

Supplementary Results

Cis-element analysis of bZIP1 targets and relevance to N-signaling

Cis-analysis of bZIP1 regulated vs bound genes. Genes induced (470 genes) (Fig. S4B) or bound (850 genes) (Fig. S4D) in response to DEX-induced nuclear import of bZIP1, showed a highly significant overrepresentation of *cis*-element shown to bind bZIP1 *in vitro* including a hybrid ACGT box ($E=2.4E-39$) and a G-box ($E=1.6E-25$), respectively (Fig. S4B&D) (1). Genes repressed in response to bZIP1 perturbation are enriched with a distinct bZIP-binding motif (“GCN4 binding”) (2), as well as a W-box (Fig. S4B). Interestingly, the GCN4 motif was reported to mediate nitrogen and amino acid starvation sensing in both yeast and plants (2-4), suggesting a conserved link between bZIPs and nutrient sensing. The significant representation of a W-box ($E=7.3E-70$) suggests, a non-exclusive alternative interpretation, which is that bZIP1 may work in conjunction with a WRKY family partner to repress primary target genes. Lastly, a *cis*-motif implicated in integrating light and defense signaling (FORC^A motif) (5), was over-represented in both the 850 bZIP1 bound genes (Fig. S4D, $E=2.1E-24$), and the 470 bZIP1 induced genes (Fig. S4B, $E=6.7E-7$), consistent with known roles of bZIP1 *in planta* (1, 6, 7).

Cis-analysis of the three classes of bZIP1 targets: Poised, Stable, and Transient. *Cis*-element analysis of each of the three subclasses of bZIP1 primary gene targets - Class I “Poised”, Class II “Stable”, and Class III “Transient” – each show enrichment of known bZIP binding sites (Fig. 2). Genes that either bind to bZIP1 or are activated by bZIP1 (Class I, IIA and IIIA), have significant over-representation of the known bZIP1 binding site hybrid “ACGT” box (1) (Fig. 2). By contrast, genes that are repressed by bZIP1 do not display the canonical “ACGT” core, and instead possess the GCN4 binding motif for the bZIP family (2). Promoters of Class IIIA genes are significantly enriched with bZIP family TF binding sites [e.g. the TGA1 binding site (8), ABRE binding site (8), and GBF1/2/3 binding site (9)], and also with MYB family TF binding sites [I-box (8) and CCA1 motif (8)], GATA promoter motif (8), and the light-responsive motif SORLIP1 (8) (Fig. 2). For the Class IIIB bZIP1 target genes (primary target genes repressed by bZIP1, but no detectable bZIP1 binding), a number of *cis*-elements implicated in light and temperature signaling were significantly over-represented in their promoters, including T-box, W-box, and HSE binding site (8) (Fig. 2). A *de novo* motif identification analysis using MEME was performed on the 3' UTR of targets from Class III to identify potential RNA stability motifs. This search did not find any over-represented motifs in the 3' UTR of the Class III transient genes.

Supplementary Methods

Plant Materials and DNA Constructs.

Wild-type *Arabidopsis thaliana* seeds [Columbia ecotype (Col-0)] were vapor-phase sterilized, vernalized for 3 days, then 1 ml of seed were sown on agar plates containing 2.2 g/l custom made Murashige and Skoog salts without N or sucrose (Sigma-Aldrich), 1% [w/v] sucrose, 0.5 g/l MES hydrate (Sigma-Aldrich), 1 mM KNO₃ and 2% [w/v] agar. Plants were grown vertically on plates in an Intellus environment controller (Percival Scientific, Perry, IA), whose light regime was set to 50 $\mu\text{mol m}^{-2}\text{s}^{-1}$ and 16h-light/8h-dark at constant temp of 22°C. The bZIP1 (At5g49450) cDNA in pENTR was obtained from the REGIA collection (10) and

was then cloned into the destination vector pBeaconRFP_GR used in the protoplast expression system (11) by LR recombination (Life Technologies). The pBeaconRFP_GR vector is available through the VIB website (<http://gateway.psb.ugent.be/>).

Protoplast Preparation, Transfection, Treatments and Cell Sorting.

Root protoplasts were prepared, transfected and sorted as previously described (11-13). Briefly, roots of 10-day-old seedlings were harvested and treated with cell wall digesting enzymes (Cellulase and Macerozyme; Yakult, Japan) for 4 h. Cells were filtered and washed then transfected with 40 μ g of pBeaconRFP_GR::bZIP1 plasmid DNA per 1×10^6 cells facilitated by polyethylene glycol treatment (PEG; Fluka 81242) for 25 minutes (11). Cells were washed drop-wise, concentrated by centrifugation, then resuspended in wash solution W5 (154 mM NaCl, 125mM CaCl₂, 5mM KCl, 5mM MES, 1mM Glucose) for overnight incubation at room temperature. Protoplast suspensions were treated sequentially with: 1) a N-signal treatment of either a 20 mM KNO₃ and 20 mM NH₄NO₃ solution (N) or 20 mM KCl (control) for 2 h, 2) either CHX (35 μ M in DMSO, Sigma-Aldrich) or solvent alone as mock for 20 min, and then 3) with either DEX (10 μ M in EtOH, Sigma-Aldrich) or solvent alone as mock for 5h at room temperature. Treated protoplast suspensions were FACS sorted as in (13): approximately 10,000 RFP-positive cells were FACS sorted directly into RLT buffer (QIAGEN) for RNA extraction.

RNA Extraction and Microarray.

RNA from 6 replicates (3 treatment replicates and 2 biological replicates) was extracted from protoplasts using an RNeasy Micro Kit with RNase-free DNaseI Set (QIAGEN and quantified on a Bioanalyzer RNA Pico Chip (Agilent Technologies). RNA was then converted into cDNA, amplified and labeled with Ovation Pico WTA System V2 (NuGEN) and Encore Biotin Module (NuGEN), respectively. The labeled cDNA was hybridized, washed and stained on an ATH1-121501 Arabidopsis Genome Array (Affymetrix) using a Hybridization Control Kit (Affymetrix), a GeneChip Hybridization, Wash, and Stain Kit (Affymetrix), a GeneChip Fluidics Station 450 and a GeneChip Scanner (Affymetrix).

Analysis and filtering of microarray data

Analysis of microarray data with CHX treatment:

Microarray intensities were normalized using the GCRMA (<http://www.bioconductor.org/packages/2.11/bioc/html/gcrma.html>) package. Differentially expressed genes were then determined by a 3-way ANOVA with N, DEX and biological replicates as factors. The raw p-value from ANOVA was adjusted by False Discovery Rate (FDR) to control for multiple testing (14). Genes significantly regulated by the N-signal and/or DEX-induced bZIP1 nuclear localization were then selected with a FDR cutoff of 5%. Genes significantly regulated by the interaction of the N-signal and bZIP1 (N-signal x bZIP1) were selected with a p-val (ANOVA) cutoff of 0.01. Only unambiguous probes were included. Heat maps were created using Multiple Experiment Viewer software (TIGR; <http://www.tm4.org/mev/>). The significance of overlaps of gene sets were calculated using the GeneSect (R)script (15) using the microarray as background. Hypergeometric distribution was used in one case (specified in the manuscript) to evaluate the enrichment of gene sets, when a specific background - N-responsive genes identified in different root cell types (16) – was needed.

Filtering bZIP1 targets for the effects of protoplasting, and response to CHX or DEX:

In this step, we filtered out genes whose expression states respond to protoplasting, or to treatments of DEX or CHX that are not related to the bZIP1 mediated regulation in our analysis, in the following three steps: **Filter 1: DEX-response filter: Genes responding to DEX independent of TF.** Genes significantly induced/repressed by DEX-treatment in protoplasts transfected with the empty pBeanconRFP_GR plasmid (ANOVA analysis; $FDR < 0.05$), were excluded from analysis (1.6% genes filtered). **Filter 2: Protoplast-response filter: Genes induced by protoplasting.** Genes that are induced by root protoplasting (17) were removed from the list of bZIP1 targets (12.3% genes filtered). **Filter 3: DEX x CHX interaction filter. Genes whose DEX-regulation is modified by CHX.** This filter removes genes from our analysis in cases where the effects of DEX-induced TF nuclear import on gene regulation are affected by CHX treatment. To do this, we performed a 3-way ANOVA (Factors Nitrogen, DEX, and CHX) and identified bZIP1 primary targets whose gene expression regulation by the DEX-induced nuclear import of bZIP1 is different between +CHX and -CHX conditions (FDR cutoff of interaction term $CHX * DEX < 0.05$). This will eliminate genes that are regulated by bZIP1 in the presence of CHX, but not in the absence of CHX. This gene set may contain bZIP1 targets under a self-control negative feedback loop, and bZIP1 targets for which the half-lives of the transcripts affected by CHX. While the first case is potentially interesting, the second case represents the CHX artifact to be removed. Since it is difficult to differentiate between the two outcomes, these CHX-sensitive DEX-responsive genes dependent on bZIP1 were eliminated from the list of bZIP1 target genes (17.4% genes filtered), thus increasing precision over recall.

Micro-Chromatin Immunoprecipitation.

For each combination of protoplast treatments (see above), an unsorted suspension of protoplasts containing approximately 5,000-10,000 GR::bZIP1 transfected cells was fixed for ChIP analysis, using an adapted version of the micro-ChIP protocol by Dahl et al (18). The advantage in a ChIP analysis from protoplasts is that short-lived interactions would likely be missed in *in planta* assays, as effective protein-DNA cross-linking in intact plant tissues requires prolonged (for a minimum of 15 minutes) infiltration under vacuum (19). Cells were incubated with gentle rotation in 1% formaldehyde in W5 buffer for 7 minutes, then washed with W5 buffer and frozen in liquid N₂. μ ChIP was performed according to Dahl et. al. (18), with a few modifications below. The GR::bZIP1-DNA complexes were captured using anti-GR antibody [GR (P-20) (Santa Cruz biotech) bound to Protein-A beads (Life Biotechnologies)]. A washing step with LiCl buffer [0.25M LiCl, 1% Na deoxycholate, 10mM Tris-HCl (pH8), 1% NP-40] was added in between the wash with RIPA buffer and TE (18). After elution from the beads, the ChIP material and the Input DNA were cleaned and concentrated using QIAGEN MiniElute Kit (QIAGEN). The protoplast suspension used for micro-ChIP was not FACS sorted in order to maintain a comparable incubation time between the samples that were used for microarray analyses and for micro ChIP. Importantly, while FACS sorting of transformed cells is required for microarray studies, it was not required to identify DNA targets using ChIP-seq.

ChIP-Seq library prep.

The ChIP DNA and Input DNA were prepared for Illumina HiSeq sequencing platform following the Illumina ChIP-Seq protocol (Illumina, San Diego, CA) with modifications. Barcoded adaptors and enrichment primers (BiOO Scientific, TX, USA) were used according to the manufacturer's protocol. The concentration and the quality of the libraries was determined by

the Qubit Fluorometric DNA Assay (Invitrogen, NY, USA), DNA 12000 Bioanalyzer chip (Agilent, CA, USA) and KAPA Quant Library Kit for Illumina (KAPA Biosystems, MA, USA). A total of 8 libraries were then pooled in equimolar amounts and sequenced on two lanes of an Illumina HiSeq platform for 100 cycles in paired-end configuration (Cold Spring Harbor Lab, NY).

ChIP-Seq Analysis.

Reads obtained from the four treatments (with DEX and N in the presence of CHX) were filtered and aligned to the *Arabidopsis thaliana* genome (TAIR10) and clonal reads were removed. The ChIP alignment data was compared to its partner Input DNA and peaks were called using the QuEST package (20) with a ChIP seeding enrichment ≥ 3 , and extension and background enrichments ≥ 2 . These regions were overlapped with the genome annotation to identify genes within 500bp downstream of the peak. The gene lists from multiple treatments were largely overlapping sets, and hence were pooled to generate a single list of genes that show significant binding of bZIP1. Due to technical issues, the experimental design used for ChIP-Seq precludes the observation of significant differences between the genes bound by bZIP1 under the different treatment conditions. This is because the samples fixed for ChIP included a variable number of transfected cells that were not sorted by FACS.

Our ChIP-seq studies were performed using a micro-ChIP protocol on $\sim 10,000$ cells, which result in a low DNA input, compared to standard ChIP studies. It has been shown that peak discovery from ChIP data becomes more challenging as the number of cells goes down [Fig. 3 in Gilfillan et al., 2012 (21)]. Therefore, ChIP libraries made from these very low input-DNA samples have a higher level of background noise, necessitating lower peak calling thresholds. However, even with this caveat for micro-ChIP studies, we were able to recover 850 targets including several previously validated bZIP1 targets (ASN1 and ProDH) (22).

Time-series ChIP-seq: The ChIP time-series samples were pre-treated with a N-signal treatment of 20 mM KNO₃ and 20 mM NH₄NO₃ solution (N) for 2 h, followed by CHX (35 μ M in DMSO, Sigma-Aldrich) for 20 min. Protoplasts were then treated with DEX (10 μ M in Ethanol, Sigma-Aldrich) and samples were harvested at 1, 5, 30 and 60 min after the start of the DEX-induced bZIP1 nuclear localization.

Cis-element Motif Analysis.

1 Kb regions upstream of the TSS (Transcription Start Site) for target genes were extracted based on TAIR10 annotation and submitted to the *Elefinder* program (all promoters from the genome as background) (23) or MEME (against a randomized dinucleotide background) (24) to determine over-representation of known cis-element binding sites (different parameters used in specific cases were notified in the paper if applicable). The E-value of significance for each motif was used to cluster the occurrence of motifs in the various subsets using the HCL algorithm in MeV (25). Motifs that show a higher specificity to a particular category or a sub-group were identified with the PTM algorithm in MeV. *De novo* motif identification was performed on 1Kb upstream sequence of the genes regulated by bZIP1 from microarray and ChIP-Seq data separately using the MEME suite (24).

Supporting References

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Supplementary Figures

Supplementary Figure S1. Experimental scheme for TF (A) and N-signal perturbation (B), and parallel RNA-Seq and ChIP-Seq analysis (C & D) of bZIP1 primary targets. (A) A GR::TF fusion protein is overexpressed in protoplasts and its location is restricted to the cytoplasm by Hsp90. DEX-treatment releases the GR::TF from Hsp90 allowing TF entry to the nucleus, where the TF binds to and regulates its target genes (11, 13, 26). CHX blocks translation. Thus, when DEX-induced TF import is performed in the presence of CHX, changes in transcript levels are attributed to the direct interaction of the target with the TF of interest, as we have shown previously for the well-studied TF, ABI3 (11). (B) Prior to DEX-induction of GR::TF nuclear import, pre-treatment with a signal (e.g. N-nutrient signal) could result in post-translational modifications of the TF and/or transcriptional/post-translational effects on its TF

partners (e.g. TF2). Genes whose response to TF-induced regulation (by DEX) is altered by CHX treatment were removed from our study, to eliminate potential side effects of CHX (see Materials and Methods). (C) Experimental design for identification of primary bZIP1 targets by either Microarray or ChIP-Seq analysis in the cell-based *TARGET* system (11, 26). CHX: cycloheximide; DEX: dexamethasone; N: nitrogen; GR: glucocorticoid receptor. (D) Bioinformatics pipeline to identify bZIP1 primary targets based on transcriptional response or TF binding. bZIP1-regulated genes were identified by ATH1 arrays. bZIP1-bound genes were identified by ChIP-Seq analysis. The integrated datasets were analyzed for the functional significance of classes of genes grouped based on TF-binding and/or TF-regulation.

Supplementary Figure S2. Nitrogen-responsive genes in the cell-based *TARGET* system. A heat map showing the expression profiles of 328 nitrogen (N)-responsive genes in the *TARGET* cell-based system (11) as identified by microarray in our study. The GO terms over-represented (FDR adjusted $p\text{-val}<0.05$) were identified for the genes up-regulated or down-regulated in response to the N-signal perturbation.

Supplementary Figure S3. Validation of N-response in *TARGET* system. The 328 N-responsive genes in the cell-based *TARGET* system show significant overlaps with previously reported N-response gene in roots of whole plants (27-29) and in seedlings (30). The significance of overlap between any two of these N-responsive sets is determined by the Genesect tool in VirtualPlant Platform (www.virtualplant.org) (15).

Supplementary Figure S4. Primary targets of bZIP1 are identified by either TF-activation or TF-binding. (A) Cluster analysis of bZIP1 primary target genes identified by their up-regulation or down-regulation by DEX-induced bZIP1 nuclear import in Arabidopsis root protoplasts sequentially treated with inorganic N, CHX and DEX. bZIP motifs and other *cis*-motifs are significantly over-represented in the promoters of bZIP1 primary target genes identified by transcriptional response (B), or by bZIP1 binding (D). (C) Examples of primary targets bound transiently by bZIP1 based on time-course ChIP-Seq.

Supplementary Figure S5. Genes influenced by a significant N-signal x bZIP1 interaction in the cell-based *TARGET* system. Genes regulated in response to DEX-induced bZIP1 nuclear import (FDR<0.05) and with a significant N-signal*bZIP1 interaction ($p\text{-val}<0.01$) from ANOVA analysis. Heat map showing four distinct clusters of genes regulated by a N-signal x bZIP1 interaction. Note that two of the “early response” genes shown to bind transiently to bZIP1 (NLP3 and LBD39, see Fig. 1C), are in cluster 1 of the genes regulated by a N-signal x bZIP1 interaction.

Supplementary Figure S6. Over-represented GO terms in each of the bZIP1 target Classes. The set of genes from each class of bZIP1 targets were analyzed for over-representation of GO terms using the BioMaps feature of VirtualPlant (www.virtualplant.org) (15). All classes of bZIP1 targets have an over-representation of GO terms related to “Stress” and “Stimulus”. When sub-divided by direction of regulation, Class IIA loses all significant GO terms. In addition to the stress terms, Class I is over-represented for genes responding to “biotic stress” and “divalent ion transport”. Class IIIA shows specific enrichment of GO terms for “Amino acid metabolism”,

hence showing an enrichment of genes related to the N-signal. Class IIIB has specific enrichment of genes related to cell death and phosphorus metabolism.

Supplementary Figure S7. bZIP1 as a pioneer TF for N-uptake/assimilation pathway genes.

Global analysis of bZIP1 targets reveals that it regulates multiple genes encoding for the N-uptake/assimilation pathway. Multiple genes encoding nitrate transporters and isoenzymes in the N-assimilation pathway are represented by hexagonal nodes. The nodes targeted by bZIP1 are connected with red arrows. Thickness of the arrow is proportional to the number of genes in that node that are targeted by bZIP1. The IDs of the targeted genes are listed adjacent to the node. This pathway overview suggests that bZIP1 is a master regulator of the N-assimilation pathway. The pathway was constructed in Cytoscape (www.cytoscape.org) based on KEGG annotation (www.genome.jp/kegg/). Node abbreviations: NRT: Nitrate transporters; AMT: Ammonia transporters; GDH: Glutamate dehydrogenases; GOGAT: Glutamate synthases; GS: Glutamine synthetases; ASN: Asparagine synthetases.

Supplementary Figure S8. A network of biological processes represented by Class III transient bZIP1 targets.

The set of genes from Class III “transient” bZIP1 targets were analyzed for over-representation of GO terms using the Bingo plugin in Cytoscape (31). In addition to terms related to “Stress” and “Stimulus” which are found in all 3 classes of bZIP1 targets, the Class III transient targets also shows class-specific enrichment of GO terms both for “nitrogen metabolism” and the “regulation of nitrogen compound metabolism”, hence showing an enrichment of genes related to the N-signal. Class III transient targets also show over-representation of genes involved in “defense response”, “phosphorylation” and “regulation of metabolism”.

Supplementary Tables:

Table S1. N-responsive genes (FDR<0.05) in root protoplasts used in the *TARGET* system.

Table S2. Overlap of N-responsive genes in root protoplasts (this study) vs N-response studies performed *in planta* (Krouk et al., 2010; Gutierrez et al., 2008; Wang et al., 2003; Wang et al., 2004).

Table S3. Genes identified to be ZIP1 targets based on ANOVA analysis of transcriptome and/or by ChIP-Seq analysis.

Table S4. bZIP1 primary targets identified as genes up-regulated or down-regulated by DEX-induced nuclear import of bZIP1 (FDR<0.05).

Table S5. Significantly over-represented GO terms (FDR <0.01) identified for genes up-regulated or down-regulated by DEX-induced nuclear import of bZIP1 (FDR<0.05).

Table S6. Genes regulated by DEX-induced nuclear import of bZIP1 (FDR<0.05) and by the interaction of N-signal and DEX-induced nuclear import of bZIP1 (*p-val*<0.01).

Table S7. Genes bound by GR::bZIP1 as detected by ChIP-seq with anti-GR antibody.

Table S8. 3 Classes of bZIP1 primary targets: Class I, Poised; Class II Stable (IIA induced; IIB repressed); and Class III transient (IIIA induced, IIIB repressed) listed as 5 subclasses. Gene annotations are from TAIR10.

Table S9. bZIP1 primary targets in the N-assimilation pathway.

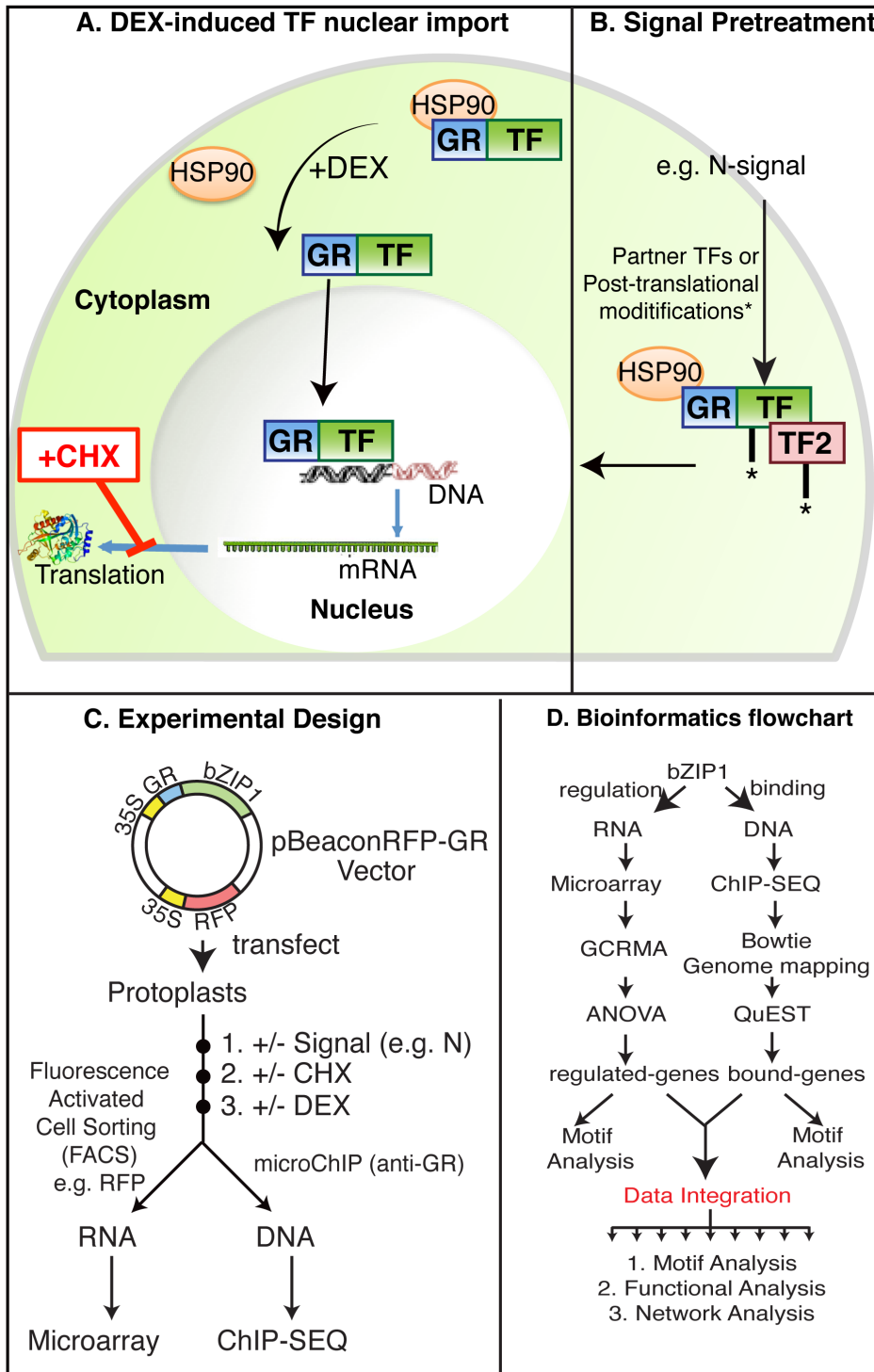
Table S10. Significantly over-represented GO terms (FDR adjusted $p\text{-val}<0.01$) identified for genes in each of the five subclasses of bZIP1 primary targets.

Table S11. Class IIIA bZIP1 primary targets that transiently and rapidly up-regulated by N.

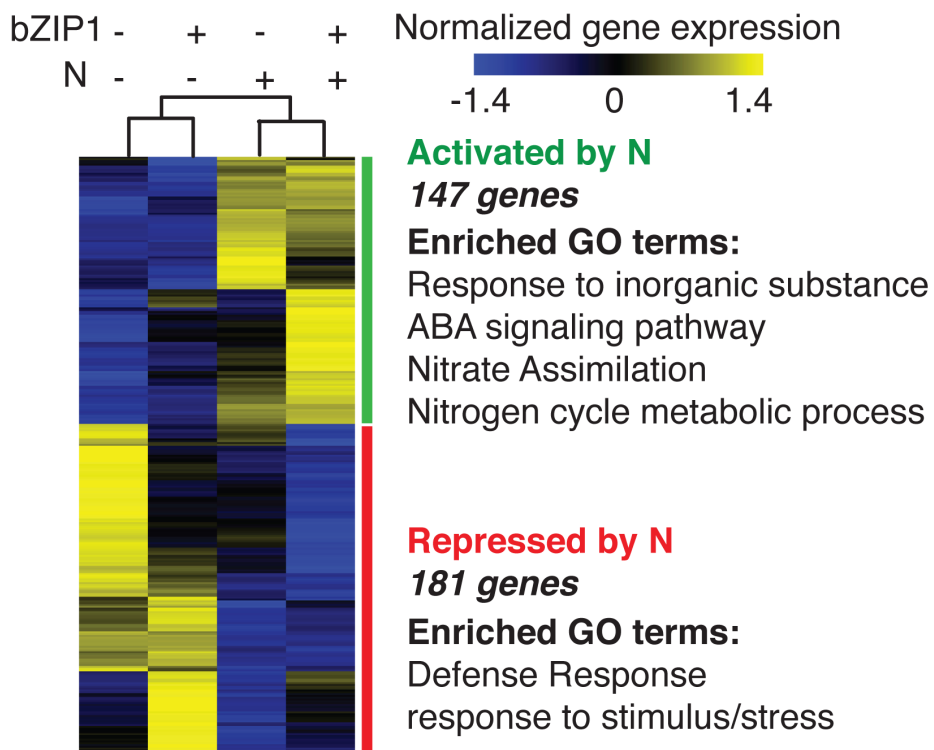
Table S12. Class III bZIP1-regulated genes that show evidence of bZIP1 binding at early (1, 5, 30 or 60 min), but not at a 5hr time point.

Table S13. bZIP1 protein-protein interaction partners.

Supplementary Figure S1. Experimental scheme for TF (A) and N-signal perturbation (B), and parallel RNA-Seq and ChIP-Seq analysis (C & D) of bZIP1 primary targets.



Supplementary Figure S2. Nitrogen-responsive genes in the cell-based *TARGET* system.



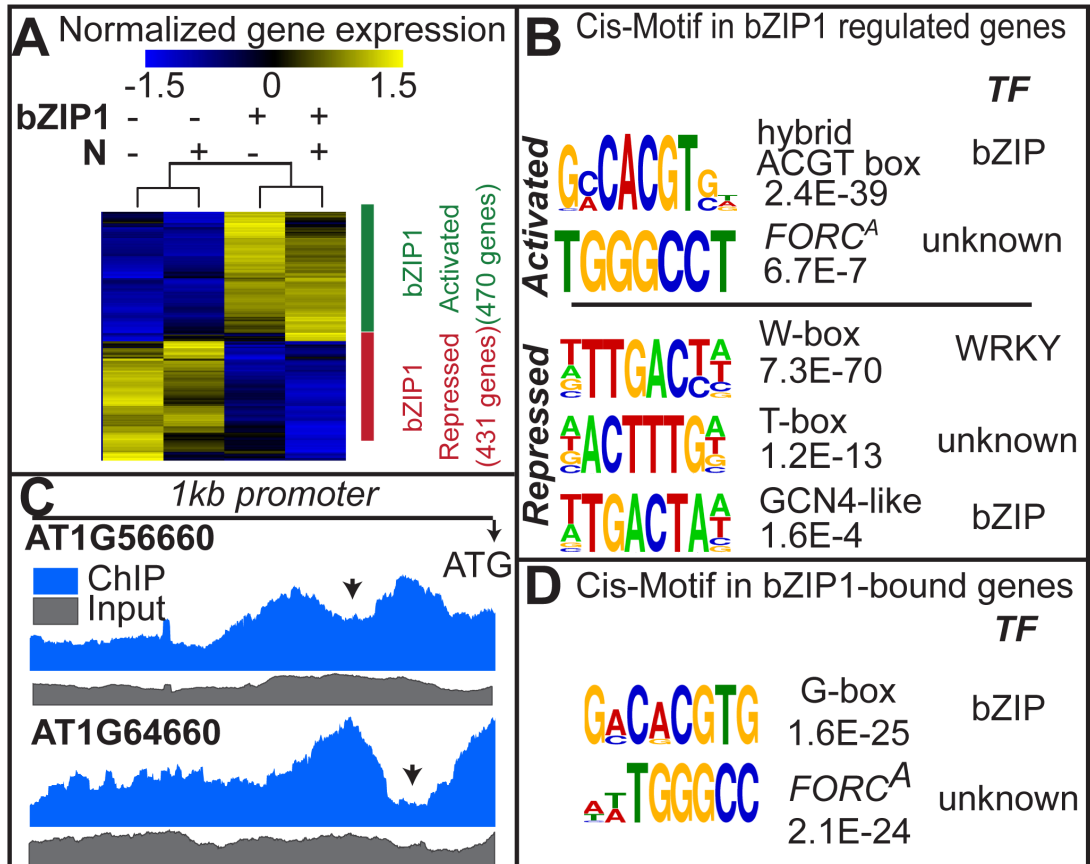
Supplementary Figure S3. Validation of N-response in TARGET system.

Genes overlapped
*** pval<0.001

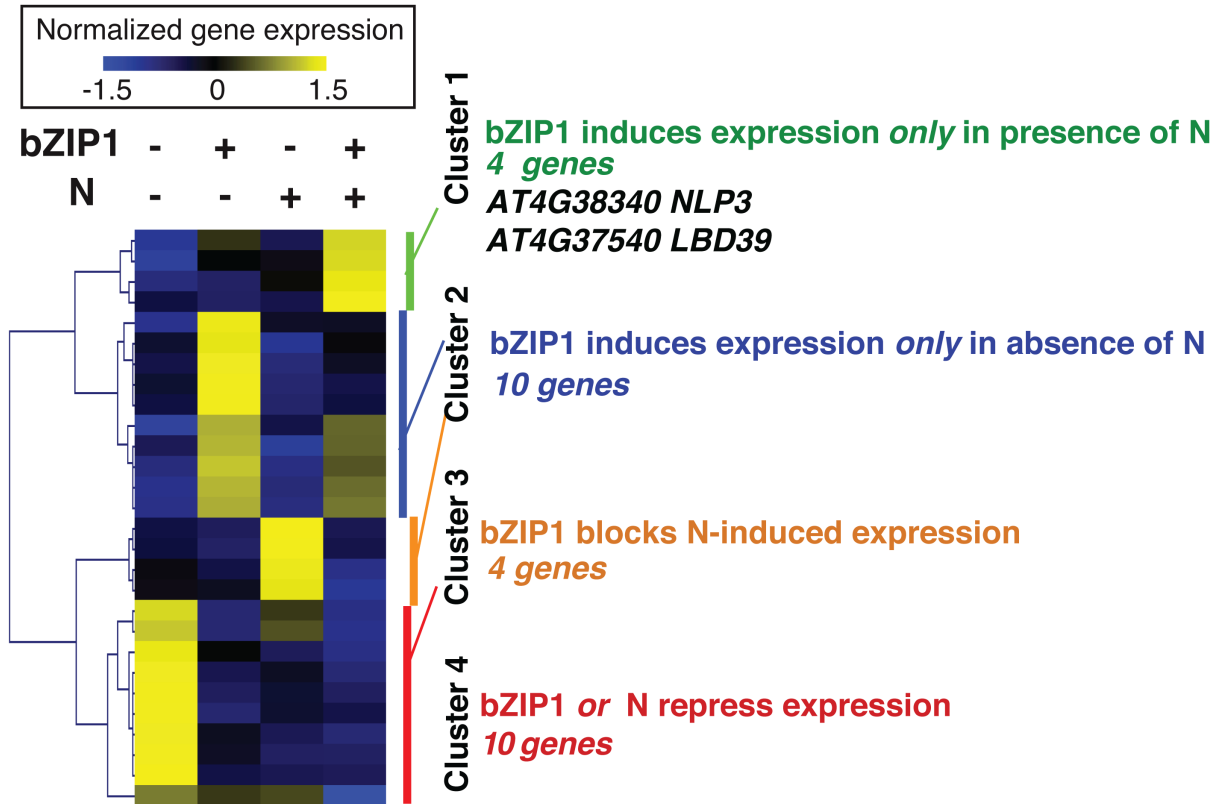
N-responsive genes
This study
(328)

	Union of other N-studies	Krouk et al., 2010	Gutierrez et al., 2008	Wang et al., 2004	Wang et al., 2003
	121***	43***	38***	79***	75***

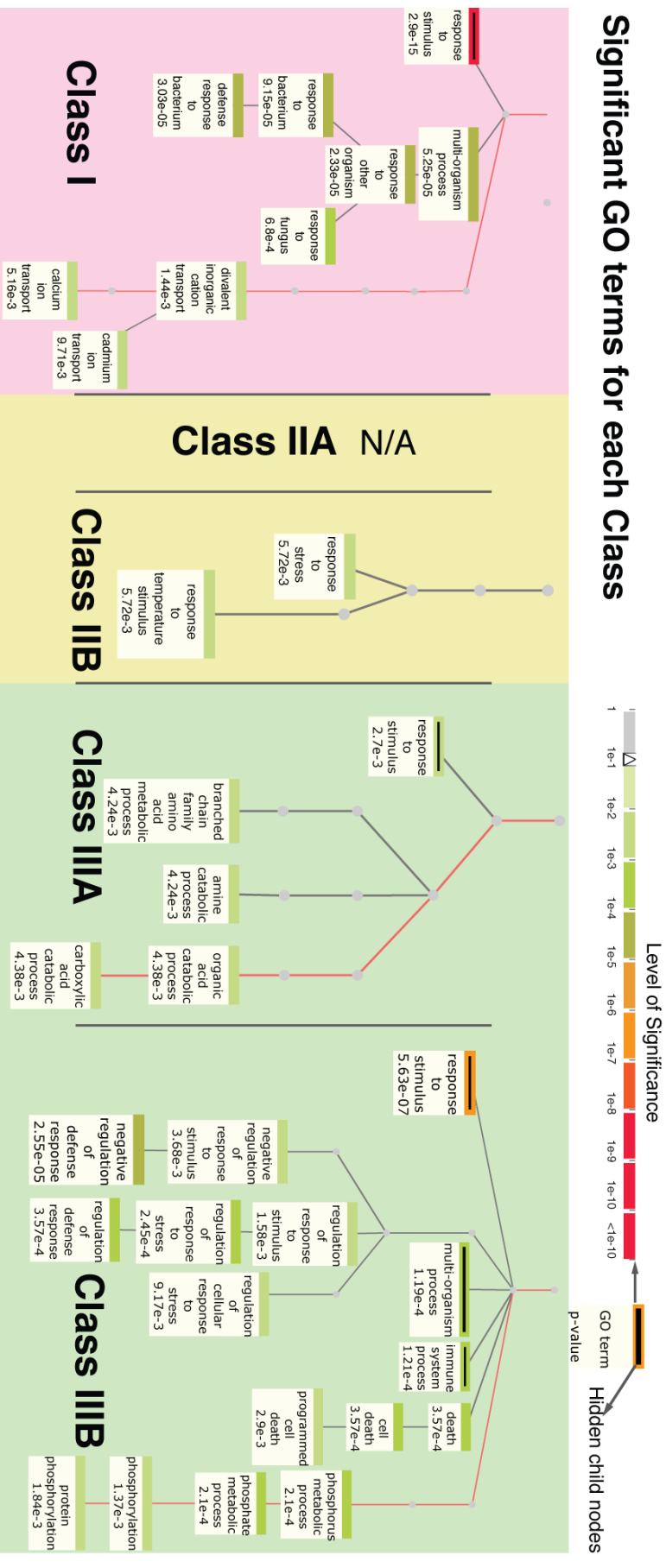
Supplementary Figure S4. Primary targets of bZIP1 are identified by *either* TF-activation *or* TF-binding.



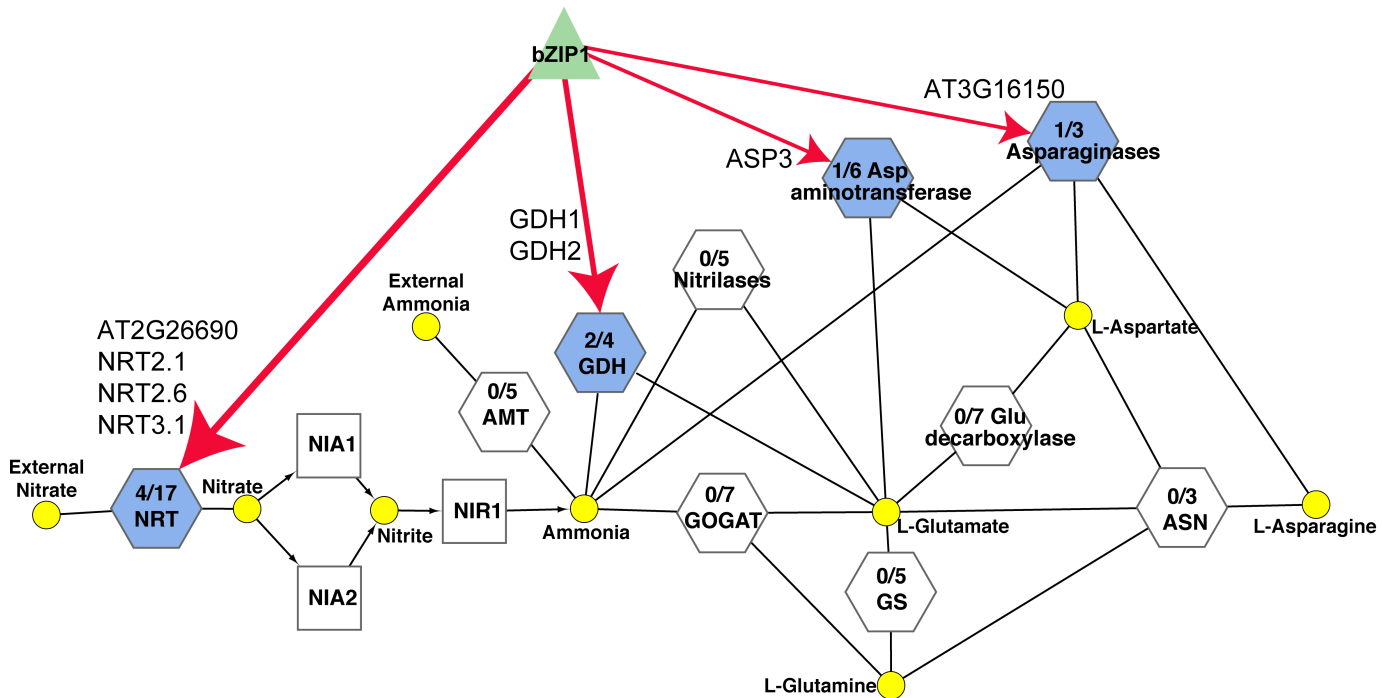
Supplementary Figure S5. Genes influenced by a significant N-signal x bZIP1 interaction in the cell-based *TARGET* system.



Supplementary Figure S6. Over-represented GO terms in each of the bZIP1 target Classes.



Supplementary Figure S7. bZIP1 as a pioneer TF for N-uptake/assimilation pathway genes.



Supplementary Figure S8. A network of biological processes represented by Class III transient bZIP1 targets.

