**(a) SIGNIFICANCE** Modeling predictive Gene Regulatory Networks (GRNs) from comprehensive gene expression datasets is both a major goal *and* challenge in fundamental and applied research in Systems Biology. In this grant, our overall goal is to infer a causal genetic network, effectively the circuit diagram underlying the metabolic regulation of N-assimilation pathway, to predict network states under untested conditions, and in response to perturbation, a holy grail of Systems Biology. Computational inference of biological networks is an inherently difficult problem, mainly due to limitations of genomic data (e.g. presence of too few time-point measurements, many genes, measurement errors and noise) [Jaeger and Monk 2010][ De Smet and Marchal (2010) Nature Reviews: Microbiology, vol 8, p 717-729] However, progress has been made using machine learning approaches to successfully predict network states under untested conditions in microbes [Bonneau Cell 2007] and yeast [REFS], and more recently in higher eukaryotes including animals [REF???] plants [Krouk 2010]. Our recent work in this area, applied a machine learning approach to time-series transcriptome data to model how nitrate signals control the N-assimilatory network, and these learned networks enabled us to accurately predict gene expression states on “left-out” experimental data [Krouk 2010]. In this renewal, we propose to build on and improve the predictive power of our inferred networks, by developing *NetWalk: An iterative high throughput experimental/machine learning approach to infer causal networks.* The experimental novelty, employs a high-throughput TF perturbation system to rapidly validate TF🡪 targets genome-wide. This data will validate a new network inference approach, whose novelty resides in combining diverse genomic datasets into a single pipeline. This approach, which is not currently possible in existing frameworks, improves network prediction in preliminary studies. Broadly applied, NetWalk, will illustrate a combined experimental/informatics approach to the discovery of causal networks for any gene, metabolic pathway, process, or trait, with applications across a wide range of problems in biology, medicine and agriculture. In our case, the inferred networks controlling N-assimilation can suggest targeted interventions to reduce nitrogen fertilizer use, with implications for health, energy and the environment.

**(b) INNOVATIONS**  from a close collaboration between Biologists and Computer Scientists at NYU Courant.

**Experimental: NetWalk Targets: A high-throughput method to validate TF🡪network targets.**  This approach provides a systems view of a TF function *in vivo*, based on transient TF perturbation*.* The system involves TF overexpression, coupled with inducible TF nuclear import, to identify effects on TF targets genome-wide. Its advantages are: i) *High throughput*: acceleration of TF perturbation studies to weeks, compared to stable transgenics (~4-6 mos), ii) *TF Redundancy*: transient overexpression and TF activation overcomes problems of TF redundancy encountered in T-DNA mutants, iii) *Validation*: TF🡪 targets can be validated genome-wide using transcriptome *and* ChIP-seq approaches. The TF🡪target data generated in Aim 1, will train and validate our the NetWalk pipeline inference approach in Aim 2, as below.

**Computational:** **NetWalk Inference: A computational pipeline for network inference combining multiple data-types.** Current network inference methods are limited in that they can only accept specific transcriptome data-types (e.g. steady state or time -series) Bonneau (2007) Cell] [ Bonneau Genome Biol 2006] [Wang Y,. Bioinformatics 2006] [Shimamura. BMC Syst Biol 2009]. This road-block to data integration prevents the identification of “high confidence” TF🡪target edges required to train and validate machine learning approaches. To overcome this problem, we will develop a computational network inference pipeline with the capacity to “learn” from multiple genomic data types (e.g. time-series data, steady state data, and TF perturbation data), to validate and identify “high confidence” edges in our networks. This pipeline will combine the DFG algorithm (Dynamic Factor Graph), a form of “state space” analysis we used previously on time-series transcriptome data [Krouk et al 2010], with the MCZ algorithm (Median Corrected Z-score method), a simple but successful approach to infer the effects of TF perturbations on targets using steady state mutant data [Greenfield & Bonneau]. Preliminary results suggest that this new pipelined approach to network inference will increase the quality of our predictions, as judged using left-out data. As the NetWalk approach is iterative, the networks inferred from this new computational pipeline in Aim 2, will drive a further round of experimentation, on new TFs and TF interactions in Aim 3, in a true Systems Biology cycle [Gutierrez 2005].

**Practical Significance: A model for metabolic regulatory networks.** The N-assimilatory pathway is well-suited for metabolic regulatory network modeling studies, owing to its exquisite transcriptional regulation by nutrient cues, and its importance to plant growth and development [Ruffel S, 2010]. The N-metabolite inputs (nitrate) and outputs (Glu/Gln), each serve as “N-signals” to regulate N-assimilation pathway genes according to supply and demand of inorganic [Ruffel 2011] and organic-N signals [Gutierrez 2008]. Our current regulatory networks derived from transcriptome data [Krouk 2010 [Gutierrez 2008], support a model in which inorganic-N (nitrate) induces expression of genes involved in nitrate uptake, reduction and assimilation into Glu/Gln (Fig. X). The organic-N products, in turn feed back repress expression genes involved in Gln synthesis, and activate genes involved in Gln-dependent synthesis of Asn, an inert carbon-efficient amino acid used to transport/store N (e.g. in seeds). This may represent an “energy sensing” mechanism to save ATP, reducing equivalents, and C-skeletons when levels of organic-N are abundant. As as altering transcription of key genes in the N-assimilation pathway (GLN1 or ASN1) affects N-use in transgenic plants [Oliveira (2002)][Lam, (2003)], our inferred network models may predict targeted interventions of master TF regulators affecting multiple target genes to effect changes in N-use efficiency, a goal of significant import to agriculture, energy, and the environment.

**PROGRESS REPORT**: ***Overview:*** Our goal to create dynamic, predictive models for the N-assimilatory pathway has been achieved during the current cycle, using a new machine learning approach to network inference. To put this in context, during the previous NIH cycle, our static network models were based on analyzing steady-state transcriptome data in the context of an Arabidopsis multinetwork [Gutierrez 2007, 2008]. That approach, uncovered TFs and a novel hypothesis for interactions between N-assimilation and the circadian clock [Gutierrez 2008]. During the current funding cycle, by creating new dynamic network models, we achieved the the ultimate goal of Systems Biology - predict network states under untested conditions. To do this, we generated time-series data (Aim 2B) and used a machine learning approach, to infer dynamic network models for the nitrate-control of N-assimilation (Aim 4) [Krouk 2010]. Predictions from this “learned” network were validated *in silico*, by predicting gene expression states in an experiment that wasn’t used in the inference of the network, and then comparing our predictions with the data from that “left-out” experiment, and also experimentally for selected TFs [Krouk 2010]. To accelerate the experimental validation of our TF🡪 target predictions (Aim 2A), we developed a rapid high through-put system to perturb (e.g. induce) TF function and monitor changes in target gene expression [Bargmann et al, 2012, submitted]. This transient TF perturbation system successfully uncovers changes in expression of direct TF targets, as validated using ABI3, a well-studied TF for which network targets are known [Vernoux 2011]. We are currently this transient TF expression system to validate other TF hubs involved in our nitrate [Krouk et al 2010] and organic-N [Gutierrez 2008] networks controlling N-assimilation, to accelerate our studies, and overcome problems with TF redundancies that we have encountered (Aim 1). Finally, our studies of post-translational responses to nitrate and Glu/Gln sensing were conducted collaboratively as part of an NIH Fogarty Award t0 R. Gutierrez [Vidal 2010].

*Note: This Progress Report specifically highlights progress most relevant to our Research Strategy.*

**Prior Aim 1. Test hypotheses for TFs controlling targets in N-assimilation. *Publications*:** Nero (2009a) [*In silico* Evaluation of Predicted Regulatory Interactions in Arabidopsis thaliana.](http://www.ncbi.nlm.nih.gov/pubmed/20025756?itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum&ordinalpos=1) ***BMC Bioinformatics***, 10(1): 435; Nero (2009b) “[A system biology approach highlights a hormonal enhancer effect on regulation of genes in a nitrate responsive "biomodule".](http://www.ncbi.nlm.nih.gov/pubmed/19500399?itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum&ordinalpos=4) ***BMC Syst Biol***., 3:59; Obertello M (2010) “Modeling the global effect of the basic-leucine zipper transcription factor 1 (bZIP1) on nitrogen and light regulation in Arabidopsis.” ***BMC Syst Biol***., 4:111.

***A regulatory network controlling N-assimilation in response to nitrogen sensing*.** Our regulatory networks aim to uncover the mechanisms by which the input (nitrate) and output (Glu/Gln) of the N-assimilation pathway act as N-signals to regulate pathway gene expression. Nitrate signaling is well documented [Wang et al 2004] [Krouk, (2010) Current Opin Plant Biology] and recent studies have identified a nitrate “transceptor” [Ho 2009,] **[Gojon** 2011]. There is also ample, though less direct, evidence to support Glu/Gln signaling [Gutierrez 2008] [Rawat 1999], but the receptor(s) remain elusive [Hsieh (1998)][ Lam (1998)] [Forde & Lea 2007, J Exp Bot. 58 pp2339]. To identify the downstream components of N-signaling, we modeled regulatory networks using a multinetwork approach on steady-state transcriptome data under conditions shown to induce nitrate [Nero et al 2009a,b] or organic-N signaling [Gutierrez 2008]. More recently, we used time-series transcriptome data and machine learning approaches to infer *dynamic* regulatory networks for nitrate [Krouk 2010] (See Aim 2B and 4, below). Combined, these studies support a model in which nitrate signaling induces expression of TF hubs (e.g. HRS1, HHO1,2,3) predicted to activate genes involved in nitrate uptake, reduction, and assimilation into organic-N (Glu/Gln) (Fig. XA) [Krouk et al 2010]. In turn, the organic-N products of nitrate assimilation (Glu/Gln) repress expression of TF hubs (CCA1, GLK1, WRKY1) that activate genes involved in Gln synthesis, in a negative feed back loop. This TF repression in turn leads to the induction of bZip1, an activator of ASN1, catalyzing the Gln-dependent synthesis of Asn an inert, C-efficient amino acid used for N-transport/storage (Fig. XB). This may represent an “energy conservation” mechanism to save ATP, reducing equivalents, and carbon skeletons, when levels of organic-N are abundant. Indeed there is experimental support for a role of bZip1🡪ASN1 in an “energy sensing” mechanism [Baena Gonzales]. ***Validation of TF🡪target predictions in T-DNA mutants and transgenics***: Our validation studies involved TF perturbations in plants. Owing to TF functional redundancy, analysis of double mutants or transgenic overexpressors were usually required for TF🡪 target validation. All of the TF🡪target relationships for the organic-N network shown in Fig. X, have been confirmed using the following lines: CCA1 (35S::CCA1) [Gutierrez 2008], GLK1 (glk1/2) [Para, unpublished]. In the case of WRKY1, T-DNA mutant studies confirm its role as “toggle switch” between Gln and Asn synthesis. Three independent wrky1 T-DNA lines (SALK\_016954; SALK\_136009; SALK\_070989) showed decreased expression of genes predicted to be targets of WRKY1 activation (NIA2 & NRT2.1), and increased expression of genes predicted to be targets of WRKY1 repression (bZIP1 & ASN1) (Fig. X). Interestingly, the opposite expression patterns are observed when WRKY1 is transiently over-expressed in protoplasts, using the transient system described in Prior Aim 2A, below. Owing to functional redundancy, a bZip1 T-DNA mutant could not confirm the TF🡪target relationship [Obertello 2010], but independent transient expression of bZip1 [Baena-Gonzales confirm the bZip1🡪ASN1 relationship, as do preliminary bZip1 ChIP-seq studies (see Aim 1C of Plan). With regard to the nitrate-activated TF hubs identified in our dynamic networks [Krouk 2010] (HRS1, HHO1-3) (see Aims 2B/4 below), our collaborator, Dr. Krouk (INRA, France) has generated and is testing double mutants for validation. The T-DNA mutants will be useful in our studies of TF interactions described in Aim 3 of the Plan.

**Prior Aims 4 & 2B. Creation of a time-dependent dynamic network model for the control of N- assimilation. *Relevant Publication***: Krouk *et al.* (2010) “Predictive network modeling of the high-resolution dynamic plant transcriptome in response to nitrate.” ***Genome Biology,*** 11 (12), R123.

***Summary:*** The analysis of fine-scale time-series transcriptome data generated in (Aim 2B), using a machine learning approach called “State Space modeling” (Aim 4), enabled us to predict network responses under untested conditions- the ultimate goal of Systems Biology. Network predictions were validated using left-out data and experimentally for select TFs, as described in [Krouk et al 2010] and below.

***A predictive network model for nitrate-control of the N-assimilation network.*** Because causality moves forward in time, time-series experiments are a particularly promising source of structure to derive predictive networks. We thus generated time-series transcriptome data from nitrate-treated plants (0,3,6,9,12,15,20 min) (Aim 2B) and identified 550 genes whose expression was regulated by nitrate, as a function of time. This analysis identified >200 genes (some induced as early as 3 min) not previously identified in steady state transcriptome data [Wang 2004]. We used this time-series data to drive a machine-learning, network inference approach called “State-space analysis”, with several adaptations. The State-Space model synthesizes Bayesian and Markovian approaches (in which each gene’s expression value at a time *t* is assumed to depend directly only on the state of potentially all the genes at the previous time point and indirectly on values from previous time points (Dennis- is this part in blue correct?) [Mirowski, P., et al. Clin Neurophysiol, 2009]. In a departure from previous state-space frameworks, we implemented a noise-mitigation approach that uses hidden variables to represent an idealized, “true” sequence of gene expressions **z**(*t*) that would be measured if there were no noise (e.g. in transcriptome data). The goal is to *learn* the function ***f***, that determines the change in expression of a target gene as a linear (or if needed non-linear, to account for TF interactions) combination of the expression of a relatively small number of TFs (typically up to three or four) (Fig. XA). To test the ability of “State Space” to generate accurate *predictive* regulatory networks, we used the 0, 3, 6, 9, 12, 15 min transcriptome data (as a training set), and then used the “learned” network to *predict* the direction of gene expression change from 15🡪20 min, and validated these predictions using the “left-out” 20 min transcriptome data. Surprisingly, based only on these relatively few time point measurments, the coherence of the regulatory network model generated is good enough that it is able to predict the direction of gene change (up or down regulation) on future data points. State Space predictions of gene regulation at the 20 min time-point, were correct for 74% of the genes in a sub-network of 76 genes associated with the N-assimilation pathway. As comparison, the *"naive trend forecast"* test was correct for only 52% of the genes, just slightly better than random, p-val<0.006. When compared with other network inference approaches [Bonneau, Genome Biol, 2006. **7**(5): p. R36] [Bonneau, Cell, 2007. **131**(7): p. 1354-65.] [Wang, Bioinformatics, 2006.] [Shimamura, BMC Syst Biol.], our State-Space method showed a slight improvement in accuracy, and had a better signal-to-noise ratio using the same data. Further, our adapted method reduces the importance of initial parameters by using random starting points and bootstrapping, thus offering a principled way to deal with uncertainty and avoid over-fitting in microarray measurements. We next used the model to predict the “most influential TFs” in the network (e.g. ones that are predicted to influence the most genes in the network), and prioritized them for validation testing (Fig. X). Our studies of T-DNA mutants for TFs were hampered by issues of functional redundancy, we thus turned to TF overexpression constructs. For the master TF gene, SPL6, we examined overexpression of mRNA using an spl9 mutant impaired in miRNA binding [Wang Cell 2009, 138:738-749]. The effect of SPL9 over-expression evaluated over time, validated the vast majority of the genes in the regulatory network that we tested [Krouk 2010]. To accelerate testing of other TFs hubs, we developed a transient expression approach described below.

**Prior Aim2A. Use of genome-wide approaches to validate causal TF->target relationships mediating N-regulation of N-assimilation networks. *Publication*: “***A transient transformation system for genome-wide transcription factor target assessment”.* Bargmann et al., 2012. (Submitted) **Summary**: This rapid transient expression system enables identification of direct TF targets in genome-wide within 2 weeks. This system was developed and validated using a well-studied TF (ABI3), for which the targets uncovered in our system could be validated by external data.

**Background**: A critical component of Gene Regulatory Network (GRN) inference is experimental validation of TF🡪target predictions. While TF🡪targets can be identified by Chromatin immunoprecipitation TF perturbation studies have advantages because: (i) while a Chromatin-IP can confirm protein:DNA binding, it does not guarantee functional activation/repression [(Zheng *et al*, 2009)], and (ii) TF perturbation studies can identify the effect of regulatory components that may not bind directly to DNA. TF perturbation in plants can be accomplished using T-DNA mutants or overexpressors, but both approaches are problematic. Functional redundancy of TFs is a built-in feature of GRNs that makes them robust, and as such molecular phenotypes in T-DNA mutants of TFs are rare [Cutler and McCourt 2005]. As an alternative, transcriptomic analysis of transgenic TF over-expressors (35S::TF) can suggest the TF🡪 targets [Suzuki et al, Reeves], but it cannot reveal whether changes in transcript levels are a *direct* consequence of TF manipulation, or caused by indirect or possibly ectopic effects. A better approach is to use an inducible system that relies either on conditional expression, or regulated nuclear entry of the TF [*e.g*. Hachez 2011, Bustos 2010]. Indeed, regulated nuclear translocation of a TF (using DEX), combined with the use of protein synthesis inhibitors (cycloheximide, CHX), has previously been used to eliminate the effects of secondary transcriptional regulators, and to identify direct from indirect TF targets, based on changes in target gene expression [Bustos].

As indirect TF1 targets (e.g. TF1🡪TF2🡪target) require protein translation (e.g. of TF2), their activation by DEX-induction of TF nuclear localization is blocked by CHX. Only direct TF1🡪target genes are activated in +DEX+CHX conditions (see Fig. X). We adapted this +DEX/+CHX approach to identify direct TF🡪targets in a transient system described in [Bargmann 2012] and below.

**The System: A DEX-inducible transient TF perturbation system**: To accelerate validation of TF🡪target relationships in our networks using TF perturbation, we adapted our transient expression system [Bargmann 2009], to overexpress any TF, selectively induce its translocation to the nucleus, and identify changes in the expression of target genes based on RNA analysis (Q-PCR or transcriptome analysis). Technically, protoplasts are transfected with a 35S-driven Gateway expression vector harboring a TF fused to the rat glucocorticoid receptor (GR). In this system, HSP90 binding to GR, restrains TF-GR location to the cytoplasm, until the addition of the GR ligand dexamethasone (GR), which displaces HSP90 and allows nuclear import of the TF (Fig. X). Following transfection and DEX treatment, a positive fluorescent selection marker (RFP) on the vector allows isolation of successfully transformed protoplasts using Fluorescence Activated Cell Sorting (FACS) [Bargmann et al 2009]. Subsequent RNA analysis (by Q-PCR or transcriptome) can identify genes whose expression is affected (induced or repressed) upon TF nuclear localization induced by DEX. If the effects of DEX are measured in the presence of the translational inhibitor cycloheximide (CHX), only direct targets of the TF of interest (for details of this concept see) [ Lloyd et al (1994) *Science*.] [Sablowski RW, Meyerowitz EM (1998). *Cell*].

**The Results:** As ***a proof-of-principle***, this transient TF perturbation system was used to investigate the targets of the well-studied TF, ABI3 (ABSCISIC ACID INSENSITIVE3), a master regulator of ABA signaling networks [Vernoux *et al*, 2011 Mol Syst Biol. 2011 Jul 5;7:5089) where known ABI3 direct targets can be used to validate our system. Protoplasts were transformed with a 35S::GR::ABI3 expression vector, and sequential treatments of +/-CHX, and +/- DEX (to induce nuclear localization of TF) were performed. FACS selection of positive transformants [Bargmann 2009] and subsequent RNA analysis enabled us to identified genes whose expression was altered by DEX treatment in the protoplasts expressing the GR::ABI3 constructs, compared to empty vector controls. This analysis showed that known direct ABI3 target genes (e.g. CRU1, PER1) are specifically induced by ABI3 nuclear localization (e.g. +DEX), and this DEX-induction was enhanced under +CHX conditions, possibly by eliminating repressors (Fig. X). Further confirmation that other genes induced under +DEX +CHX are direct ABI3 targets includes: (i) significant over-representation of GO-categories known to be controlled by ABI3 (e.g. “response to abscisic acid stimulus”, “seed development”, “dormancy process”) and (ii) significant overrepresentation of *cis*-elements known to directly bind to ABI3 (e.g. the RY-repeat motif (CATGCA)) identified using (CISPROM) or de novo using MIME [Bailey and Elkan, 1994)]. These findings validate that this transient expression system can be used to identify direct TF targets. This transient assay system is adapted to high through put testing of TFs in the N-assimilatory network (see Plan). **Prior Aim 3. Determine the role(s) of post-transcriptional mechanisms in mediating N-regulatory network. *Relevant publications*:** Vidal EA, Araus V, Lu C, Parry G, Green PJ, Coruzzi GM, Gutiérrez RA (2010). “[Nitrate-responsive miR393/AFB3 regulatory module controls root system architecture in Arabidopsis thaliana.](http://www.ncbi.nlm.nih.gov/pubmed/20142497?itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum&ordinalpos=1)” ***Proc. Natl. Acad. Sci.*** ***USA***, 107(9): 4477-82. ***Summary***: This aim explored the role of miRNAs in mediating nitrogen-metabolite signaling, and the use of 15N-tracers to monitor N-use efficiency. The miRNA studies were conducted in collaboration (R. Gutierrez, Chile), a Fogarty Award recipient connected to this NIH grant. That study identified how a miRNA-TF module was regulated in response to sensing inorganic nitrate (externally) or N-assimilated internally (Glu/Gln) [see Vidal 2010].

**RESEARCH STRATEGY:**  Our strategy builds on our prior success in inferring N-regulatory networks able to predict gene responses under untested conditions – the ultimate goal of Systems Biology [Krouk 2010]. We now aim to expand on and improve the predictive power of our learned networks using an approach called “*NetWalk”: an iterative high throughput experimental/machine learning approach to infer causal networks.* This approach is novel, in that it combines diverse types of genomic datasets (time series, steady state and TF perturbation data) generated in Aim1, into a pipelined network inference analysis in Aim 2, to improve the predictive power of the networks, and to infer the next TFs and TF pairs for perturbation in Aim 3, in an iterative Systems Biology cycle of high through put experimentation, analysis/modeling, and validation.

**Aim 1. NetWalk Targets: High-throughput validation of TF🡪network targets**. ***Rationale***: We will generate diverse genomic datasets in Aim1, that will drive a the new NetWalk network inference pipeline in Aim 2. Combining diverse datasets into one analysis pipeline, will enable us to identify “high confidence” edges to validate and refine our network inference pipeline and to increase its predictive power. The data we will generate consists of: i) new time-series transcriptome data (Aim 1A), ii) steady state transcriptome data, and iii) TF perturbation data in which TF->targets will be validated by transcriptomic (Aim 1B) and ChIP-seq (Aim 1C) analysis. In each subaim, we provide preliminary data for one TF, as proof-of-principle for the feasibility of the approach.

**Aim 1A. Generation of time-series transcriptome datasets for organic-N signaling.**  Time-series transcriptome data from nitrate-treated plants, was very valuable as it enabled us to (i) identify >200 new nitrate regulated genes (including TFs induced as early as 3 min) compared to steady state data [Wang 2004], and (ii) to derive dynamic, predictive network models based on State-space machine learning [Krouk 2010]. We will now use the same approach to generate time-series transcriptome data (0, 3, 6, 9, 12, 15, 20, 25, 30 min) under conditions shown to elicit an organic-N response (growth on 1mM nitrate, transient treatment with 40mM ammonium/nitrate vs. control KCl) [Gutierrez 2008]. The analysis of this new time-series data, for organic-N signaling, should enable us to uncover new genes including TF and generate network models (using machine learning approaches [Krouk 2010] and using the new pipeline in Aim 2, to prioritize TF hubs among the first TFs to be induced, for validation studies described below.

**Aim 1B. “NetWalk”: Generation of high throughput, high confidence TF🡪network target data.** *Prioritization of TF hubs for testing.* Our current network models have identified TF hubs predicted to regulate genes in the N-assimilatory pathway by nitrate [Krouk 2010] or organic-N [Gutierrez 2008] signals (Fig. X). TF hubs in the organic-N networks (CCA1, GLK1, WRKY1, bZip1), were identified from steady state data, and TF🡪targets were based on correlation (>0.8) and representation of *cis*-elements (Fig. X) [Gutierrez 2008]. In a machine learning approach, time-series data and state-space modeling, enabled us to generate dynamic, predictive networks for nitrate regulation of the N-assimilation pathway, and to identify TF hubs induced early (e.g. HRS1, HHO1, HHO2, HHO3) and at the top of the cascade (Fig. X) [Krouk 2010]. Unexpectedly, the TF hubs predicted to mediate either nitrate or organic-N regulation of N-assimilation - and identified in separate studies - comprise two subclades of a single myb gene family (see Fig. X), a member of which was previously associated with phosphate signaling [Liu 2009]. This finding underscores the biological relevance of these prioritized TFs in nutrient sensing, which is supported by preliminary studies below.

*“NetWalk”: Generation of TF🡪network target data using transient TF perturbation.*To identify network targets of the prioritized TFs genome-wide, we will adapt the transient system described in Progress (Prior Aim2A) [Bargmann 2012]. Briefly, this TF perturbation system currently comprises the following components; i) transient expression (using 35S CaMV promoter) of a GR-TF fusion in protoplasts (the vector expresses RFP as transfection control allowing FACS sorting of successfully transfected cells), ii) use of an inducible Dexamethasone (DEX) system to inducibly control translocation of the TF into the nucleus, iii) use of the translation inhibitor Cycloheximide (CHX), to identify primary targets of the TF. We will now add an additional layer to the DEX-concept, by iv) including N-treatments to precondition cells (prior to TF nuclear localization by DEX) to allow N-regulated post-translational modifications of TFs, and/or N-regulated transcriptional activation of potential TF cooperators. The ***prioritized TFs*** will be subjected to transient perturbation studies and Fig. X shows progress in Gateway cloning and transient expression. For each TF, the N-pretreatments will be tailored for either nitrate signaling (growth in ammonium succinate, treatment with nitrate) or organic-N signaling (growth in 1mM nitrate, and treatment with 40mM ammonium/nitrate). Indeed, we show using a ***proof-of-principle*** example TF (HRS1) that (i) nitrate-preconditioning affects DEX-induced target gene activation and (ii) that the TF🡪targets uncovered in this transient assay have relevance to whole plants.We initially hypothesized a role for the myb TF HRS1 in nitrate signaling, as its expression is induced within 9 min of NO3- treatment, it is predicted to be the top most controlling and top most controlled TF hub in the N-assimilation network [Krouk et al 2010]. Intriguingly, HRS1 was previously associated with phosphate signaling, based on phenotypes of transgenic 35S::HRS1 plants [Liu 2009]. Our preliminary analysis of transcriptome data from 35S::GR-HRS1 activation in the transient DEX system, revealed four distinct gene clusters whose expression is influenced by a combination of HRS1 nuclear import (by DEX) and nitrate-treatment (Fig. X). Genes in Cluster 4, defined as primary targets of HRS1 (e.g. activated by +DEX+CHX), are specifically induced in +DEX/+NO3 conditions. Further, GO-term analysis of these HRS1 nitrate-dependent HRS1 direct targets, reveals significant overrepresentation of genes involved in phosphate transport (p-val 8.14 E-6), reminiscent of the phosphate signaling phenotype observed in 35S::HRS1 plants [Liu 2009]. This result provides evidence that TF🡪targets identified in the transient DEX protoplast system have relevance to whole plants, and further suggest that some of the TFs in our nitrate-regulated networks may coordinate responses to diverse nutrients. Following this example, transcriptome analysis will identify specific genes, clusters and biological processes controlled by each prioritized TF. The TF🡪target data from transcriptome analysis will be complementary to the Chromatin-IP (ChIP) data (in Aim 1C) and has several advantages: (i) ChIP can confirm protein:DNA binding, but it does not indicate functional regulation [Eilers 2008], and (ii) transcriptome analysis from the DEX-inducible TF system allows one to identify the effect of regulatory components that may not bind directly to DNA [ Lee, J, 2007].

**Aim 1C. Genome-wide validation of TF🡪network targets using Chip-Seq.** Chromatin immunoprecipitation followed by sequencing analysis (ChIP-seq) can reveal the binding of a TF to the promoter of a target gene, but does not indicate if this results in actual gene activation/repression [(Zheng *et al*, 2009)]. Thus, we will perform ChIP analyses on protoplasts from TF-perturbation studies, and compare TF🡪targets identified by ChIP, with target genes identified by transcriptional analysis (Aim 1B) [Zhu 2012]. To test the feasibility of performing ChIP-Seq and transcriptome analysis on the same samples, we performed a ***proof-of-principle*** study that identified a genome-wide map of TF binding sites for the TF bZip1, using ChIP seq on samples from the transient protoplast expression system. We adapted the micro-ChIP protocol from [Dhal and Collas, 2008], which requires a relatively small number of cells (1,000 cells). After transformation of protoplasts with a 35S::GR::bZip1 construct, transformed protoplasts (~5-8,000 cells), were treated with 1% formaldehyde for 10 min, quenched with 100mM glycine for 5 min. Cells were washed in W5 buffer, pelleting by centrifugation (2,500 rpm for 2 min) and snap-frozen at -80C. Anti GR (sc-1002) antibodies (Santa Cruz Biotechnology Inc.) were used to capture the GR::bZip1 protein:DNA complexes, and ChIP DNA was purified after reverse cross-linking using the MiniElute kit (QIAGEN). For ***Illumina sequencing*** **of the ChIP-DNA**, paired-end libraries were constructed using the sample prep guide (Illumina, San Diego, CA), with the following modifications for low amounts of starting DNA (~1ng): (i) adaptor oligo mix was further diluted 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; (iii) SYBR gold agarose gel size selection was performed after PCR enrichment, to remove adaptor self-ligation product from the library. Libraries were separately constructed from the immunoprecipitated DNA and the input DNA as control. The libraries were sequenced on the Illumina GAIIx platform. ***Bioinformatic analysis* of ChIP Seq reads** was used to identify network targets of bZIP1 binding genome-wide. The sequence reads were filtered for quality, trimmed to remove adapter sequences, and aligned to the TAIR10 assembly of the *A. thaliana* genome. Genomic regions significantly enriched in the immunoprecipitated sample relative to the input DNA, were identified using the QuEST peak-calling algorithm [Valouev et al., Nature methods 5, 829-834 (2008)]. This analysis identified approximately 300 genic regions that show increased peak heights (individual peak heights **>4** fold, overall genic region **>2** fold) compared to the background (input DNA), indicating significant binding (p<1E-8) of bZIP1 binding sites in these 300 genes. **To validate that the ChIP samples were *direct* bZip1 targets**, we performed an analysis of cis-regulatory motifs. 500bp immediately upstream of the transcription start site (TSS) of genes with the highest normalized read count for bZIP1 binding, were analyzed with MEME [Bailey and Elkan, Proc. of ISMB,1994 pp28-36]. The most significant cis-motif found (p<1.9e-6) in this set was G[C/A]CACGT[G/C] which includes the G-box motif (CACGTG), a known bZIP1 binding site [Kang et.al. *Mol. Plant (2010) 3 (2): 361-373*]. As further validation, we intersected of bZip1🡪target data from ChIP studies with the bZip1 targets predicted from our multinetwork analysis (e.g. based on TF🡪target correlation > o.8 and over-representation of bZIP1 cis-binding elements) [Gutierrez 2008]. Of the 30 predicted bZip1 targets in this organic N-regulatory network, 16 show significant enrichment in the bZip1-ChIP studies, and the significance of this overlap is p<0.001. These bZIP1 targets include the prioritized TFs in our networks: CCA1, GLK1, WRKY1 whose regulation by bZip1 is depicted in Fig. X., as well as the N-assimilation target gene ASN1.

**Outcome of Chip-Seq**: **Identifying gold-standard TF🡪target paire and predicting TF interactions:** The TF🡪targets identified by ChIP seq data will be intersected with targets identified by transcriptome analysis (Aim 1B). This will identify “gold standard” TF🡪target examples where direct TF binding results in gene activation (or repression). This high-quality information will be used to train and validate our inference pipeline in Aim 2. In addition, as we perform ChIP studies across the TFs in our network, the collective ChIP studies will provide information about TF1 and TF2 binding (e.g. promoter location, target genes etc), that will help to direct our studies on TF interactions (in Aim 3B). Once we have Chip-Seq data from several TFs in the network, we can start looking at combinatorial regulation. This approach is fully described in Aim 3.

**Data integration and analysis**: The distinct sets of data generated in Aim 1 (i) time-series transcriptome, (i) steady state transcriptome, (iii) data from TF perturbations, (a) transcriptome and (b) ChIP-Seq, will be combined into a single pipeline for network inference in Aim 2.

**Expected results, limitations and alternate approaches (Aim 1)**: Aim 1 will produce distinct datasets to “feed” and validate the Pipelined Network Inference approach in Aim 2. Some new outcomes will be, for example, the identification of new TFs invoked to operate early in the network based on new time-series data for organic-N signaling (Aim 1A), and validations of direct TF1🡪TF2 relationships the effects of which can be further explored in Aim 3. TF interactions can also be predicted from ChIP seq experiments performed across several TFs. For each subaim (Aim 1B, C, and D), we have proof-of-principle examples to demonstrate the feasibility of the approach, so we do not anticipate technical problems in data generation or computational interpretation. In addition, the coordinated analysis of the distinct data sets (e.g. based on transcriptome (Aim 1B) vs ChIP seq (Aim 1C) will cross-validate our TF🡪targets identified.

**Aim 2: NetWalk Inference: A computational pipeline for network inference combining multiple data-types. *Rationale***: Our ultimate goal is to build 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 quality and predictive power of such networks, by feeding our network learning algorithms both more time series data as well as TF perturbation data. The transcriptome data generated from these TF perturbation experiments will uncover both direct TF targets as well as indirect ones. Direct relationships will also be validated by ChIP-seq. The refined network models derived from this pipeline in Aim 2, will in turn suggest new TFs on which to experiment (in Aim 3), refueling the iterative systems biology cycle of model building, experimentation and model refinement.

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

**The Network Model**: The nodes in the causal network will be genes and the edges between genes represent positive and negative influences. A positive coefficient associated with an edge implies that the edge is inductive. 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 gene 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 roughly 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, our initial model will be “linear” as opposed to quadrtic. (A quadratic model would include terms of the form d\*B\*C, where B and C would be gene expressions. We consider a quadratic model in the next aim.). Surprisingly, linear models explain behavior well. A model explains behavior if it can predict the state of one gene given the state of other genes at 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].

**Building a Pipelined Network Inference Pipeline of Machine Learning Algorithms to integrate learning from distinct data-types.** One reasonable approach to combining multiple forms of evidence to derive network edges is to establish a machine learning pipeline (Fig. X, figure dfg.eps) that analyzes different kinds of data using different algorithms. In one such pipeline, the GENIE3 algorithm is used on steady state data (resulting from some intervention such as the addition of a nutrient), the MCZ algorithm (short for the Median Corrected Z-score method [Greenfield, (2010]) is used for mutation data, followed by the DFG algorithm (short for Dynamic Factor Graph) for time-series data [Krouk (2010]. In this pipeline, the GENIE3 and MCZ algorithms assign initial weights to certain edges that are then refined by the DFG algorithm.

GENIE3 (Gene Network Inference with Ensemble of Trees) [**[Inferring Regulatory Networks from Expression Data Using Tree-Based Methods](http://www.plosone.org/article/info%3Adoi/10.1371/journal.pone.0012776)**

Huynh-Thu VA, Irrthum A, Wehenkel L, Geurts P (2010) Inferring Regulatory Networks from Expression Data Using Tree-Based Methods. PLoS ONE 5(9):e12776. doi:10.1371/journal.pone.0012776] uses a collection of regression trees (which are generalized decision trees) to perform inference. The algorithm creates a collection of regression trees, ranks the edges from each ones, and then creates a global ranking.

In the MCZ algorithm, if gene \[x\_j] influences \[x\_i], then perturbing \[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-perturbation experiments.

 In preliminary work, done for a book we have written called *Network Inference in Molecular Biology*, [Lingeman/Shasha 2012], we show using data from the DREAM benchmark (Dialogue for Reverse Engineering Assessments and Methods) [Schaffter, (2011)] to show that the GENIE3🡪MCZ🡪DFG pipeline is better than using any single machine learning algorithm by itself.

 An crowdsourcing approach we will try is to apply several algorithms that each predicts edges and then use a “voting” approach to determine the best one. In that scheme, illustrated in the figure Pipelined.eps, in addition to the Median-Corrected Z-score and Dynamic Factor Graph algorithms, we use the NIR (Network Identification by Multiple Regression [Gardner, (2003)]) and CLR (Context Likelihood of Relatedness [Faith, (2007)]) on steady state data. We also use BANJO (Bayesian Inference with Java Objects [Yu,J (2004)]), Time-Delay ARACNE [Zoppoli, (2010)], and Inferelator [Greenfield, (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 of the Pipelined Network Inference Approach:** 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 2010] built on time-series data with the GENIE3🡪MCZ🡪DFG pipeline . 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. On the other hand, using just the data up to 12 minutes, we were able to predict the values at 15 minutes correctly only 57% of the time. As the following table shows, new data and the computational pipeline improves these results at least for this small sample of genes.

|  |
| --- |
| **TABLE X: NETWALK MACHINE LEARNING PIPELINE (Preliminary Results)** |
| **STEPS** | **1. GENIE** | **2. MCZ** |  **3. DFG** | Prediction Accuracy | Prediction Accuracy |
| **DATA** | **Steady State** | **TF perturbation** (35S::HRS1 +DEX) | **Time Series** | **12-15 min** | **15-20 min** |
| **Test 1** | No | No | **Yes** | 57% | 74% |
| **Test 2** | **Yes** | No | **Yes** | 66% | 74% |
| **Test 3** | **Yes** | **Yes** | **Yes** | 68% | 76% |

Caption: The 12-15 min column shows the correctness of predicting whether expression will increase or decrease between 12 minutes and 15 minutes depending on the data up to 12 minutes. Using time series data alone gives just 57% accuracy, this increases substantially with steady state data and still further given just one DEX gene target. The improvements from 15 to 20 minutes are less impressive, partly because the model is already so good (for these 76 genes) with the time series data alone. The single DEX gene helped, even though that experiment gave only the direct targets of that gene.

**Intended Approach**: Based on our preliminary results, we will iterate the following steps: (i) identify the genes relevant to nitrogen treatments based on steady state and time-series transcriptome data resulting in just over 200 genes (including regulated members of the 66 genes in the N-assimilatory pathway as well as TFs), (ii) develop a Pipelined Network 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 predicted regulatory network of the genes controlling the N-assimilation pathway. That regulatory network will suggest new genes on which to try DEX experiments (e.g. putative TF hubs) (in Aim 3). Those validations will in turn 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 TF perturbations. 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 pertain to the most important genes or which will clarify inaccuracies in our model.

**Data integration and analysis**: Aim 2 integrates the four sets of data generated in Aim 1- (i) time-series transcriptome, (2) steady state transcriptome, and data from TF perturbations (iii) transcriptome, and (iv) ChIP-Seq into a single pipeline for machine learning (Aim 2). We will test the various approaches for their ability to correctly predict network states using out-of-sample data, as exemplified for the preliminary study shown in Table X.

**Expected results, limitations and alternate approaches (Aim 2)**: Aim 2 will yield “learned regulatory network” for TFs controlling the N-assimilatory pathway, built/trained on a pipeline analysis of data sets (from Aims 1 and 3). Predictions will be performed using out-of-sample data, and we will test a number of machine learning methods to identify which combination is best. The main possible problems we may encounter, have to do with the fact that the number of possible edges is much larger than the number of experimental data points we will have. We nevertheless believe that the approach will work, because we have already seen successful results in predictive modeling using much less data, and a single machine algorithm [Krouk et al 2010]. Moreover, our preliminary results for combining machine learning approaches in a pipeline shown in Aim 2 suggest we can improve on this already successful approach to network inference.

An additional benefit of our approach is that we will discover which data type (time-series, steady state, TF perturbation) is most useful for network inference. For example, we may learn that the TF perturbation data is more informative than the steady state data and Chip-seq data combined, or that time-series is just as informative (and less expensive) compared to Chip-seq for regulatory network modeling. These findings will allow our lab and possibly others to save money and time, by focusing on generating the most informative data type(s) for network inference.

**Aim 3: NetWalk Coordination: Experimental testing of transcription factor interactions.**

***Rationale***: The refined network models in Aim 2 will suggest additional TF perturbation experiments to perform. This includes: i) new TF hubs and ii) TF interactions. These studies have the potential to identify TFs and network motifs (e.g. TF pairs) that globally regulate the N-assimilatory pathway in response to nitrate and organic-N signals, for testing in transgenic plants.

**Aim 3A. Perturbation studies for new candidate TFs**: We will functionally validate TF🡪targets for new TFs identified in Aim 2 using the transient DEX-system, as described in Aim 1B-C. These may include new TFs hubs acting early and at the top of the cascade discovered in new time series experiments (Aim1A) not previously uncovered in steady state models [Gutierrez 2008], and/or TFs that affect key target genes in the N-assimilatory pathway (e.g. GLN1 and ASN1) whose expression is known to affect N-use efficiency [Lam 2004][Oliveira].

**Aim 3B. Testing TF interactions in the control of the N-assimilatory network**

**Identification and Prioritization of TF pairs for testing**: (Dennis is this a correct explanation? I tried to clarify.). The linear models used in the NetWalk pipeline to “learn” and infer regulatory networks in Aim 2, will identify all TFs predicted to individually and additively control expression of a target gene. However, to avoid overfitting on the relatively small amount of data, they do not explicitly model TF interactions (see explanation Aim 2). However, we can use our linear network model to predict interactions among these TFs to create a reasonably parsimonious quadratic model as follows: for each target gene X in the linear model, try to fit a model for X including pairwise combinations of X’s inputs. Thus the linear model suggests which genes are relevant to X. The linear model is then refined into a quadratic model on those relevant genes.

Our current networks uncovered evidence for the TF interaction WRKY1|--|bZIP1🡪ASN1. That is supported by regression modeling and also by ChIP-seq studies on bZIP1 (see Aim 1C). As binding site information for the prioritized TF list (Fig. X) becomes available from our ChIP-Seq studies in Aim 1C, we will use algorithms, such as SpaMo [Whitington et. al., NAR 2011], that are designed to identify tightly linked motifs, whose spatial distribution could also be used to support a mechanistic basis for TF1:TF2 protein interactions.

Based on the networks generated in Aim 2 and our quadratic model, we will prioritize testing of TF pairs that: i) are predicted to interact, ii) whose binding sites co-occur in Chip-Seq studies, and/or iii) that share the most targets in the network and/or target key N-assimilatory genes shown to control N-use (e.g. GLN1 and ASN1) [Lam 2004][Oliviera].

**Approach**: We will test potential TF interactions using the transient DEX-inducible system described in Aim 1B using two complementary approaches: (i) Co-expression of TF1 and TF2 in a single vector, (ii) expression of TF1 in protoplasts from tf2 mutant background. [Given Amy’s recent problems with dex without chx, Dennis prefers (i) to (ii)] In each case, synergistic activation (or repression) of target genes when TF1 and TF2 are both present, compared to either one alone, will be evidence for interaction.

*Co-expression of TF1 & TF2***:** To test how TF interactions affect target gene expression *in vivo*, we will co-activate the TFs in the DEX transient assay system using a TF1/TF2 double expression vector, and identify genes whose expression differs synergistically compared to either TF expressed alone in a single vector. The control is the empty vector. TF target genes whose expression is synergistic in the TF1/TF2 double vector experiments (compared to single TF), would be evidence for TF interaction, while an additive effect would imply cooperation. In order to co-express two TFs (TF1 and TF2) in the same cell, we will use the MultiSite Gateway**®** Technology that allows for simultaneous cloning of multiple DNA fragments in a defined order and orientation. To generate the co-expression construct, TF1 cDNA, TF2 cDNA and the “GR cassette” (35S promoter - GR sequence- 3’ terminator) will be cloned in appropriate Gateway donor vectors, and allowed to recombine with each other and with the destination vector pBob11 [Bargmann 2009]. This results in the assembly of two consecutive expression cassettes 35S::GR:TF1 and 35S::GR:TF2 (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.

*Perturbation of TF1 activity in a tf2 mutant background*: [Dennis thinks this is less good. Less symmetric, more difficult.] As a complement to the multisite vector approach, we will use a genetic approach to examine effects of TF1/TF2 interactions on target gene expression *in vivo*. Here we will transiently express the single 35S::GR:TF1 in protoplasts made from T-DNA mutant in TF2. This will enable us to assess TF1 function in the absence of TF2. We will compare the effect on target gene expression to that observed when TF1 is expressed in protoplasts from wild-type plants (where native TF2 is present). Loss or change in target gene regulation by TF1 in a tf2 mutant protoplast (compared to wild type), will be interpreted as evidence for TF1 and TF2 cooperation and/or interaction. We will use this genetic approach for TFs that show a molecular phenotype (e.g. alteration in target gene expression) in the T-DNA mutant to explore possible TF cooperation. It is also possible that a T-DNA mutant with no alteration in a target gene (e.g. due to redundancy), could provide a sensitized background to uncover evidence (in the DEX transient system) for their interaction.

**Data integration and analysis**: The transcriptome data from TF perturbation experiments in Aim 3A will feed back into the machine learning pipeline in Aim 2, to refine network predictions. In order to analyze/integrate the transcriptome data supporting TF1/TF2 interactions (Aim 3B) into our machine learning pipeline in Aim 2, we will modify the model for TF regulation of target gene expression to include TF interactions as quadratic terms in cases where the TF1/TF2 interaction data shows synergistic effects for a target gene expression, compared to the single TF data.

**Expected results, limitations and alternate approaches (Aim 3)**: The results of Aim 3 will enable us to test predictions for new TFs identified in Aim 2, and to improve the quality of edges in the inferred network of Aim 2. It will also enable us to test how TF interaction affects target genes in the network, by studying whether two TFs affect target gene expression in a synergistic fashion *in vivo*, using the transient assay system.

**Aim 3 Postscript**: **Effects of TF perturbations on N-assimilation in whole plants**: For TFs validated to affect the N-assimilatory pathway based on our transient assays, we will collaborate with Dr. Gabriel Krouk & Sandrine Ruffel at the INRA Biology & Biochemistry of Plants on studies related to optimizing N-use efficiency. Using N15 as a tracer, the rate and amount of NO3 incorporation into total N can be measured/dry weight. We are currently testing plants for several TFs for which changes in N-assimilation target genes are observed in the corresponding T-DNA mutants or transgenic overexpressors (e.g. WRKY1 T-DNAs, 35S::CCA1). This collaboration between our genomics lab and a world-renowned plant physiology lab, will be the ultimate test of our ability to use system biology approaches to predict targeted interventions in the N-assimilatory pathway, with significance to plant biomass and N-use efficiency.

**TIMELINE**

**Year 1-2**

**Year 3-4**

**CUT SECTION:**

Our aims are:

Aim 1: NetWalk Targets: High throughput experimental validation of TF🡪target genome-wide.

Aim 2: NetWalk Inference: A computational pipeline for network inference combining multiple data-types.

Aim 3: NetWalk Coordination: Experimental testing of transcription factor coordination.

**Aim 3C. Testing effects on N-assimilation of TF perturbations in whole plants.** For selected TFs, we will validate TF🡪targets using TF perturbations *in planta* using mutants or transgenics, as follows. **T-DNA mutants**: As mentioned above, TF redundancies underlie the robustness of the N-assimilation network, soT-DNA mutants in TFs could not be used to validate TF🡪target relationships for bzip1 [Obertello 2010], glk1 [Gutierrez 2008], hrs1, hho1, 2 and 3 [Medici and Krouk, unpublished] (Table X). In the case of WRKY1, a TF predicted to act as a toggle switch between activating genes involved in nitrate assimilation vs. its conversion into Asn (see Fig. X), three independent T-DNA wrky1 mutant lines (SALK\_016954; SALK\_136009; SALK\_070989) showed decreased expression genes predicted to be targets of WRKY1 activation (NIA2 & NRT2.1), and increased expression of genes predicted to be targets of WRKY1 repression (bZIP1 & ASN1) (Fig. X). Interestingly, the opposite expression patterns are observed when WRKY1 is transiently over-expressed in protoplasts, see Aim 1B. Single and double mutants (e.g. glk1/2, hho1/2, cca1/lhy1) that show alteration regulation of targets in the N-assimilation pathway, will be used in TF interaction studies described in Aim 3. **Transgenic lines**: For some TFs, we already have data from stable 35S::TF transgenic lines which support TF🡪target relationships predicted in our network models, e.g. 35S::CCA1 [Gutierrez et al 2008], 35::GLK1 [XXXX], 35S::HRS1 [Liu 2009]. For selected TFs, we will produce transgenic Arabidopsis plants that express a TF::GR fusion under the control of the TF native promoter, to enable inducible expression and identification of target genes. To do this, the genomic region encompassing the coding sequence and the promoter (1Kb upstream the ATG or up to the upstream gene) of the TF will be cloned into a pENTR vector and then transferred into a plasmid called “pDEX”, a Gateway destination vector we created (to enable rapid TF-GR cloning) by inserting the GR sequence downstream the Gatewaycassette of pMDC 99 [Brand et al.,2006. [Plant Physiol. 141:1194-1204](http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=pubmed&cmd=Retrieve&dopt=AbstractPlus&list_uids=16896232)].

**Aim 1D. Effects of TF perturbations in whole plants.**  ***Transgenic Studies***: Based on results in our transient assays (Aims 1B and C), we will select TFs for perturbations *in planta*. For some TFs, we already have data from stable 35S::TF transgenic lines which support TF🡪target relationships predicted in our network models, e.g. 35S::CCA1 [Gutierrez et al 2008], 35S::GLK1 [XXXX], 35S::HRS1 [Liu 2009], so transcriptomic analysis to identify genes whose expression is activated (or repressed) compared to wild-type controls, will be straight forward. However, such lines will not allow us to identify direct targets. Therefore, in selected examples prioritized by our transient studies, we will produce transgenic Arabidopsis plants that express a TF::GR fusion under the control of the TF native promoter?, to enable inducible expression and identification of target genes. To do this, the genomic region encompassing the coding sequence and the promoter (1Kb upstream the ATG or up to the upstream gene) of the TF will be cloned into a pENTR vector and then transferred into a plasmid called “pDEX”, a Gateway destination vector we created (to enable rapid TF-GR cloning) by inserting the GR sequence downstream the Gatewaycassette of pMDC 99 [Brand et al.,2006. [Plant Physiol. 141:1194-1204](http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=pubmed&cmd=Retrieve&dopt=AbstractPlus&list_uids=16896232)].

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