Computational inference of gene regulatory networks: approaches, limitations and opportunities

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Abstract

Gene regulatory networks lie at the core of cell function control. In *E. coli* and *S. cerevisiae*, the study of gene regulatory networks has led to the discovery of regulatory mechanisms responsible for the control of cell growth, differentiation and responses to environmental stimuli. In plants, computational rendering of gene regulatory networks is gaining momentum, thanks to the recent availability of high-quality genomes and transcriptomes and development of computational network inference approaches.

Here, we review current techniques, challenges and trends in gene regulatory network inference and highlight challenges and opportunities for plant science. We provide plant-specific application examples to guide researchers in selecting methodologies that suit their particular research questions.

Given the interdisciplinary nature of gene regulatory network inference, we tried to cater to both biologists and computer scientists to help them engage in a dialogue about concepts and caveats in network inference. Specifically, we discuss problems and opportunities in heterogeneous data integration for eukaryotic organisms and common caveats to be considered during network model evaluation.

Keywords: computational systems biology, gene regulatory network inference,

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1. Introduction

Gene regulatory networks are central to all biological processes of an organism. In their most basic form, they describe the complex web of transcription factor proteins that bind regulatory sequences of target genes in order to affect
5 their spatial and temporal expression [1].

Understanding gene expression regulation has an immediate impact in biology and medicine as many traits and diseases are associated with mutations in regulatory sequences or dysfunctional transcriptional regulators [1]. In agriculture, changes in plant transcriptional regulation shaped modern crops such as
10 maize, rice and wheat and enabled yield increases [2]. Elucidation of transcriptional regulatory systems could help improve metabolite production rates and resilience against environmental stresses [3, 4].

Owing to their sessile nature, plants are subject to variations in their environment that drive adaptation. Yet, how gene-regulatory networks are rewired
15 to control the adapted traits or drive adaptation is largely unknown. Despite extensive insights into the core components of the transcriptional machinery, how specificity is encoded during the highly dynamic process of transcriptional regulation remains an open question [5].

Given the interdisciplinary nature of the problem, biologists and computer
20 scientists need to engage in a dialogue to solve the network inference problem.

In this review, we aim to: i) introduce the basic concepts and procedures used for gene expression based regulatory network inference, which are borrowed from several disciplines including statistics, information theory, graph theory and machine learning; (ii) discuss limitations of network inference based only
25 on transcriptional datasets; (iii) present data types and strategies used for integrative network inference and causal link predictions; and (iv) describe caveats and solutions for the evaluation and selection of statistically and biologically relevant regulatory interactions.

2. Methods for gene regulatory network inference from expression data

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Gene expression data generated by high-throughput technologies such as microarray or RNAseq still serve as one of the main sources for the development of gene regulatory networks. Therefore, we start our review by highlighting some of the main concepts, methods and limitations in inferring regulatory networks from different types of gene expression data. For an in-depth comparison of the state-of-the-art network inference methods using expression data, we refer the reader to [6, 7, 8, 9]. For an overview on gene expression normalization, filtering and pre-processing steps for network inference, see [10, 11].

2.1. Correlation and information theoretic approaches

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Approaches within this category employ statistical analyses of dependencies between expression patterns. The most basic models, called co-expression networks, exploit correlations between expression profiles of genes [12]. A popular correlation measure is Pearson’s correlation coefficient r [10], i.e.

$$r_{E_i, E_j} = \frac{\text{cov}(E_i, E_j)}{\sigma(E_i) \cdot \sigma(E_j)} \quad (1)$$

Here, E_i, E_j denote gene expression profiles of two genes i, j with covariance $\text{cov}(E_i, E_j)$ and standard deviations $\sigma(\cdot)$. Other correlation measures include Spearmans correlation [10] or the more recently introduced weighted correlation coefficient [13]. More sophisticated correlation based approaches aim to distinguish direct from indirect, spurious correlations between genes by using partial correlations [14, 15, 16].

50

Information theoretic concepts [17, 18] extend correlation to capture more complex statistical dependencies between expression patterns. This approach led to the development of a specific kind of association network called a relevance network [19]. Relevance networks define relationships between two genes i, j based on an information theoretic property, called mutual information [19],

55 based on their respective gene expression profiles E_i and E_j . Mutual information is defined as:

$$I(E_i, E_j) = \sum_{e_i \in E_i} \sum_{e_j \in E_j} p(e_i, e_j) \log \left(\frac{p(e_i, e_j)}{p(e_i)p(e_j)} \right) \quad (2)$$

where $p(e_i, e_j)$ is the joint probability distribution of E_i and E_j , and $p(e_i)$ and $p(e_j)$ denote the marginal probabilities.

Relevance networks are built by first constructing a fully connected graph
60 for all gene pairs using mutual information to weight each link. Links whose associated weights lie below a certain threshold are removed from the network. The threshold, according to Butte and Kohane [19], is estimated by first randomizing the expression data, and then re-computing mutual information values to obtain a reference null distribution.

65 Various refinements of this idea have been proposed to discriminate direct from indirect effects [17, 20, 18, 12, 21]. The most prominent methods include ARACNE (algorithm for the reconstruction of accurate cellular networks) [22], CLR (context likelihood of relatedness) [21], MRNET (minimum redundancy, maximum relevance) [20] and C3NET (conservative causal core) [17].

70 The ARACNE algorithm [22] adjusts the construction of a relevance network by applying a constraint known as Data Processing Inequality to filter indirect interactions. The Data Processing Inequality states that, if gene i interacts with gene j via gene k , then the following inequality holds with respect to their corresponding mutual information values: $I(E_i, E_j) \leq \min(I(E_i, E_k), I(E_k, E_j))$,
75 i.e. the smallest of the mutual information scores $I(\cdot)$ within this inequality indicates an indirect regulatory interaction [22]. As a consequence, ARACNE evaluates all possible gene triplets and prunes individual (indirect) interactions within each triplet, if the Data Processing Inequality is violated beyond a certain tolerance threshold.

80 The CLR algorithm [21] first estimates the pair-wise mutual information values for all gene pairs. Then, it estimates the statistical likelihood of each mutual information value I_{ij} for a particular pair of genes (i, j) by comparing this mutual information value to a background distribution. For each gene pair

(i, j), two z-scores are obtained, one for gene i and one for gene j , by comparing
85 the mutual information value I_{ij} with gene-specific distributions, p_i and p_j .
Here lies CLR’s major advantage over the relevance network approach by Butte
and Kohan [19] or ARACNE, as individual thresholds can be established by
considering an individual background for each pair of genes. This is in contrast
to relevance networks or the ARANCE approach, which use a global threshold
90 for graph pruning.

The MRNET algorithm [20] incorporates a feature selection methodology,
called Minimum Redundancy Maximum Relevance (MRMR), to infer interac-
tions between genes. This algorithm first places each gene as a target gene
with all remaining genes as its putative regulators. The MRMR method is then
95 applied to select the best subset of regulators.

More recently, C3NET [17] has been proposed. This approach consists of
two main steps. First, a relevance network is constructed and non-significant
edges are pruned according to a chosen significance level. Then, only the most
significant link for each gene, i.e. the highest mutual information value among
100 the neighboring edges, is selected. This implies that the highest possible number
of edges that can be inferred by C3NET is equal to the number of genes in the
network.

In addition to aforementioned variations of the relevance network approach,
concepts such as three-way and conditional mutual information have been pro-
posed to directly address the problem of indirect interactions within gene triplets.
105 For instance, the MI3 algorithm [23] uses three-way mutual information for in-
ference, hypothesizing that gene regulation commonly involves more than one
regulatory gene. Soranzo *et al.* [12] use only the conditional mutual information
(CMI) to infer regulatory networks between gene triplets, pruning links that fall
110 below a chosen threshold.

2.1.1. Limitations

In general, co-expression and relevance networks are designed to help ex-
plore co-functionality of genes on a systems level [13]. In this context, the no-

tion of separating direct from indirect regulatory effects between genes should
115 not be confused with directionality or even causality. In general, correlation-
based relationships are symmetric, i.e. bidirectional, although recent efforts
to exploit time-delay effects using time-course expression data might allow for
asymmetric relationships [24, 25, 26]. For an in-depth comparison of time-lagged
information-theoretic approaches, see [25].

120 However, for most of the common approaches within this group of method-
ologies, directionalities between genes can only be assumed if regulator genes are
known in advance. As a consequence, large-scale mining for biologically relevant
graph-patterns, such as feedback or feed-forward loops, should be exercised with
caution.

125 In general, correlations between gene expression profiles are used as an in-
dicator of co-regulation [27], and a plethora of clustering approaches has been
developed based on that premise [28, 29, 30, 31, 32]. Complementing cluster-
ing with various gene regulatory network inference approaches have been pro-
posed to find a more robust method for identifying condition-specific regulators
130 [33, 31]. Advantages and limitations of these ideas are discussed in [32]

2.1.2. Application examples in plant science

An in-depth tutorial on how to apply co-expression analysis for plant systems
biology is given in [10]. The authors *et al.* [10] not only describe methodologies
but also discuss statistical issues including how normalization of gene expression
135 data can influence co-expression results. A more recent extensive overview on
the construction and application of co-expression networks in plant species with
agricultural importance, including soybean, rice, maize and tomato is given in
[34]. Given the importance of removing indirect links for causal network struc-
ture prediction, application of indirect link removal methodologies were also
140 used within plant systems biology [35, 36]. For example, the concept of Data
Processing Inequality to filter indirect interactions, established based on mutual
information [22], was used to construct an *A. thaliana* root transcriptional reg-
ulatory network [36]. The predicted network successfully recovered previously

identified root transcriptional modules as well as proposed new transcription
 145 factors involved in the regulation of root secondary cell-wall synthesis. The concept of partial correlations was used to analyze expression time series datasets in order to investigate the impact of the diurnal cycle on starch metabolism regulation in *A. thaliana* [35].

2.2. Boolean network approaches

150 Boolean networks represent relationships among regulators and putative target genes based on Boolean logic functions [37, 38]. Boolean networks assume each gene g at a time point t to be in one of two states, active or inactive, according to its gene expression profile at time t . Hence, the gene expression profiles of all genes are discretized before network inference. The challenge of
 155 generating a Boolean network is to find a Boolean function per gene g so that the observed, discrete data can be explained by the network model. One of the earliest algorithms for Boolean network inference is REVEAL (REVerse Engineering ALgorithm) [39].

2.2.1. Limitations

160 Since gene expression patterns are generally more complex than having only two states, data discretization often leads to information loss. In addition, being time-dependent, Boolean networks suffer from the small number of time points in gene expression time-series, making large-scale applications difficult. Nevertheless, Boolean networks can be interpreted intuitively. In addition, various
 165 extensions have been proposed to allow for multiple gene expression states and stochastic events during gene regulation [38], which are promising directions.

2.2.2. Application examples in plant science

Several examples of network inference using Boolean networks to describe small-scale *A. thaliana* developmental regulatory networks are provided in [40].
 170 For instance, Savage *et al.* [41] used a Boolean framework to explore the regulatory mechanisms underlying patterning of *A. thaliana* root epidermis. They developed two competing network models describing different transcription factor

interactions to regulate the patterning process. While one model proposed self-activation feedback loop mechanism for one of the transcription factors involved, the other described to a mutual support mechanism between the transcription factors. These simulations were then used to design multiple experiments that led to the confirmation of the mutual support mechanism.

2.3. Bayesian network approaches

Bayesian networks were among the first approaches for generating gene regulatory networks from gene expression data [42, 43]. Bayesian networks represent conditional dependencies between random variables, genes $x_i \in X$ (nodes), using a directed acyclic graph structure $G(X, E)$. Edges E between nodes indicate putative causal dependencies given their gene expression levels [8].

$$P(x_1, x_2, \dots, x_n) = \prod_{i=1}^n P(x_i | Pa(x_i)) \quad (3)$$

The random variables are drawn from conditional probability distributions $P(x_i | Pa(x_i))$, where $Pa(x_i)$ is a set of parents for each node x_i . A Bayesian network implicitly encodes the *Markov assumption* which states that, given its parents, each variable is independent of its non-descendants. Based on the Markov assumption, a Bayesian network specifies a decomposition of the joint distribution over all random variables down to the conditional distribution for each node. This probabilistic framework is appealing for modeling causal relationships [44].

Modeling using Bayesian networks involves two steps: (i) model selection, also referred to as structure learning; and (ii) parameter learning. Model selection involves constructing a specific network structure to identify the network topology that best explains the observed data from all possible network structures. In contrast, parameter learning involves estimating probability values associated with each node in the network. For model evaluation, Bayesian scoring metrics such as Bayesian information criterion (BIC) or Akaike's information criterion (AIC) are used [45]. For each possible model, the metric defines a score that represents the logarithm of the probability that the model correctly describes a given set of data.

2.3.1. Limitations

A fundamental practical problem of learning the structure of Bayesian networks is that the number of possible network topologies increases super-exponentially with the number of genes, which makes it not feasible to compute the likelihood of all possible networks [46]. Hence, exact Bayesian network inference methods are applicable only to small networks or networks with built-in constraints. An example of such a heuristic would be to place an upper limit on the number of parents per node [47]. In this case, locally constrained search techniques can be applied, which make the computational complexity manageable [42]. Other approaches include approximate sampling strategies such as Markov Chain Monte Carlo (MCMC) [48].

Traditional Bayesian models do not allow for modeling cycles, i.e. regulatory feedback loops, which occur frequently in biological networks [49]. To circumvent this limitation, dynamic Bayesian networks were introduced [50, 51], whose main rationale is to unfold cyclic processes by mapping them onto a sequence of acyclic events [52]. A prominent example of this category are variational Bayesian state space models (VBSSM) [53, 8].

2.3.2. Application examples in plant science

Needham *et al.* applied a Bayesian network approach to learn the most plausible *A. thaliana* network structure based on a large compendium of individual gene expression datasets [54]. Their model was validated based on its ability to recover a well-studied regulatory network of the circadian clock. Penfold and colleagues [55] applied variational Bayesian state space models to infer gene regulatory networks across multiple experimental conditions in *A. thaliana*. They used their model to integrate complementary data from several stress conditions and to infer potential transcriptional switches in *A. thaliana* stress response. An in-depth comparison between different Bayesian network variations for inferring a network controlling lateral root initiation in *A. thaliana* was shown recently [8].

230 2.4. Regression and differential equation based models

Regression-based approaches to gene regulatory network inference are based on the assumption that the expression profiles of the transcription factors that directly regulate a target gene are the most informative, among all transcription factors, to predict the expression profile of the target gene. In this respect, gene regulatory network inference is reformulated as a feature selection problem that
235 seeks to find the most predictive subset of transcription factors for each target gene. In order to infer the global gene regulatory network, a regression model is formulated to predict a set of putative direct regulators among all transcription factors $r_1, \dots, r_N$ for each target gene g :

$$\sum_{i \in M} (e_i^g - f(\mathbf{e}_i^{r_1, \dots, r_N}))^2, \quad (4)$$

240 Here $f(\cdot)$ denotes a mapping function to combine the expression profiles $\mathbf{E}_{r_1, \dots, r_N}$ (based on M individual expression samples) of transcription factors to approximate the expression profile E_g of a target gene, e.g. by minimizing the square error loss. Several alternative approaches have been proposed for implementing $f(\cdot)$, resulting in feature selection strategies such as stepwise selection [56], least
245 angle regression [57], ridge regression [58], and least absolute shrinkage and selection operator (LASSO) [59]. Among all these methods, LASSO has emerged as the most popular in gene network reconstruction [7]. Unlike ridge regression, which aims to shrink insignificant coefficients close to zero, Lasso exploits L_1 regularization to shrink insignificant coefficients to be exactly zero. This leads
250 to a much sparser linear model, i.e., only a few predicted regulators per target gene.

Recently, tree-based ensemble regression methods have been proposed [60, 61, 62, 63], including the GENIE3 algorithm [60]. In contrast to linear regression models, tree-based approaches do not make any assumptions about the nature
255 of gene regulation. They can, therefore, handle combinatorial and non-linear interactions.

Random forest regression is one of the most prominent examples of a tree based approach [64]. The main rationale is that for each gene g , a number of

decision trees are grown over different bootstrapped samples of the complete
 260 gene expression dataset. Within each decision tree, the method recursively
 splits the data sample. It applies binary tests per node in the tree based on
 a random subset k_r of regulators, trying to reduce the variance of the target
 gene expression profile. The logic, similar to linear regression approaches, is to
 select the set of regulators that best explains the expression profiles of the target
 265 gene. Each tree-based model yields a separate ranking of the genes as potential
 regulators r of a target gene g . Averaging these individual predictions over
 several trees leads to a final prediction of regulators for each target gene. Since
 its initial implementation, extensions have been proposed to handle time-series
 data [61, 62] and integrate heterogeneous data [63].

270 Differential equation based approaches represent complex physical regres-
 sion models that attempt to mimic the biological mechanism of transcriptional
 regulation [65, 66, 67]. These models are typically based on systems of ordi-
 nary or stochastic differential equations. They are continuous and deterministic
 modeling formalisms, capable of describing non-linear and emerging phenomena
 275 of complex dynamical systems. Therefore, differential equation based models
 of gene networks are based on rate equations, quantifying the rate of change of
 gene expression as a function of the expression profiles of other genes. Equation
 5 illustrates a first-order differential equation model, where $e_g(t)$ denotes the
 expression of gene g at time t and $\mathbf{e}_{r_1, \dots, r_N}(t)$ denote the expression levels of
 280 putative regulators of g . The general model can be expressed as:

$$\frac{de_g(t)}{dt} = \alpha_g + f_g(\mathbf{e}_{r_1, \dots, r_N}(t)) - \lambda_g e_g(t), \quad (5)$$

where α_g denotes the basal transcription rate, f_g is an unknown regulation
 function and λ_g is the decay rate of the transcript. Specific forms of the node
 functions $f_g(\cdot)$ typically come from biochemical considerations and modeling
 components of the genetic system [65]. In general, these functions are non-linear
 285 because, among other things, all concentrations become saturated at some point
 in time. A benefit of differential equation models is that they can be approxi-
 mated as discrete-time (linear) models, also known as difference equations [68],

which makes them solvable based on well-established methods of linear algebra, such as singular value decomposition (SVD) [68].

290 2.4.1. Limitations

Linear models yield reasonable predictions when the gene expression experiment is performed in a slowly changing system or around a steady state [69]. LASSO sparse regression tends to run the risk of producing inconsistent results with respect to feature (e.g. a regulator) selection. Therefore, a procedure
295 known as stability selection, has been proposed within the TIGRESS algorithm [70].

2.4.2. Application examples in plant science

In-depth reviews about using dynamic approaches to analyze small-scale *A. thaliana* developmental regulatory networks are given in [71] and [40]. For
300 instance, a linear regression model was used to refine an initial predicted regulatory network of the photosynthetic light acclimation response [72]. In this approach, an initial network had been constructed based on Pearson’s correlation. This network was subsequently pruned by applying a regression model on time-series gene expression datasets.

305 3. Handling an under-determined problem: Caveats in gene regulatory network inference based solely on gene expression data

In this section, we discuss caveats of inferring gene regulatory networks from gene expression data alone. In the next section, we highlight one solution to the problem through integrating multiple, heterogeneous datasets.

310 3.1. Gene expression and the curse of dimensionality problem

Gene expression data can be divided into different experimental types such as steady state, time course or perturbation. Marbach *et al.* [7] compared different types of experimental data for their usefulness in regulatory network inference. They concluded that direct transcription factor perturbations such as

Figure 1: Integrating inferred (i) physical (DNA-binding) regulatory models and (ii) functional (influential) regulatory models to reconstruct condition-specific causal networks using an integrative classifier. Experimental validation can be used as a feedback mechanism to provide new datasets for network inference refinement and model training.

315 knockout or over-expression experiments, although typically sparse, are among the most informative data [7]. However, this particular type of experimental data often limits network inference to finding regulatory effects downstream of specific regulators [5, 73]. It does not allow the search for regulatory cascades upstream of certain target genes of interest [74]. Furthermore, a given gene
320 expression dataset drives the selection of a suitable method and scale of the inference. For instance, while Bayesian methods perform well on perturbation or time-course data for small regulatory sub-networks, they scored below average in a recent large-scale study on multi-factorial biological data in *S. cerevisiae* and *E. coli* [7].

325 In practice, all available types of gene expression data suffer from ‘the curse of dimensionality’, i.e. the number of genes (and in turn the number of putative regulatory interactions) dramatically outnumbers the number of gene expression samples [6, 32]. As a consequence, this may lead to many different Bayesian network structures or regression models that can explain the observed gene

330 expression data equally well, making the decision for choosing the most plausible
underlying regulatory network infeasible.

This high dimensionality problem is particularly acute for time-series data,
which is appealing for establishing causal models of transcriptional regulation
[67]. This type of information is limited, especially in plant science, notwith-
335 standing a few examples in [75, 76, 77]. Given their limited number of time
points, they contain insufficient information for the realistic reconstruction of
large-scale gene regulatory networks [32]. Therefore, only small regulatory sub-
network prediction tasks can be conducted. In addition, if sampling between
individual time points is too coarse, important biological events may occur be-
340 tween the time points and the data set is incapable of inferring any time-delay
related effects. To overcome this problem, time point interpolation approaches
such as Gaussian process regression have been proposed for inferring a net-
work to describe *A. thaliana* leaf senescence [77]. In their study, Breeze *et al.*
[77] used Gaussian process regression [78] as pre-processing step to interpolate
345 time series expression data of a single leaf over a 3-week period to senescence.
They then identified the rate of change in gene expression over time using the
interpolated time points in order to cluster sets of co-regulated genes.

3.2. The computational complexity of network structure learning

Another challenge lies in the computational complexities of inference algo-
350 rithms. For example, as already mentioned, network structure learning with
Bayesian networks is limited, as the number of possible network topologies in-
creases super-exponentially with the number of genes. This makes it not feasible
to compute the likelihood of all possible networks [46]. As a consequence, prob-
lems such as Bayesian network structure learning, in complexity theory, are
355 generally referred to be NP-hard (Non-deterministic Polynomial time) prob-
lems. This means that these problems cannot be solved in polynomial time and
can be used as a way of showing that certain classes of problems are not solvable
in realistic time [79].

3.3. Non-linearity and hidden layers of transcriptional regulation

360 Some methodologies such as (partial) correlation based methods (Section 2.1) or linear regression approaches (Section 2.4) assume linear relationships between the expression levels of individual transcription factors and their putative targets. In contrast, information theoretic concepts (Section 2.1) or tree-based regression models (Section 2.4) do not make such an assumption, which makes
365 them better suited to model non-linear effects in regulatory systems. In addition, (partial) correlation as well as information theoretic approaches (Section 2.1) model individual relationships between a single regulator and its putative target. In contrast, Boolean (Section 2.2) and Bayesian networks (Section 2.3) as well as regression based approaches are capable of modeling combinatorial or
370 epistatic effects of multiple transcription factors on a common target.

A bigger challenge is the general assumption that transcript levels of regulators and their targets display some degree of mutual dependency. It has recently been shown that this hypothesis cannot be taken for granted, especially for higher organisms. Studies by Kueffner *et al.* [80] and Marbach *et al.* [7] highlight that dependencies between expression profiles of transcription
375 factors and their known target genes tend to exceed the dependencies observed between non-interacting gene pairs for *in silico* and *E. coli* data but not for eukaryotic data such as those for *S. cerevisiae* [80]. In fact, the dependency distributions of non-interacting and interacting gene pairs for the *S. cerevisiae*
380 data were found to be almost identical, underscoring the difficulty to detect true regulatory interactions based on dependencies derived from gene expression data alone. Similar observations were made for supervised plant developmental regulatory network inference in *A. thaliana* [81]. The study by Yu *et al.* [81] proposed a supervised classification scheme to predict novel targets of known
385 transcription factors by using gene expression profiles of known target genes as training set. In the study, the expression profiles of genes in positive and negative classes showed nearly identical distributions. This observation of almost identical distributions for non-interacting and interacting gene pairs can be explained, in part, by the roles of chromatin remodeling, post-transcriptional and

390 post-translational processes [82] on gene expression regulation.

More recently, additional mechanisms have been linked to transcriptional regulation in plants. For example, enhancers represent important cis-regulatory DNA elements that regulate transcription programs by recruiting transcription factors and directing them to the promoters of target genes [83]. In general, 395 enhancers are difficult to identify given their unpredictable positions relative to their corresponding promoters [84]. Another example is transposable elements. Although transposable element insertions within introns or untranslated regions of genes have minimal impact on expression level or splicing, there are examples in which transposable element insertions within genes can lead to novel 400 transcripts or promoters, which can result in changes of expression patterns [85].

All these additional layers of regulation make inference of causal dependencies between genes from gene expression data alone more difficult. Therefore, when working with complex organisms such as plants and animals, it is crucial to first verify whether a particular method has been evaluated for species 405 of similar complexity as the organism under scrutiny. Furthermore, as one of the major goals in regulatory network inference is to establish causal relationships between a regulator and a target gene, gene expression data can only support the inference process and is necessary but not sufficient to achieve this 410 goal. Therefore, it is important to extend the gene regulatory network modeling to use other types of experimental data besides gene expression measurements [86, 87, 88]. In the next section, we discuss different data types that have proven helpful for gene regulatory network inference, as well as strategies and caveats with respect to their integration for network inference.

415 **4. Gene regulatory network inference based on integration of multiple data types: Searching for causality**

In order to infer causal regulatory relationships between genes, Gardner and Faith [89] emphasize two complementary types of data-dependent inference

strategies: (i) Physical models that describe real physical interactions, which are
420 reconstructed based on experimental or predicted sequence-based DNA binding;
(ii) Functional influence models that describe regulatory effects of a transcrip-
tion factor on its putative downstream targets, including indirect influences.
Reconstruction of high-quality, genome-scale regulatory relationships requires
the integration of both physical and functional networks [87]. Figure 1 illus-
425 trates such an integrative inference approach. Figure 1 also highlights the ex-
perimental feedback loop [74] to include experimental validation in the inference
process. An early example of network inference to integrate physical and func-
tional models is the combined use of gene expression data with gene promoter
analysis to identify cis-regulatory elements of putative groups of potentially co-
430 regulated genes [90, 31]. Examples in plant science include the reconstruction
of a regulatory network for photosynthesis [91] and flower development [92].
Yu *et al.* in [91] used a two-step pipeline to elucidate the detailed regulatory
networks controlling the construction and maintenance of the photosynthetic
machinery. First, the promoter regions of genes known for their involvement
435 in photosynthesis were analyzed for known cis binding motifs to identify puta-
tive regulators. Subsequently, a mutual information based approach was used
to determine putative interactions between these regulators and targets. In
[92], Yu *et al.* derived a gene regulatory network of *A. thaliana* flower develop-
ment. Here they extended their earlier proposed classification framework [81]
440 to combine gene expression and cis-binding motif information into a support
vector machine classifier model to identify novel targets of known transcription
factors.

Here we present different data types that have proven helpful for gene regu-
latory network inference, as well as strategies and caveats with respect to their
445 integration for causal gene regulatory network inference.

4.1. Data types for integrative gene regulatory network inference

The most crucial types of information for gene regulatory network inference
are transcription factor DNA binding site information generally derived from

chromatin immunoprecipitation experiments and predictions based on motif sequence matching [87]. A disadvantage of transcription factor binding data is that it can generate many false positives if no additional filtering (e.g. based on expression data) is applied [87]. However, given that multiple transcription factors are often capable to bind the same motif, expression based filtering might also disregard true binding predictions. This would be the case if one of the transcription factor binding is not active within a specific expression experiments, and, as a consequence, lacks correlation with putative target's expression profile. In general, without any additional filtering, DNA binding information can be primarily harnessed to generate a regulatory blueprint i.e. a network of all possible regulatory interactions across different developmental stages or organ types (Figure 1, left). However, only a small subset of this space of possible regulatory relationships is active and observable in a specific condition (Figure 1, right). Accordingly, Mejia-Guerra *et al.* [74] coined the term gene regulatory grid in order to distinguish this static regulatory blueprint of all theoretically possible interactions from the highly dynamical nature of a condition-specific gene regulatory network instantiation.

Both experimental and predicted DNA binding information are becoming available for plants [93, 94, 95, 96, 97, 98, 99, 100]. In the most recent release of the JASPAR database of experimentally validated binding motifs, the number of curated plant motifs was superseded only by that of vertebrates[95]. The most recent release of the PlantPAN (Plant Promoter Analysis Navigator) database [99] reported binding motifs for 17673 transcription factors across 76 plant species. However, the majority of PlantPAN's DNA binding motif datasets are based on predictions that have not (yet) been described within plants and some predictions have even been inferred from information in different kingdoms. In contrast, purely experimental motif datasets for *A. thaliana* are available for about 530 out of the approx. 2000 transcription factors [93, 94, 95, 96, 97, 98, 99, 100, 101]. Given that a typical binding element is 6-12bp long, the quest for functional transcription factor binding motifs in a 3000bp promoter region is prone to a high number of false positive predictions. This

Figure 2: DNA binding information generates a regulatory blueprint, i.e. a network of all possible regulatory interactions between transcription factors (red) and other genes (green) across different developmental stages or organ types (left). Only a small subset of this space of possible regulatory relationships is active in a specific condition (right).

480 makes computational identification of cis-regulatory elements on a genome-scale challenging [102, 103, 104, 105].

Since transcriptional regulation of a target gene is often orchestrated by the combinatorial action of several transcription factors [104], the inference of cis-regulatory modules, i.e. combinations of individual cis-regulatory elements, can
 485 lead to more robust predictions than single-element inference [106, 107]. This allows the incorporation of additional predictive features such as the order, relative spacing or distance to the transcription start site of the cis-elements [108]. An example for supervised cis-regulatory module prediction in *A. thaliana* is given in [109]. Recent advances in deep learning [107] have been applied to the
 490 identification of regulatory elements. Deep learning refers to neural networks with more than one hidden layer. The depth of these types of multi-layered

neural networks allows for the construction of feature sets of increasing abstraction, which is particularly suited for learning complex representation and patterns such as combinatorial regulatory elements [107].

495 A large-scale comparison of cis-regulatory module detection approaches concluded that methods considering evolutionary conservation have a stronger predictive power than methods designed to be run on a single genome [110]. In addition, conserved non-coding sequences in promoter regions are reliable pointers to regulatory elements controlling gene expression [87, 111]. Recent examples
500 of conserved regulatory element recovery in plant science include comparative, phylogenetic footprinting approaches by [112, 113, 111]. Yu *et al.* studied the positional distribution of transcription factor binding sites in *A. thaliana* and three additional closely related species [114]. The key assumption in these approaches is that mutations within functional regions of genes are likely to accumulate more slowly than those in regions without sequence-specific function
505 and, therefore, the comparison of conserved sequences from orthologous genes can indicate segments that might direct transcription [102]. These approaches predicted hundreds of conserved non-coding sequences upstream of *A. thaliana* genes. The length, specific positioning and enrichment for transcription factor
510 binding sites suggest these conserved non-coding sequences play a functional role in transcriptional regulation. Other plant specific examples of conserved non-coding sequence inference include [115, 116].

Besides conserved non-coding sequences, the elucidation of expression Quantitative Trait Loci (eQTL) in the form of single nucleotide polymorphisms
515 (SNPs) and small insertions and deletions (INDELs) appears promising for identifying functional binding sites, given their enrichment for transcription factor binding sites [117, 93]. A recent example in *A. thaliana* suggests a certain pathogen response to be dependent on a SNP present in a specific target genes promoter regulated by the transcriptional repressor VNI2 [93].

520 Another type of experimental data that can assist gene regulatory network inference is chromatin conformation mapping data from sequencing approaches such as DNase-seq, ATAC-seq and FAIREseq [118]. These experimental meth-

ods reveal regions of open chromatin, which is accessible to regulatory proteins [119, 120]. An overview of these methods is given in [118]. For example, DNase I hypersensitivity site information has been used to validate predicted gene regulatory networks in *A. thaliana* [113]. In their study, Van de Velde *et al.* observed that two-thirds of the regulators analyzed acted through binding events located in conserved non-coding regions coinciding with DNase I hypersensitive sites [113]. In *A. thaliana*, mapping of DNase I hypersensitivity sites from leaf and floral tissues has been useful for identifying cis-regulatory elements and elucidating the roles of transcription factors on leaf patterning and flower development [94]. Recently, efforts to curate plant-specific open chromatin maps have begun [121]. In addition, correlations between transcription factors and putative targets in chromatin modification [122] have been used to infer regulatory relationships between regulators and their target genes [87].

4.2. Supervised versus unsupervised approaches for dataset integration

The selection of an appropriate integrative classification framework to infer causal relationships remains challenging [88, 123, 124, 63]. Recent work on *D. melanogaster* demonstrated that multiple datasets can be integrated in a supervised fashion by learning the importance of each dataset in network inference [87]. In general, a great variety of supervised machine learning methods exists, including Support Vector Machines, Random Forests and Neural Networks [125]. In their analysis using *D. melanogaster* data, Marbach *et al.* showed that even a much simpler binary classifier (e.g. a logistic regression-based generalized linear model) can perform supervised data integration in inferring gene regulatory networks [87]. Interestingly, a simple, unsupervised feature aggregation yields similar results to supervised feature importance learning [87]. Alternatively, pipeline approaches, such as the INFERELATOR [126], are useful for combining individual algorithms used for different data types.

The competitive results of unsupervised approaches are encouraging, especially for plant science where gold standard data for model training is scarce. In addition, as unsupervised approaches, except for a few model parameters, do

not require any training of a large set of weight parameters for data integration, they are less prone to overfitting. Overfitting occurs when a supervised
555 model becomes too complex, i.e. it has too many parameters relative to the number of observations in the training data. Such a model has poor predictive performance on an unseen test set, as it is extensively tuned towards the training examples, and, as a consequence, overreacts to minor fluctuations from the training examples as given through unseen test examples.

560 4.3. Caveats in dataset integration

The consistency of the heterogeneous datasets is another important element in data integration. In contrast to working with prokaryotic organisms, datasets from multicellular organisms represent different developmental stages, organ/tissue/cell types and treatment conditions. Therefore, it is important to
565 integrate only the same condition or organ/tissue/cell-specific data when constructing a specific regulatory network [87], or combine condition-independent data such as transcription factor binding predictions with more condition-specific data, as in [5, 127].

The integration of multiple inference methods on gene expression data has
570 also been made [7]. Examples in plant science include the inference of gene regulatory networks for oxidative stress [128] and secondary cell wall synthesis [129]. However, a recent analysis on network-based gene function inference highlights that, while data aggregation exhibits enormous improvements, algorithm aggregation provides only modest benefits [130]. Therefore, algorithm aggregation
575 might provide an additional layer of robustness when gold standard data for evaluation and validation of individual methods is limited.

5. Assessing the quality of predicted gene regulatory networks

An important aspect of gene regulatory network inference is the experimental and *in silico* validation of the predicted regulatory relationships. Experimental
580 validation can include a variety of approaches, including genetic and chemical

perturbations of the regulators and observing changes of target gene expression
 and have been discussed in [131]. Here we focus our discussion on *in silico* vali-
 dation. A general approach for evaluating the quality of an inferred network *in*
silico is to interpret the problem as a binary classification task. Therefore, edges
 585 are evaluated as being present or absent. Standard performance metrics such
 as receiver operating characteristic or precision versus recall curves are used to
 measure algorithms performance [132]. Precision (P) and recall (R) are defined
 as $P = \frac{TP}{TP+FP}$ and $R = \frac{TP}{TP+FN}$, respectively. TP and FP denote the number
 of true and false positives, while FN is the number of false negatives. The
 590 receiver operator characteristic metric highlights how the number of correctly
 classified positive examples varies with the number of incorrectly classified neg-
 ative examples. Although receiver operator curves are typically recommended
 when evaluating binary decision problems, they can paint an overly optimistic
 picture of an algorithm’s performance if there is a large skew in the class dis-
 595 tribution [133]. Since this is the case for gene regulatory network inference
 where the number of putative true negatives by far outnumbers the most likely
 true positives, precision versus recall is generally more appropriate than receiver
 operator characteristic [88].

Here, we focus on three major challenges to consider when treating gene
 600 regulatory network evaluation as a binary classification problem. First is the
 general lack of negative data in gold standard data that consists of manually
 curated, experimentally proven regulatory relationships. Therefore, these cu-
 rated lists typically only contain positive class labels. Several strategies have
 been proposed to generate negative samples, including (i) random selection of
 605 regulator and target gene pairs; (ii) selecting genes without known binding sites
 of specific transcription factors in their promoters [134] as well as (iii) boot-
 strapping [135]. These samples, however, are not equivalent to true, biologically
 validated non-interaction and likely include false negatives.

Second, a major challenge lies in the extreme sparsity of gold standard data
 610 for model training and evaluation. As a consequence, precision values rapidly
 approximate scores expected by random guessing with increasing recall and the

highest precision value achieved by an algorithm is often defined by only few (e.g. 1-10) recalled gold standard links. This low density of recalled links over all predictions typically renders statistical significance tests between different inference methods meaningless. It also means that many computational models
615 are likely to perform similarly with respect to the sparse gold standard.

Finally, curated gold standard data contain experimentally validated regulatory links with respect to a plethora of experimental conditions. Given a specific condition under scrutiny, it is unlikely that the entire gold standard will
620 be recovered during inference. Accordingly, to exclude false positives, only a subset of the gold standard data, those corresponding to the conditions of the datasets employed for inference, can be used for model evaluation.

In lieu of substantial gold standard data, it is becoming common practice to assess the performance of an inference method with respect to the biological relevance of the inferred network [18, 87]. An example of this approach is
625 given for conserved regulatory network inference in *A. thaliana* [113]. In general, it is assumed that genes in a gene regulatory network are preferentially linked to genes involved in similar biological processes [136]. An example of how this assumption can be implemented to evaluate gene regulatory networks is given in [87]. Here the number of resulting co-regulated gene pairs with similar experimental co-function annotation (Gene Ontology) is used to evaluate the biological relevance of a specific inference method. Co-regulation is defined as gene pairs being regulated by shared regulators (generally defined by a Jaccard's coefficient higher than 50 %) [87]. Here, the Jaccard's coefficient computes the
630 intersection (shared) over the union of involved regulators per gene pair. An evaluation strategy that utilizes biological information to assess the performance of an inference method could help quantify the biological relevance of the inferred network. Such a strategy will help distinguish multiple methods, which perform similarly on the regulatory evidence alone, and assist in the selection
640 of more biologically relevant sub-networks for experimental validation. Validation could provide larger and more accurate gold standard data, which, in turn, could serve as training sets to improve prediction algorithms [5, 137].

6. Conclusion

With the emergence of large-scale transcriptional datasets, the mathematical
645 modeling of gene regulatory networks has played a major role in deciphering
the gene regulatory code. Computational inference of gene regulatory networks
can help prescreen *in silico* potential interactions and thus limit the extent
of experimentation needed [27]. With the advent of more powerful inference
frameworks such as deep learning [107], the infrastructure needed for large-scale
650 regulatory network inference is becoming established.

However, in gene regulatory network inference, there is still a great need
for consistent, large-scale, multiple data types. Currently, most datasets have
limited information content for specific cell types and developmental stages. As
a consequence, one of the major goals in plant systems biology should be the
655 generation of high-resolution spatio-temporal data, applicable to the task of
network inference [5, 137, 27, 11].

The decision about what biological questions can be answered and which
methods should be employed to answer the questions given a certain com-
pendium of biological data will most likely be in the hands of an interdisciplinary
660 team of biologists and computer scientists. It will therefore become increasingly
important for this diverse group of scientists to engage in a dialogue to solve
problems together. This requires the biologist to gain a reasonable understand-
ing about the underlying concepts and rationales of the computational methods
involved. The computer scientist, on the other hand, must be able to understand
665 the biological question, evaluate the opportunities and limitations of available
data and assess whether a particular computational concept can be applied
to gain biologically meaningful insights. To this end, community efforts such
as the DREAM challenges (<http://dreamchallenges.org/>) provide a promising
platform in bringing researchers together to present computational solutions
670 for biological problems, fostering collaboration and building communities in the
process. This will help advance our understanding of gene regulatory network
inference as well as systems biology.

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Competing interests

The authors declare that they have no competing interests.

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