5) can be approximated as:

$[C]_A \approx [A]_0$ … (10)

$[C]_B \approx [B]_0$ … (11)

$[C]_{AB} \approx [A]_0 + [B]_0$ … (12)

From (10), (11), and (12),

$[C]_{AB} \approx [C]_A + [C]_B$ … (13)

Therefore, under the conditions of $k_A, k_B >> 1$ (meaning [A],[B] << [C]), the additive model without interaction describes the system well.

The discussion in this supplemental text demonstrates that whether the averaging model or the additive model describes the no interaction state better in a two-input system depends on the system. Since we can only observe the output of the system, [C], (but not [A] or [B]), we cannot determine which of the averaging and additive models would be better for a particular system.