**(c) Approach**

Describe overall strategy, methodology and analyses used. Include how data will be collected, analyzed, and interpreted as well as any resource sharing plans

If the project is in its early stages of development, describe any strategy to establish feasibility, and address the management of any high risk aspect of the work

**EXPERIMENTAL PLAN**

**Motivation & Significance**: Our ultimate goal is find a causal genetic network, effectively the circuit diagram regulating the N-assimilatory pathway in plants. By analogy to an electrical network, such a gene regulatory network would enable us to infer the causal relationships between genes. The nitrate assimilation pathway in Arabidopsis is a great system to develop methods to model the transcriptional regulatory control of metabolic networks, where the models can suggest targeted interventions to reduce fertilizer usage with implications for human health, energy and the environment. Importantly, this work will illustrate an experimental/informatics approach to the discovery of the causal network for any gene (or potentially any trait) of interest across a wide range of problems in biology and medicine. In the true spirit of the Systems Biology cycle [Gutierrez 2005], the high through put data generated in Aim 1, will drive network modeling in Aim 2, and the resulting network models will in turn predict new experiments to perform in Aim 3, as outlined below:

Aim 1. Experimental Innovation: “Network Walking”: Generation of high through put, high confidence TF🡪target datasets for TF hubs in the N-assimilatory network.

Aim 2. Computational Innovation: A pipeline machine learning approach to find causal links in regulatory networks using many kinds of genome-scale data.

Aim 3. Re-fuel the Systems Biology Cycle: The feedback from analysis to subsequent experiment.Testing new candidate TF hubs and TF cooperation suggested by new/refined network models.

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**Aim 1. Experimental Innovation: “Network-Walking”: Generation of high through put, high confidence TF🡪target datasets for hubs in the N-assimilatory network.**

***Rationale*:** This aim will produce high-confidence TF🡪target data genome-wide, which will be used to refine and train a machine learning pipeline (Aim 2), to model predictive regulatory networks controlling N-assimilation. The machine learning pipeline in Aim 2 will combine four distinct types of datasets we will generate in Aim 1: (i) steady state transcriptome data, (ii) time-serie transcriptome data, and data from TF perturbation studies including (iii) transcriptome and (iv) ChIP seq. For the TF perturbation studies, we will use a “Network Walking” approach, a transient, high throughput approach to perturb the expression of TF hubs associated with N-assimilatory networks, and examine alterations in target responses genome-wide. For selected TFs we will also examine effects of perturbations in planta.

**Network models for the N-regulation of genes in the N-assimilation pathway**: To date, our regulatory network models for the N-assimilation pathway, indicate that inorganic-N (nitrate) signals activate genes involved in nitrate uptake, reduction, and assimilation into organic-N (Glu/Gln) (Fig. X). These genes are in turn feedback-repressed by these organic-N products (Glu/Gln) of N-assimilation. (This may represent an “energy conservation” mechanism to conserve ATP & reducing equivalents, & carbon skeletons required for N-assimilation, when levels of organic-N are abundant.) In turn, these organic-N signals (Glu/Gln), activate target genes involved in converting “reactive” Gln into “inert” Asn, a more carbon-efficient N-transport amino acid used for N-transport/storage (e.g. to seeds) (see Fig. X). Our network models [Krouk 2010, Nero 2009b, Gutierrez 2008] have enabled us to identify TFs involved in mediating the responses to nitrate and organic-N signals (see Fig. 1, Table X), which are the focus of our initial perturbation studies.

**Identification and prioritization of TF hubs for perturbation studies.** We used two different types of network modeling approaches to identify the TFs associated with the regulation of this N-assimilation network. Time-series transcriptome data and state-space modeling (e.g. Dynamic Factor Graphs- DFG) was used to derive predictions network models for nitrate control of N-assimilation genes [Krouk 2010]. Network predictions – which were validated using out of sample data –was used to prioritize TFs induced “early” and at the top in the hierarchy for experimental testing (e.g. HRS1, HHO1, HHO2, HHO3) (Table 1). By contrast, TF hubs associated with organic-N regulation of N-assimilation pathway (e.g. CCA1, GLK1/2, WRKY1, bZip1) were identified based on TF🡪target correlation (>0.8) and representation of cis-elements [Gutierrez 2008] [Nero 2009a]. Remarkably, despite the different methods for their derivation, the top-ranking TF hubs of the nitrate vs. organic-N networks comprise two sub-clades of a single myb gene family, for which one member (HRS1) was also identified to be involved in phosphate signaling [Liu 2009] (Table X). This finding underscores the biological relevance of these TF hubs, and suggests that some may be involved in coordinating responses to nutrient signals (e.g. nitrate and phosphate). This hypothesis is supported by our preliminary results of HRS1 expression in transient assays (see Aim 1B), and attests to the biological relevance of the transient system.

We will test the TFs as prioritized in Table X in the transient DEX-protoplast system, to rapidly identify their network targets genome-wide using a combined analysis of transcriptome and ChIP assays. This will enable us to: (i) validate TF🡪target network predictions genome-wide, (ii) identify biological processes co-regulated with N-assimilation and (iii) provide validated TF🡪target data to feed our machine learning pipeline in Aim 2. In each of the subaims, we provide preliminary data using one of these TFs as a proof-of-principle example for the approach. [I think we should stress the iterative nature of what we are doing]

**Aim 1A. Generation of time-series transcriptome datasets for organic-N signaling networks.** In the case of nitrate signaling networks, fine-scale time-series data enabled us to identify >200 new nitrate-regulated genes compared to steady state studies (e.g. at 20 min) [Wang 2004], and also to generate dynamic, predictive regulatory networks [Krouk 2010]. By contrast, our organic-N network models were built using steady-state transcriptome data analyzed in the context of the Arabidopsis multinetwork [Gutierrez 2007, 2008]. While those network models enabled us to identify and validate master TF genes involved in organic-N regulation of the N-assimilation pathway (e.g. CCA1) [Gutierrez 2008], we cannot use these static network models to predict network dynamics or responses under untested conditions, an important goal of Systems Biology. To enable this approach, we will generate fine-scale, kinetic transcriptome data (0, 3, 6, 9, 12, 15, 20, 25, 30 min) using conditions shown to elicit an organic-N response (growth on 1mM nitrate, treatment with 40mM ammonium/nitrate vs. control KCl) [Gutierrez 2008]. This time-series transcriptome data will be used to drive state-space modeling of organic-N regulatory networks affecting N-assimilation as described in [Krouk 2010]. The time-series data will also be combined in a pipeline with analysis of steady state and TF perturbation data in Aim 2.

**Aim 1B. “Network Walking”: Generation of high through put, high confidence TF🡪target data.** Our goal in this subaim, is to use TF perturbation studies to validate predicted TF targets genome-wide, and the data will be used to functionally test and refine our gene regulatory networks, as discussed in Aim 2. As review (see Progress for details), this TF-expression system employs transient transformation of Arabidopsis protoplasts with TF-GR fusions, and inducible nuclear re-localization of TFs by dexamethasone (DEX) treatment. Transcriptomic analysis then enables us to investigate the network targets of a given TF genome wide. This system is rapid, and this allows the discovery of network targets any given TF in less than 2 weeks. This DEX-inducible approach has several advantages over Chromatin-IP (ChIP) for a number of reasons. (i) a Chromatin-IP (Chip-Seq) approach can confirm protein:DNA binding, it does not guarantee functional regulation [Eilers and Eisenman Genes & Dev. 2008. 22: 2755-2766]. (ii) the DEX-inducible TF system allows one to identify the effect of regulatory components that may not bind directly to DNA [ Lee, J, He, K, Stolc, V, Lee, H, Figueroa, P, Gao, Y, Tongprasit, W, Zhao, H, Lee, I, and Deng, XW, *Analysis of transcription factor HY5 genomic binding sites revealed its hierarchical role in light regulation of development.* Plant Cell, 2007. **19**(3): p. 731-749.] Technically, our approach is an adaptation of the original technique [ Sablowski, RW and Meyerowitz, EM, *A homolog of NO APICAL MERISTEM is an immediate target of the floral homeotic genes APETALA3/PISTILLATA.* Cell, 1998. **92**(1): p. 93-103.] combined with transcriptome analysis described in [ Hanson, J, Hanssen, M, Wiese, A, Hendriks, MM, and Smeekens, S, *The sucrose regulated transcription factor bZIP11 affects amino acid metabolism by regulating the expression of ASPARAGINE SYNTHETASE1 and PROLINE DEHYDROGENASE2.* Plant J, 2008. **53**(6): p. 935-949.] [Wang, Y, Joshi, T, Zhang, XS, Xu, D, and Chen, L, *Inferring gene regulatory networks from multiple microarray datasets.* Bioinformatics, 2006. **22**(19): p. 2413-2420] with modifications as below.

***Network Walking*** ***Approach*:** Conceptually, our DEX transient assay approach to rapidly validate TF🡪target interactions genome-wide consists of the following components; i) transient expression of a GR-TF fusion in protoplasts using FACS selection of successfully transfected cells, ii) use of an inducible dexamethasone (DEX) system to artificially control the entry of the TF into the nucleus, iii) DEX induction of TF activation is performed +/- cycloheximide (CHX), to identify primary and secondary targets of the TF (hence the name “Network Walking”, and iv) the use of nitrogen pre-treatments (e.g. prior to DEX induction of the TF) (see Fig. X). This last step allows us to identify the primary targets of the TF when acting alone or in combination with other TFs that are N-regulated transcriptionally or post-translationally. A global view of the DEX approach is summarized in Fig. X, and described more fully in Progress and in Bargmann et al 2012.

***As proof-of-principle***, we demonstrate that the rapid protoplast DEX-inducible TF system enables us to uncover network targets with relevance to whole plants. For this, we targeted HRS1 (At1g1330), a top scoring TF hub of our “learned” nitrate-regulatory network [Krouk 2010], previously shown to be involved in phosphate signaling [Liu 2009, Journal of Integrative Plant Biology]. We hypothesize that HRS1 may also be involved in nitrogen signaling, as HRS1 is induced within 9 min of NO3- treatment in the dynamic inferred network, and it is predicted to be the top most controlling and top most controlled TF hub controlling the N-assimilation pathway [Krouk et al 2010]. Our initial analysis of transcriptome data from 35S::HRS1-GR expressed in the transient DEX-protoplast system, revealed four distinct gene clusters influenced by a combination of HRS1 induction (by DEX) and nitrate-treatment (Fig. X). Cluster 4 genes which are primary targets of HRS1 (+DEX+CHX) that are activated by DEX only in the *presence* of NO3- are significantly overrepresented in the GO-categories of phosphate transport (p-val 8.14 E-6). Thus, these HRS1 network targets observed in the transient DEX system, recapitulate the phosphate signaling phenotype observed in 35S::HRS1 overexpressing plants [Liu 2009]. These results support the notion that TF->target relationships validated in the rapid & transient TF protoplast system, are relevant to whole plants. [Kranthi-This paragraph needs a description of what happened when we did the preliminary results on HRS1 gene +DEX and +/-CHX to show that direct targets are a subset of indirect] We will subject the prioritized TFs, to the perturbations in this system (+/- DEX, +/-CHX, +/-N), where Table X lists our progress. Conditions for +/-N will be tailored to either nitrate signaling (growth in ammonium succinate, treatment with nitrate) or organic-N signaling (growth in 1mM nitrate, and treatment with 40mM ammonium/nitrate). Cluster analysis of transcriptomic data, followed by GO-term analysis using the Biomaps function in VirtualPlant [Katari 2010], will identify biological processes controlled by specific TFs. The transcriptome data will also be fed into the pipeline of machine learning algorithms developed in Aim 2, to fuel the generation and refinement of a causal predictive network.

**Aim 1C. Genome-wide validation of TF (protein)🡪targets (DNA) using Chip-Seq.** In this Aim, we will determine direct TF->target interactions using our transient TF perturbation system and compare these results to gene activation (transcriptome) studies. This will enable us to determine (i) targets to which the TF is bound, and (ii) gene targets that are transcriptionally activated. Because 35S::TF-GR (glucorticoid receptor) constructs are expressed in this system, we can use anti-GR antibodies to bind the TF-GR fusion protein. To demonstrate that perform ChIP and transcriptome studies and from the same sample, we performed a ***proof-of-principle*** study using a 35S::GR-BZip construct. To enable this approach in protoplasts, we adapted the micro-ChIP protocol from [Dhal and Collas, 2008], which requires a relatively small number of cells. After transformation and FACS cell sorting (to select successfully transformed cells), protoplasts were treated with 1% formaldehyde for 10 min and then quenched with 100mM glycine for 5 min. W5 buffer was then added to wash the cells before pelletting by centrifugation at 2,500 rpm for 2 min. Cross-linked cells were snap-frozen in liquid N and stored at -80C. The protocol for processing 1,000 cells was then followed. Anti GR (sc-1002) antibodies from Santa Cruz Biotechnology Inc. were used to capture the GR::bZip1:DNA complexes, and ChIP DNA was purified after reverse cross-linking using the MiniElute Reaction cleanup kit from QIAGEN. ChIP DNA was prepared for illumine sequencing as follows. Pair-end ChIP-seq libraries were constructed in accordance with the Illumina ChIP-seq sample prep guide (Illumina, San Diego, CA), with the following modifications to facilitate library preparation from low amounts of starting DNA (~1ng): (i) adaptor oligo mix was further diluted by 3-fold to maintain a proper adaptor to DNA insert ratio; (ii) Solid Phase Reversible Immobilization (SPRI) magnetic bead-based technology was used to size-select the library after adaptor ligation, instead of the common agarose gel size selection, to minimize DNA loss. (3) SYBR gold agarose gel size selection was performed after PCR enrichment to remove adaptor self-ligation product from the library. Libraries were separately constructed for the immunoprecipitated DNA and the input DNA (the DNA without immunoprecipitation) to serve as control. The libraries were subsequently sequenced on the Illumina GAIIx platform. The obtained sequence reads were filtered for quality and trimmed to remove adapter sequences. These filtered reads were then aligned to the TAIR10 assembly of the Arabidopsis thaliana genome. Genomic regions that are significantly enriched in the immunoprecipitated sample relative to the input DNA were identified using a peak-calling algorithm called QuEST [Valouev et al., Nature methods 5, 829-834 (2008)]. The genes immediately adjacent to the identified peak positions are considered putative targets of bZIP1. KRANTHI- RESULTS OF ANALYSIS bZIP1???????

**Aim 1D. Perturbations of TFs in whole plants: T-DNA mutants and 35S::Over-expressors.** The transient DEX-TF protoplast assay used in Aims 1B & C, is a rapid way to test how perturbations of TFs affect network targets genome-wide. It also enables us to overcome problems associated with functional redundancy of TFs encountered in reverse genetic studies [Chen HW, Bandyopadhyay S, Shasha DE, Birnbaum KD, “[Predicting genome-wide redundancy using machine learning”.](http://www.ncbi.nlm.nih.gov/pubmed/21087504). BMC Evol Biol. 2010 Nov 18;10:357] [Cutler S and McCourt P (2005) Dude, Where’s my phenotype? Dealing with redundancy in Signaling Networks. Plant Physiology (2005) v. 138, pp558-9]. Owing to functional redundancy, we have failed to see a molecular phenotype (e.g. altered expression of a target genes) in T-DNA mutants for most of the TFs listed in Table X. For example, testing of sentinel TF->target relations by Q-PCR revealed molecular phenotypes in 35S::CCA1 [Gutierrez et al 2008], and also in double mutants (e.g cca1/lhy1 and glk1/glk2), but not in single mutants (Fig. X). In rare cases, misregulation of predicted TF🡪target genes were observed in a single TF T-DNA mutant. For example, WRKY1 (At2g04880), is predicted to be a TF “toggle switch”, to induce expression of genes involved in nitrate reduction and assimilation into Gln, while simultaneously repressing expression of genes involved in converting Gln to Asn (Fig. X). Indeed all three WRKY1 T-DNA insertion mutants (SALK\_016954; SALK\_136009; SALK\_070989) revealed decreased expression of NIA2 and NRT2.1 (targets of WRKY1 activation), and increased expression bZIP1🡪ASN1 (both targets of WRKY1 repression), as predicted by the network model (Fig. X), while the opposite expression patterns are found in the DEX system where WRKY1 is over-expressed using methods described in Aim 1C. We will perform transcriptomic analysis on T-DNAs for TFs in cases where Q-PCR analysis shows mis-regulation of a predicted target gene in the N-assimilation network. Such T-DNA mutants in TFs will also be used to study TF-TF cooperation (see Aim 3B). Additionally, based on results in the DEX-transient system (Aim 1B), for selected TFs, we will create stable transformants. This will allow us to explore network targets in planta, in distinct organs, or across developmental time-points – where the context of TF partners may affect functional activation of targets.

**Aim 2. Computational Innovation: A pipelined machine learning approach to find causal links in regulatory networks using many kinds of genome-scale data**

***Rationale***: Our ultimate goal is to employ machine-learning approaches to model a causal genetic network, effectively the circuit diagram underlying the regulation of genes in the N-assimilatory pathway. To date, we have used a machine learning approach (Dynamic Factor Graph State Space Modeling, DFG for short) to generate a predictive network model for nitrate control of N-assimilatory pathway based on time-series data from wild-type plants [Krouk 2010]. In this aim, we develop methods and approaches to refine and improve the predictive power of such networks, by feeding our network learning algorithms experimental data derived from cells (or plants), in which we have perturbed expression of the TF and monitored effects on target genes. The transcriptome data generated from these transient expression experiments will support either primary or secondary TF🡪target relationships, some of which will be validated by ChIP-seq.

**Approach**: To improve the predictive power of our networks, we will create a pipeline to generate a predictive network model that makes use of four types of genomic data that are available to us, as generated in Aim 1: (i) steady state transcriptome data (e.g. N-treatments); (ii) TF perturbation transcriptome data (e.g. DEX data and T-DNA), (iii) time-series data (e.g. expression over time), and (iv) TF🡪target binding validated by ChIP seq. No single algorithm is best for machine learning using all four kinds of data, so we will use several algorithms together in a pipeline described below.

**The Network**: The causal network will consist of nodes that are genes and edges between genes that are labeled with coefficients. A positive coefficient corresponds to an inductive edge. A negative coefficient corresponds to a repressive edge. For a given target gene Z, these coefficients will be reflected in the form of an equation Z = c1\*A + c2\*B + c3\*C …. , where A, B, and C are expression levels of transcription factor genes. So, if c1 is positive, then gene Z will rise (increase in expression, because Z would be positive) as gene A rises (i.e., A to Z would be an inductive edge) assuming the other genes are held constant. If c2 were negative, then an increase in expression of B would cause a decrease in Z, all else being equal. Thus, B to Z would be a repressive edge. Typically, machine learning algorithms to infer such equations will include a regularization factor that will limit the number of additive products on the right hand side to three or four, but this still means that even for 100 genes, our algorithms would have to find the proper 400 edges out of a possible 5,000 and assign coefficients to those edges. Regularization is a form of parsimony: we want to find the simplest model that explains the behavior. Simpler models tend to be more robust to noise because they avoid overfitting. In fact, as part of our quest for simplicity, we prefer “linear” models to quadratic ones (in which there would be terms of the form d\*B\*C, where B and C would be gene expressions). Surprisingly, the resulting models explain behavior well. A model explains behavior if it can predict the state of one gene given the state of other genes at the same or previous time points. For example, we used this approach to successfully predict regulatory edges in a network generated from time-series data in nitrate-treated roots [Krouk et al 2010].

**Pipeline of Machine Learning Algorithms to integrate learning from distinct data-types.**

One reasonable approach is to establish a pipeline (Fig. X, figure dfg.eps) that analyzes different kinds of data using different algorithms. In one such pipeline, the MCZ algorithm (short for the Median Corrected Z-score method [Greenfield, A., Madar, A., Ostrer, H., Bonneau, R.: DREAM4: Combining Genetic and Dynamic Information to Identify Biological Networks and Dynamical Models. PloS one (2010]) is used for both the steady state and mutation data, followed by the DFG algorithm (short for Dynamic Factor Graph used for time-series data [Krouk,G.,Mirowski,P.,LeCun,Y.,Shasha,D.E.,Coruzzi,G.M.:Predictivenetworkmodeling of the high-resolution dynamic plant transcriptome in response to nitrate. Genome Biology 11(12), R123 (2010]). In this pipeline, the MCZ algorithm assigns initial weights to certain edges that are then refined by the DFG algorithm. The basic idea of the MCZ algorithm is that if gene \[x\_j] influences \[x\_i], then knocking out \[x\_j] should change the value of \[x\_i] in a significant way. We will measure the significance based on the number of standard deviations from the median value of \[x\_i] over all non-mutation experiments.

In preliminary work, done for a book we have written called “Network Inference in Molecular Biology”, [Jesse Lingeman and Dennis Shasha 2012 *Network Inference in Molecular Biology -- a hand-on framework*, Springer 2012], we show with data from the DREAM benchmark (Dialogue for Reverse Engineering Assessments and Methods) [Schaffter, T., Marbach, D., Floreano, D.: GeneNetWeaver: in silico benchmark generation and performance profiling of network inference methods. Bioinformatics 27(16), 2263–2270 (2011)] that using the MCZ🡪DFG pipeline is better than using any single machine learning algorithm by itself.

Alternatively, we can posit a collection of algorithms that each predicts edges and then use a “voting” approach to determine the best one. In that scheme, illustrated in the figure consensus.eps, in addition to the Median-Corrected Z-score and Dynamic Factor Graph algorithms, we use the NIR (Network Identification by Multiple Regression [Gardner,T.S.,DiBernardo,D.,Lorenz,D.,Collins,J.J.:Inferring Genetic Networks and Identifying Compound Mode of Action via Expression Profiling. Science 301(5629), 102–105 (2003)]) and CLR (Context Likelihood of Relatedness [Faith, J.J., Hayete, B., Thaden, J.T., Mogno, I., Wierzbowski, J., Cottarel, G., Kasif, S., Collins, J.J., Gardner, T.S.: Large-scale mapping and validation of Escherichia coli transcriptional regulation from a compendium of expression profiles. PLoS biology 5(1), e8 (2007)]) on steady state data. We also use BANJO (Bayesian Inference with Java Objects [Yu,J.:AdvancestoBayesiannetworkinferenceforgeneratingcausalnetworksfromobservational biological data. Bioinformatics 20(18), 3594–3603 (2004)]), Time-Delay ARACNE [Zoppoli, P., Morganella, S., Ceccarelli, M.: TimeDelay-ARACNE: Reverse engineering of gene networks from time-course data by an information theoretic approach. BMC Bioinfor- matics (2010)], and Inferelator [Greenfield, A., Madar, A., Ostrer, H., Bonneau, R.: DREAM4: Combining Genetic and Dynamic Information to Identify Biological Networks and Dynamical Models. PloS one (2010)]. Because each machine learning algorithm ranks the TF🡪Target edges, we “weight” each vote for a TF🡪target edge depending on its rank in each of these programs. Highly ranked edges acquire a weight close to 1, and lowly-ranked edges acquire weights near 0, where the weight drops off exponentially. Algorithms on steady state data from wild-type and pertubation data (e.g. DEX-TF and T-DNA) assign greater or lesser weights to edges. Those weights are refined by one of the time-series based algorithms (e.g. Inferelator, Dynamic Factor Graphs, or Time Delay ARACNE).

**Preliminary Results:** To test the value of pipelines for predicting edges in a network, we compare the results of our previous Dynamic Factor Graph (DFG)/State Space Modeling Approach [Krouk,G.,Mirowski,P.,LeCun,Y.,Shasha,D.E.,Coruzzi,G.M.:Predictive network modeling of the high-resolution dynamic plant transcriptome in response to nitrate. Genome Biology 11(12), R123 (2010] built on time-series data, with a pipeline “weighted” approach to network inference. Our criterion for quality is how well the resulting network predicts out-of-sample data. In our previous paper [Krouk 2010], we showed that using the training data consisting of time-series data from the time of a perturbation, 3 minutes later, 6 minutes later, 9, 12, and 15, we were able to use a Dynamic Factor Graph Approach to predict the direction of expression change of 76 genes associated with the N-assimilation network between 15 and 20 minutes 74% of the time correctly. By contrast, a naïve trend forecasting method, which predicted the direction of expression change of genes between 15 and 20 minutes as being the same as between 12 and 15 minutes, was correct only 52% of the time, having a prediction accuracy marginally better than chance.

Using the new data from perturbation of TF function (using Dex) and the steady state data along with the time series data from wild-type plants, we can predict z% of the gene directions correctly. Using only the steady state data along with time series data, we can predict z’% of the gene directions correctly. Using the pipeline

approach, we obtain z’’% of the gene expression directions correctly. [this paragraph should be rendered as a table. Gloria could you set one up that has five columns with headers: Steady State, Genetic Perturbation, Time Series, Prediction accuracy, p-value

**Intended Approach**: Based on our preliminary results, we will iterate the following steps: (i) identify the genes relevant to nitrogen treatments (on the order of a few hundred) based on steady state (unmutated) and time series data resulting in just over 200 genes, (ii) develop a pipeline inference approach based on steady state, TF perturbation experiments (e.g. DEX experiments or T-DNA mutants), and time-series data.. This will result in a regulatory network of the genes involved in N-assimilation. That regulatory network will suggest new genes on which to try DEX experiments (e.g. putative TF hubs). Those validations will be used to refine a new network in an iterative approach. As usual, our criterion of goodness (for network predictions) will be the ability to predict well on out-of-sample data, both missing time points and missing mutations. Ultimately, we would like to learn the network model well enough that we choose the next DEX experiment to try based on which TF🡪target relationships will be the hardest for our learned network to predict.

DENNIS- I THINK THE TIME-SERIES DATA SHOULD BE THE FIRST STEP, AS IT WILL IDENTIFY TFS THAT ARE MISSED IN THE STEADY STATE DATA. YOU MAY REMEMBER THAT GABS TIME SERIES DATA IDENTIFIED >200 NEW N-REGULATED GENES, AS PREVIOUS STEADY STATE DATA WAS FROM LONGER TIME POINTS, WHEN THESE GENES WERE ALREADY DOWN. DO YOU AGREE??? IF SO< YOU NEED TO REFINE THE INTENDED APPROACH PARAGRAPH ABOVE. Done.

**Aim 3. Re-fuel the Systems Biology Cycle: The feedback from analysis to subsequent experiment. Testing new candidate TF hubs and TF cooperation suggested by new/refined network models.**

**Rationale**: In the spirit of the Systems Biology cycle [Gutierrez 2005], the network models from Aim 2 will in turn predict new network perturbation experiments to perform in Aim 3. These predictions include identification of new TF hubs to test using the high throughput approaches described in Aim 1. This may include, for example, TFs for which TF🡪target relationships are the hardest for our learned network to predict. Our network models in Aim 2 will also give suggestions for TF partners that may act in concert (or antagonistically) on genes in the N-assimilatory network. Thus, in this aim, we will develop high throughput experimental approaches to test the cooperation of TFs in the learned network and these will enhance the network models leading to new predictions [Gloria we need a figure showing this cycle of experiment to modeling to more experiments to more modeling].

**Approach**: We will test TF🡪target relationships for new candidate TF hubs identified in Aim 2 using the transient DEX-system for TF activation described in Aim 1B. We will also use two complementary approaches to test for TF-TF cooperation: (i) Co-expression of TF1 and TF2 in the transient protoplast DEX-system, (ii) Testing of TF cooperation using a genetic approach in the transient expression system. For the latter approach, expression of TF1 in wild-type protoplasts, will be compared to expression of TF1 in protoplasts made from T-DNA mutants in TF2. Targets regulated by TF1 in protoplasts wild-type for TF2, that *are mis-*regulated by TF1 in protoplasts made from tf2 T-DNA mutants, will suggest TF1-TF2 dependencies - including additive or synergistic effects on target genes in the networks.

**Aim 3A. Perturbation studies for new candidate TFs**: In this subaim, network models derived in Aim 2, will be used to identify new TFs for targeted for perturbation studies using approaches described in Aim 1B and C. For example, new time-series data for organic-N signaling generated in Aim 1A, will refine our current network models derived from steady state data) and will identify new TFs that are activated early in the cascade (e.g. within 3-9 min). The prediction that we will find new TFs involved in organic-N signaling is based on our time-series analysis of nitrate-responsive networks where we identified >200 new nitrate-responsive genes in a 3-9 min window [Krouk et al 2010]. , not previously observed in steady state studies performed at 20 min [Wang 2004]. The new network models will also identify which TFs may have the most influence based on their out-degree (“hubbiness”) in the network, or based on the phenotypic importance of their targets. These will be the new candidate TFs to target for perturbation studies.

**Aim 3B. Testing TF cooperation in the N-assimilatory network.** Our new and refined network models generated using the pipeline in Aim 2, will suggest TF pairs that may cooperate to regulate key target genes in the network. We will use two complementary approaches to test how selected TF pairs may cooperate in the regulation of target genes. In one approach, we will co-express the two TFs in the transient DEX-protoplast system using a single vector, and compare regulation of target genes to those observed for expression of each single TF. Target genes regulated differently in the TF1/TF2 double expression vector, compared to expression of each TF alone, will be evidence for cooperation. In a complementary high through-put genetic approach, we will express TF1 in protoplasts made from a tf2 T-DNA mutant using the DEX-transient activation system described in Aim 1B. Evidence for TF cooperation, will be loss of target regulation by TF1 in a tf2 mutant background, compared to wild-type. Transcriptomic analysis will identify target genes for which TF1/TF2 cooperation can explain additive or synergistic expression. Synergistic regulation of target gene expression would be functional evidence for TF cooperation, which could involve either direct or indirect TF interaction.

**Co-expression of TF1 and TF2 and genetic perturbation:** In order to co-express two TFs (TF1 and TF2) in the same cell, we are using the MultiSite Gateway**®** Technology that allows one to simultaneously clone multiple DNA fragments in a defined order and orientation. To generate the co-expression construct, TF1 cDNA, TF2 cDNA and the “GR cassette” (that includes a terminator, 35S promoter and the GR sequence) were cloned in appropriate donor vectors, and allowed to recombine with each other and with the destination vector pBob11. This resulted in the assembly of two consecutive expression cassettes 35S::TF1::GR and 35S::TF2::GR (Fig. X). Expressing both TFs from the same vector has a few advantages: (i) it eliminates the problem of dealing with multiple vectors that could have different transfection efficiencies due to their inert TF size; (ii) when multiple vectors are transfected, each cell is likely to contain a random number of copies, while a single co-expression vector will result in homogeneous transgenic content of protoplasts. Where target gene expression is synergistically regulated in the TF1/2 vector, compared to either single vector will identify targets of TF cooperation.

As a complement to the multisite vector approach, we will explore TF cooperation by transiently expressing the single 35S::TF1-GR in protoplasts made from a T-DNA mutant in tf2, compared to expression in protoplasts wild-type for TF2. Loss, or change in target gene regulation by TF1 in a tf2 mutant protoplast (compared to wild type), would indicate cooperation of TF1 and TF2. This would provide functional evidence for targets of TF cooperation- which could involve direct or indirect TF interactions. We will use this genetic approach for TFs that show a molecular phenotype in the single T-DNA mutant (e.g. WRKY1 T-DNA). For TFs that are functionally redundant, we will use double mutants (e.g. glk1/2, cca1/2) to explore TF cooperation in transcriptional networks. For example, as glk1/2 are in the same myb family as HRS1, we will express HRS1 in a glk1/2 double mutant background, and determine the effects on target gene activation or repression. These perturbation results will be fed into the machine learning pipeline (Aim 2), to refine our network predictions.