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**The NAC24 transcription factor in maize:
from aquaporin regulation to plant development**

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du grade de Docteur en Sciences Agronomiques et Ingénierie
Biologique

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Juin 2025

ACKNOWLEDGEMENTS

Before thanking all the wonderful people who supported me on this thesis journey, I'd like to start by expressing my heartfelt thanks to the jury members: Prof. Charles Hachez, Prof. René Rezsohazy, Prof. Lieven De Veylder, and Prof. Micaela Carvajal. Thank you for taking the time to read this work and for giving me thoughtful and in-depth feedback. Our discussion during the private defense was a real pleasure—it was inspiring and rewarding to hear your perspectives. I would also like to thank the president of the jury, Prof. Michel Ghislain, for taking the time to expertly lead the private defense.

Maintenant, préparez-vous pour des remerciements sous forme d'analogie (parfois un peu bancale) qui m'a souvent accompagnée pendant cette thèse et dont j'exploite enfin tout le potentiel ici.

Rédiger une thèse, c'est un peu comme courir un ultra-trail : c'est long, souvent solitaire, mais ça ne se réussit pas sans un mental d'acier et un entourage solide. Il y a des moments d'effort intense, des passages remplis de doutes, mais aussi de belles rencontres qui donnent tout son sens à l'aventure.

Tout d'abord, j'aimerais remercier mon "coach" : François, mon promoteur. Sans lui, je n'aurais même pas osé me lancer sur ce sentier escarpé qu'est la thèse. Il a été là dès le début, pour la préparation, et surtout pour me guider tout au long de la course. Ses conseils ont été essentiels pour avancer, même quand la pente se faisait rude.

Un grand merci aussi aux compagnons de course avec qui j'ai pris le départ : Maxime, Louche et Nicolas. On a emprunté le même parcours, chacun à son rythme, chacun avec ses propres défis... mais toujours en partageant les galères (dans la bonne humeur).

Bien sûr, il y a aussi les "anciens", ceux qui sont passés sur le parcours avant nous et qui montrent la voie. Xavier et Lolo, le duo infernal, avec qui j'ai passé plus d'une nuit dehors (comme en ultra, hihi), toujours présents pour partager leur expérience et, surtout, leurs précieuses « techniques » de détente et de décompression.

Et puis, au fil du temps, j'ai aussi eu la chance d'accueillir de nouveaux coureurs (chercheurs) dans cette aventure : Marie, avec qui j'ai partagé beaucoup de ravitaillements ; Jérôme, avec qui on a retourné le sens de ce qu'on fait plus d'une fois ; Mathilde, toujours partante pour des discussions passionnantes sur le parcours ; et Manon, toujours partante pour n'importe quel défi. J'espère que j'ai pu, à ma façon, les guider un peu dans cette drôle d'aventure.

Je voudrais aussi saluer les “ravitailleurs” : Joseph et Catherine. Toujours là aux points de ravitaillement, avec des conseils précieux et un soutien sans faille. Parfois, ils ont même fait un bout de chemin avec moi, trouvant toujours les mots justes — ou un petit ragot pour me divertir — quand la fatigue ou le doute menaçaient de me faire abandonner.

Même si, comme l’ultra, la thèse peut paraître individuelle, j’ai eu la chance d’avoir des coéquipiers incroyables au sein de notre team AQP. Lei, Romina, Kim, Nicolas C. et Jahed étaient toujours prêts à m’aider et à partager leurs trucs et astuces. Mention spéciale à Laurie, qui m’a encadrée lors de mon mémoire et qui a été une vraie guide, m’apprenant toutes les bases. Et puis, les deux petits nouveaux de l’équipe, Jean et Joséphine, qu’on n’aurait pas pu mieux choisir pour renforcer le groupe. Votre présence quotidienne au labo a été un vrai vent de fraîcheur et un rayon de soleil qui illuminait mes journées. Enfin, un grand merci à celles et ceux qui, tels les bénévoles qui font tourner les courses dans l’ombre, font vivre le laboratoire FYMO au quotidien : Michèle, Catherine B., Daniela, Monique, Ben, ... Leur travail peut paraître discret, mais il a été indispensable à la réussite de cette course.

Et puis, il y a la famille : maman, papa, Alex et Nico, et les amis : Clara, Vincent, Corry et Delphine. Eux suivaient mon avancée de loin, comme sur le live tracking d’une course, m’envoyaient des messages pour savoir où j’en étais (et pourquoi je faisais ça, hihi), mais toujours là pour me soutenir, quoi qu’il arrive.

Et enfin, je garde la personne la plus importante pour la fin : mon compagnon de route, Quentin. Je pense pouvoir dire que tu as partagé 99,999 % de cette aventure avec moi. Je suis extrêmement reconnaissante de t’avoir eu à mes côtés pendant tout ce temps ; tu es clairement la clé de ma réussite.

Comme dans un ultra-trail, cette thèse n’est pas seulement le résultat d’un effort personnel, mais celui d’une aventure collective. À tous ceux qui ont contribué à ce parcours (je n’ai pas pu citer tout le monde), de près ou de loin, je vous remercie du fond du cœur.

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ABSTRACT

The regulation of gene expression is fundamental to plant development and adaptation to environmental challenges. Transcription factors (TFs) play a key role in transcriptional regulation by integrating developmental, tissue-specific and environmental signals, enabling complex and context-specific gene activity.

Aquaporins belonging to the plasma membrane intrinsic proteins (PIPs) subfamily are channels that facilitate water movement across the plasma membrane. They are involved in many physiological processes, such as the hydraulic regulation of roots and leaves, cell expansion, organ development and stress responses. Despite extensive research on their functional roles, the molecular mechanisms underlying the transcriptional regulation of *PIP* genes remain poorly understood.

An expression quantitative trait loci (eQTL) analysis performed in maize identified NAC24 as a potential regulator of *PIP2;5* expression. NACs constitute one of the largest plant-specific TF families and are involved in many developmental processes as well as stress responses.

Here, we demonstrated that NAC24 localized in the nucleus and activated the *PIP2;5* promoter in *transactivation* assays. Overexpression of *NAC24* in maize suspension cells increased *PIP2;5* expression, while maize *nac24* knockdown plants showed reduced *PIP2;5* expression accompanied by stunted growth, abnormal leaf morphology, and reduced pollen fertility. These pleiotropic defects are probably due to a broader disruption of gene regulation rather than a reduction of *PIP2;5* expression alone. DNA affinity purification sequencing (DAP-seq) identified NAC24-bound genomic regions, including promoters of genes involved in auxin and ethylene signaling, such as *Aux/IAA14*, *PILS6*, and *ARGOS1*. NAC24 bound directly to a palindromic consensus sequence in the promoters of *ARGOS1* and *LDSH5*, activating them, while repressing *Aux/IAA14*. Our study revealed that NAC24 as a key regulator of *PIP2;5* expression and maize growth, potentially influencing organ size through the regulation of *ARGOS1* and *LDSH5* expression.

ABBREVIATIONS

ABA	Absciscic acid
ABRE	ABA responsive element
ACR	Accessible chromatin region
ALOG	Arabidopsis Light-Dependent Short Hypocotyls1/ <i>Oryza sativa</i> G1
AP2/ERF	APETALA2/ethylene responsive factor
ATAC-seq	Assay for transposase-accessible chromatin with sequencing
ATAF	Arabidopsis transcription activation factor
AQP	Aquaporin
BES1	BRI1-EMS-Suppressor1
BMS	Black Mexican Sweet
BR	Brassinosteroid
bZIP	Basic leucine zipper
cDNA	Complementary DNA
CDS	Coding sequence
ChIP	Chromatin immunoprecipitation
CRE	<i>Cis</i> -regulatory element
CRM	<i>Cis</i> -regulatory module
CTD	C-terminal domain
CUC	Cup-shaped cotyledon
CY3	Cyanine 3
DAG	Days after germination
DAP	DNA affinity purification
DBD	DNA binding domain
DDR	DNA damage response
DLR	Dual Luciferase® reporter
DREB	Dehydration responsive element binding protein
EB	Elution Buffer
EMSA	Electrophoretic mobility shift assay
ER	Endoplasmic reticulum
ERF	Ethylene-responsive factor
eQTL	Expression quantitative trait locus
FDA	Fluorescein diacetate
FLuc	Firefly Luciferase
Fw	Forward
GCM	Glial cells missing
GCN	Gene co-expression network
gDNA	Genomic DNA
GFP	Green fluorescent protein
GST	Glutathione-S-transferase
GRN	Gene regulatory network
GWAS	Genome-wide association study
IPTG	Isopropyl-β-d-1-thiogalactopyranoside

JA	Jasmonic acid
kb	Kilobase(s)
KD	Knockdown
KO	Knockout
LEA	Late embryogenesis abundant protein
Lp _r	Root hydraulic conductivity
LSH	Light-Dependent Short Hypocotyls
MCS	Multicloning site
MEME	Multiple EM for motif elicitation
MYB	Myeloblastosis
mRNA	messenger RNA
<i>Mu</i>	<i>Mutator</i>
NAC	NAM, ATAF 1/2 and CUC2
NACBS	NAC binding site
NAM	No apical meristem
Ni-NTA	Nickel-nitrilotriacetic acid
NIP	Nodulin 26-like Intrinsic Protein
OE	Overexpression
OD	Optical density
<i>p35S</i>	35S promoter from the Cauliflower Mosaic Virus
PAGE	Polyacrylamide gel electrophoresis
PANTHER	Protein Analysis Through Evolutionary Relationships
PBM	Protein binding microarray
PBS	Phosphate buffered saline
PCD	Programmed cell death
PCV	Plant cell volume
PEG	Polyethylene glycol
PIP	Plasma membrane intrinsic protein
POL II	RNA polymerase II
PWM	Position weight matrix
RLuc	<i>Renilla</i> Luciferase
ROS	Reactive oxygen species
RT-qPCR	Real-time quantitative PCR
Rv	Reverse
SA	Salicylic acid
SCW	Secondary cell wall
SDS	Sodium dodecyl sulfate
SEC	Size exclusion chromatography
SELEX	Systematic Evolution of Ligands by EXponential enrichments
SOG1	SUPPRESSOR OF GAMMA RESPONSE 1
SNP	Single nucleotide polymorphism
TE	Transposable element
TEV	Tobacco Edge Virus
TF	Transcription factor
TFBS	Transcription factor binding site

TIP	Tonoplast intrinsic protein
TIR	Terminal inverted repeats
tNOS	Terminator nopaline synthase
TRD	Transcription regulatory domain
TSS	Transcription start site
TTS	Transcription termination site
UTR	Untranslated region
VND	VASCULAR-RELATED NAC-DOMAIN
WRKY	Tryptophan-arginine-lysine-tyrosine
WT	Wild type
Y1H	Yeast one-hybrid
YFP	Yellow fluorescent protein

INTRODUCTION

The regulation of gene expression is fundamental to plant development and their ability to adapt to environmental challenges. Unlike animals, plants are sessile organisms that must fine-tune their gene expression to respond dynamically to an ever-changing environment, while coordinating intricate developmental programs in various tissues.

Transcriptional regulation, the first step of gene expression regulation, allows plants to integrate a wide range of developmental, tissue-specific, and environmental cues, enabling complex and context-specific gene activity.

For example, photosynthesis-related genes are mainly expressed in leaf tissues in response to light signals, while genes involved in nutrient uptake are specifically activated in roots, where their expression is influenced by soil conditions and nutrient availability (Hibberd & Covshoff, 2010; Yaschenko *et al.*, 2022). Many genes also respond to environmental triggers such as drought, cold, or salt stress, while others are activated when the plant is confronted to pathogens. Under normal, stress-free conditions, these stress- and defense-related genes remain largely inactive. This selective transcription is largely controlled by both *cis*-regulatory elements (CREs) and *trans*-acting factors, such as transcription factors (TFs) (Singh *et al.*, 1998).

However, identifying these gene regulatory components is challenging, especially in plant species with large genomes and low gene density, where CREs may be distant from their targets or located in introns, untranslated regions (UTRs), or even coding regions (Spitz & Furlong, 2012). Despite these complexities, understanding the transcriptional regulator remains a central goal in plant biology in order to build accurate models of gene expression, which are key for developing more resilient and productive crops.

Transcriptional gene regulation

Transcription is the first step in gene expression, during which genetic information encoded in the genome is transcribed into messenger RNA (mRNA) by RNA polymerase II (Pol II), to be transmitted to the protein-producing platform. In plants, as in other organisms, a significant portion of the genome is devoted to regulating this process, which determines when, where, and how much mRNA is produced (Grotewold & Springer, 2009).

The regulation of gene transcription is orchestrated by the interaction between *cis*-regulatory elements (CREs) and *trans*-acting factors, primarily transcription factors (TFs). Truly understanding this transcriptional regulation can be viewed as assembling a complex puzzle, in which one set of pieces

comprises the CREs, and the other consists of TFs. As these pieces gradually come together, the broader regulatory landscape begins to emerge. However, the assembly of this puzzle is further complicated by additional regulatory layers, such as DNA structure, chromatin accessibility, and epigenetic modifications, which add complexity to the transcriptional regulation (Springer *et al.*, 2019).

Cis-regulatory modules

Cis-regulatory elements, typically short nucleotide sequences, function as blueprints for translating cellular signals into transcriptional outcomes by serving as binding sites for DNA-binding proteins such as TFs (Marand *et al.*, 2023). The appropriate transcriptional response is mediated by the recruitment of these TFs, either individually or in combination, to CREs, typically followed by their interaction with the basal transcriptional machinery, although the sequential order of events may vary (Springer & Grotewold, 2009; Kaufmann *et al.*, 2010; Yang *et al.*, 2016).

Like protein-coding regions, CREs are embedded within the genomic DNA. However, their interpretation and accessibility are highly context-dependent, as the genomic range in which a CRE can function is determined by the three-dimensional organization of chromatin within the nucleus (Grotewold & Springer, 2009). Nucleosome packaging can cause tight interactions between histone and DNA, rendering CREs inaccessible to the DNA-binding domains of TFs. In contrast, active CREs are typically found within accessible chromatin regions (ACRs), characterized by nucleosome depletion that facilitates interactions between TFs and their target sites (Marand *et al.*, 2023).

CREs are distributed throughout the genome, but it is the specific combination and clustering of these elements that define *cis*-regulatory regions, also referred to as *cis*-regulatory modules (CRMs) (Marand *et al.*, 2023). These may reside near the gene, such as promoters, or at more distant locations, like enhancers, silencers and other long-range regulatory elements. In addition to the presence of CREs, their number, arrangement, and relative position within a module influence the interaction with multiple TFs, other *trans*-acting factors, and the basal transcriptional machinery, ultimately allowing for the fine-tuning of transcriptional outcomes (Levo & Segal, 2014).

Proximal regulatory modules: promoters

CRMs upstream of a gene are referred to as the proximal promoter, or simply the promoter, and can be further subdivided into the core promoter and proximal promoter elements, based on their function and relative distance from the transcription start site (TSS) (Yasmeen *et al.*, 2023).

The core promoter, also known as the basal promoter, is the minimal DNA sequence required for transcription initiation through the recruitment of the basal transcriptional machinery, including Pol II and general TFs (Butler and Kadonaga, 2002). Although the length of a core promoter must be defined experimentally, it is generally defined as the ~100 nucleotide region around the TSS and includes multiple conserved motifs (TATA box, initiator elements, ...) (Molina and Grotewold, 2005). However, in practice, core promoters are highly variable: there are no universally conserved motifs, and even the TATA box is present in only a minority of genes in both plants and mammals (Roy & Singer, 2015). Furthermore, the heterogeneity of TSSs in plants underscores the flexibility of sequences capable of supporting transcription initiation (Morton *et al.*, 2014). A detailed overview of core promoter motifs and components of the basal transcriptional machinery in plants can be found in Springer and Grotewold (2009) and will not be discussed in further detail here. In plants, the proximal promoter region extends arbitrarily several hundred base pairs upstream of the core promoter and contains various CREs that serve as binding sites for TFs. In this manuscript, we use the term “promoter” to refer specifically to this arbitrary proximal promoter region. The regulatory elements within this region respond to internal and external stimuli, such as light, hormones, wounding, drought, heat, cold, oxidative stress, and anaerobic conditions, each of which is associated with specific DNA motifs (Lescot *et al.*, 2002; PlantCARE database). Plant promoters may also include transposable elements, as well as microsatellite and minisatellite repeat sequences, which have been proposed to play roles in the fine-tuning of gene expression (Yasmeen *et al.*, 2023).

In essence, while the core promoter provides the basic platform for transcription initiation, the proximal promoter elements contribute to the fine-tuning and regulation of gene expression. However, a more unified view of plant (and animal) promoter architecture has emerged, challenging this classical distinction between core and proximal promoters. Evidence from *Arabidopsis* suggests that plant promoters do not conform to this bipartite model, instead functioning as a more integrated regulatory unit (Morton *et al.*, 2014).

Distal regulatory modules

In addition to proximal regulatory elements, many CRMs, controlling gene expression, are located further from the TSS. These distal CRMs are referred to as enhancers (or silencers when they repress transcription) if they can modulate transcription independently of their orientation, position, or distance relative to the TSS (Spitz & Furlong, 2012).

Unlike promoters, enhancers lack the ability to independently initiate “productive” transcription that leads to protein production. However, like promoters, enhancer regions are also occupied by Pol II and produce noncoding transcripts known as enhancer RNAs (eRNAs). These eRNAs are increasingly recognized as functional molecules, rather than mere transcriptional noise as they play crucial roles in regulating chromatin conformation and transcriptional activation (Ye *et al.*, 2020).

In large plant genomes, such as maize, a significant proportion of enhancers are located in intergenic regions, sometimes hundreds of kilobases (kb) away from the genes they regulate. Thus, many enhancers function via chromatin loops, allowing three-dimensional interactions between enhancer- and promoter-bound protein complexes that influence transcriptional output (Ricci *et al.*, 2019). Unlike proximal regulatory elements that often exert control through a specific core promoter, enhancers can exhibit a degree of functional promiscuity, simultaneously influencing multiple gene targets (Springer & Grotewold, 2009).

Figure 1 shows a schematic overview of the proximal and distal CREs found in plants.

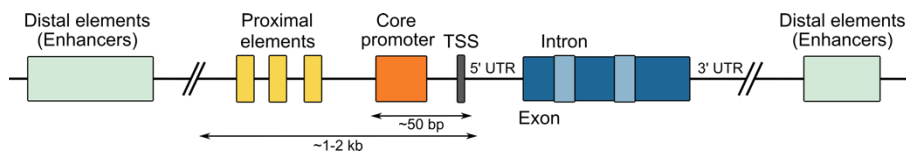


Figure 1: Cis-regulatory elements in plants. CREs are distributed throughout the genome and cluster together in cis-regulatory modules (CRMs). These modules may be located near the gene or at more distant sites like enhancers (depicted in light green). CRMs upstream of a gene are referred to as the proximal promoter and can be further subdivided into the core promoter (orange) and proximal promoter element (yellow).

Box 1: Regulatory activity of introns and UTRs¹

In plants as in animals, introns can significantly influence mRNA accumulation, acting at multiple levels, including transcription rate, mRNA processing, and stability (Rose, 2004). Regulatory effects may come from the presence of CREs within intronic regions that serve as binding sites for TFs. For example, the *AGAMOUS* gene in *Arabidopsis* contains CREs within a 3 kb intron that are bound by a homeodomain protein, resulting in transcriptional repression (Bao *et al.*, 2004). However not all effects of introns are mediated by CREs. In some cases, the presence of the intron itself enhances gene expression through a phenomenon known as intron-mediated enhancement (IME). While the mechanisms underlying IME remain unclear, it is well established that introns capable of enhancing expression are often located within 1 kb of the TSS and their function may be independent of splicing (Gallegos & Rose, 2015). Introns are now recognized not just as spacers within coding regions, but as active regulatory elements playing diverse and nuanced roles in gene regulation.

Untranslated regions (UTRs), though not translated, also influence gene expression. The 5' UTR, located upstream of the start codon, can influence transcription by harboring CREs. UTRs can modulate transcription initiation, efficiency, and even the stability of the resulting mRNA (Bilas *et al.*, 2016).

Transcription factors

TFs are central to transcriptional regulation and are usually defined as modular proteins containing a DNA-binding domain (DBD) that interacts with CREs and short linear motifs (SLiMs) - conserved short amino acid sequences found in intrinsically disordered regions that mediate protein-protein interactions. These SLiMs can be crucial for targeting specific binding partners, ultimately influencing transcriptional outcomes (Figure 2) (O'Shea *et al.*, 2017).

In addition to TFs, various proteins influence gene expression without directly binding to DNA. Known as co-activators or co-repressors, these proteins regulate indirectly transcription by modulating the activity of DNA-binding TFs (Singh *et al.*, 1998). Transcription is also influenced by other proteins that regulate chromatin structure and accessibility. These include structural chromatin components, such as histone variants, chromatin-modifying

¹Boxes offer supplementary information that complements the main text without interrupting the flow of the introduction.

enzymes such as DNA and histone methyltransferases, and chromatin-binding proteins, collectively referred to as chromatin factors (Springer & Grotewold, 2009). While essential to transcriptional regulation, chromatin factors and co-activator/co-repressors will not be discussed further in this introduction.

TFs are known to act in a combinatorial manner, enabling a relatively small set of TFs to coordinate the expression of a large number of genes in very specific spatial and temporal patterns. This precise regulation results from the coordinated activity of dozens to hundreds of TFs tethered to regulatory modules through specific protein–DNA and protein–protein interactions. Through these interactions, TFs can activate or repress transcription in a gene target specific manner (Yang *et al.*, 2016). TFs are also organized hierarchically: some regulate the expression of genes encoding other TFs, while others target genes involved in structural functions or metabolism. This hierarchical organization allows for signal amplification and fine-tuned control, facilitating the deployment of gene networks at the correct time and the right place (Kaufmann *et al.*, 2010; Strader *et al.*, 2022).

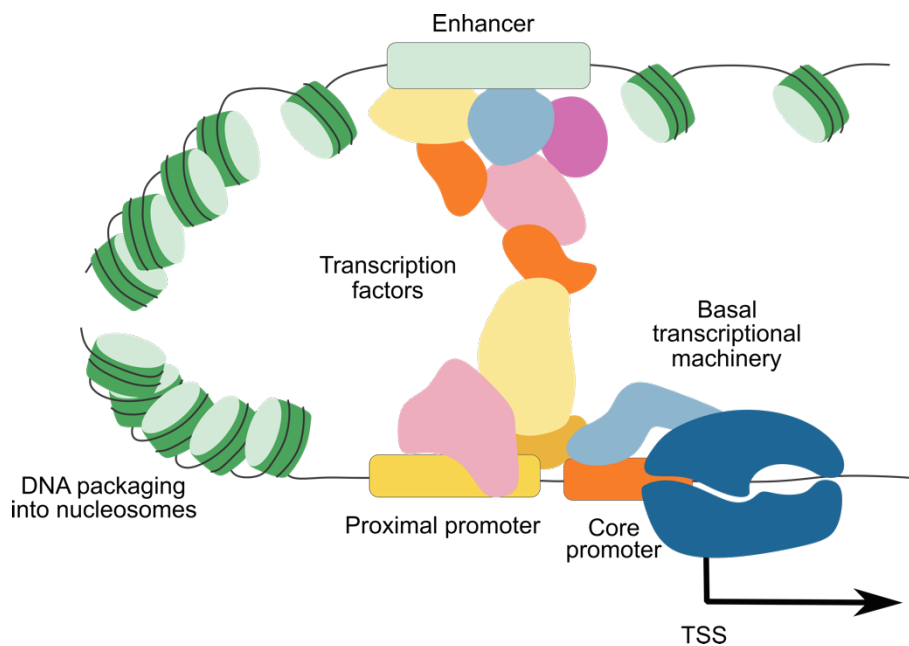


Figure 2: Simplified view of combinatorial gene transcription control. Gene transcription is regulated by interactions between TFs, CREs, the basal transcriptional machinery, and various cofactors. TFs bind accessible CREs—both proximal and distal—combinatorially through protein–DNA and protein–protein interactions. This simplified representation does not include all the elements found in transcriptional hubs (adapted from Springer *et al.*, 2019).

Plant TFs are classified into families based on their conserved DBDs, with members of the same family typically sharing sequence similarities in this region. Many families, such as AP2/ERF, WRKY, and NAC, are unique to plants, while those shared with other eukaryotes show greater expansion in plants (Shiu *et al.*, 2005). According to the Grass Regulatory Information Services (GRASSIUS) (Yilmaz *et al.*, 2009), the maize genome contains more than 2,500 DNA-binding TFs, representing 5% of its protein-coding genes, highlighting the substantial proportion of the plant genome dedicated to TFs (Springer & Grotewold, 2009).

Although the majority of TFs have been identified in key plant genomes, such as those of Arabidopsis and maize, only a small fraction of them have been functionally characterized. Yet, those that have been studied are known to regulate important (agronomic) traits, since TF knockdowns/knockouts or loss-of-function mutations often result in striking phenotypic changes (Richardson and Hake, 2022). Therefore, understanding TF binding preferences could enable major advancements in engineering genetic circuits for optimal plant fitness.

Approaches to discover TF binding preferences

Characterizing TF DNA binding preferences is an essential tool for identifying TF–CRE associations and predicting gene regulatory networks. From now on, CREs bound by TFs will be referred to as transcription factor binding sites (TFBS). Below is a non-exhaustive overview of commonly used approaches to identify TFBSs to link TFs to their target genes and provide information about their regulatory function.

Specific TFBSs can be identified using *in vitro* protein–DNA interaction techniques. However, the DNA-binding activity of a TF observed *in vitro*, which reflects its “intrinsic” binding potential when considered in isolation, does not always fully capture its promoter occupancy *in vivo* (Marand *et al.*, 2023).

Protein binding microarray (PBM) is a high-throughput *in vitro* tool used to define TF binding specificities with single-base resolution. In this approach, a large array of short DNA sequences is immobilized on microarray slides, allowing simultaneous probing of different TF binding affinities (Berger & Bulyk, 2009). Fluorescently labeled TFs are incubated with the array, and binding is quantified based on fluorescence intensity, which reflects the strength of protein–DNA interactions. PBMs have already been widely used to map TFBS across different plant species (Franco-Zorrilla *et al.*, 2014).

Systematic Evolution of Ligands by EXponential enrichment (SELEX) is an *in vitro* technique used to determine the DNA-binding specificity of proteins through an iterative enrichment process. In a SELEX experiment, a population of random double-stranded oligonucleotides, flanked by a conserved sequence, is incubated with the TF of interest. The resulting protein–DNA complexes are separated from unbound DNA and the bound DNA is then recovered, PCR-amplified, and subjected to successive cycles of selection (Tuerk, 1997).

Sequence data generated from SELEX and PBM experiments can be used to construct position weight matrices (PWMs), which quantitatively represent the binding preferences of a TF by reflecting the contribution of each nucleotide at each position. These PWMs are used to scan genomic sequences for potential TFBS and their corresponding target genes. Using these approaches, a number of TFBSs have already been identified in plants. The conclusion of these studies is that TF recognition sites are generally short (~10 bp) and degenerate, usually consisting of a highly conserved core motif flanked by more variable residues (Franco-Zorilla *et al.*, 2014).

Although *in vitro* approaches are valuable for identifying binding preferences of TFs, *in vivo*, they interact with highly dynamic chromatin rather than the naked DNA typically used in *in vitro* binding assays.

Chromatin immunoprecipitation (ChIP-seq) is a genome-wide method to identify *in vivo* interactions between TFs and genomic DNA under physiological conditions, while also assessing TF binding density at specific genomic sites (Kuo *et al.*, 1999). The experiment begins with cross-linking TF–DNA complexes in the harvested tissue, followed by chromatin fragmentation and immunoprecipitation using an antibody specific to the TF of interest. Bound DNA is recovered from the complex and used for the generation of sequencing libraries. ChIP-seq efficiently captures direct TF–DNA interactions, but it can also reveal indirectly bound targets via protein–protein interactions. However, ChIP-seq is time-consuming and costly, and relies on high-quality antibodies and efficient cross-linking. These factors, along with its limited sensitivity to low-abundance proteins, often limit the scale of ChIP-seq studies in plants (Park, 2009).

DNA affinity purification sequencing (DAP-seq) was developed to overcome these limitations of ChIP-seq. In DAP-seq, a TF expressed *in vitro* and affinity-tagged is incubated with fragmented genomic DNA. The TF–DNA complexes are then purified, and the bound DNA is recovered and sequenced to generate high-resolution, genome-wide maps of TFBSs (O'Malley *et al.*, 2016; Bartlett *et al.*, 2017).

Because the assay is performed on native genomic DNA, DAP-seq also provides insights into the methylation sensitivity of TF binding. However, since it lacks the native chromatin context, DAP-seq does not fully capture *in vivo* dynamics (Bartlett *et al.*, 2017).

To assess the *in vivo* relevance of TFBS identified by DAP-seq, one approach is to examine their overlap with accessible chromatin regions. Several genome-wide assays have been developed to profile these ACRs, including DNase-seq and ATAC-seq, which has become the preferred method due to its simplicity and efficiency. ATAC-seq uses the hyperactive Tn5 transposase that preferentially inserts sequencing adapters into open chromatin (Marand *et al.*, 2023). Integrating DAP-seq data with ATAC-seq profiles enables a more comprehensive understanding of which TFBS are truly accessible and potentially active under physiological conditions.

Recently, the integration of ChIP-seq or DAP-seq with RNA-seq (transcriptome profiling of mutant lines) has enabled the simultaneous identification of genome-wide TFBS and differentially expressed genes under TF overexpression (OE) or knockout (KO) conditions. This combined approach provides a more direct link between TF binding and transcriptional regulation by correlating binding events with gene expression changes (Chung *et al.*, 2018).

Besides TF-centered approaches that start with a TF to identify its target genes, gene-centered methods have also been developed, such as Yeast one-hybrid (Y1H) assays, to discover TFs that bind to specific genes. In Y1H, a DNA fragment of interest is cloned upstream of a reporter gene and introduced into yeast, where TFs that bind to the bait sequence activate reporter expression. Another widely used method is the electrophoretic mobility shift assay (EMSA), which detects protein–DNA complex formation based on the slower migration of these complexes during electrophoresis. As a semi-quantitative technique, EMSA can be further refined using competition assays or site-directed mutagenesis to assess binding specificity. Although both Y1H and EMSA are valuable for validating specific TF–DNA interactions, they are labor-intensive and cannot capture the complexity of regulatory interactions *in vivo* (Yang *et al.*, 2016).

Once TFBS have been identified within the promoter of a gene of interest, their regulatory function can be evaluated using reporter assays. In these assays, the promoter is cloned upstream of a reporter gene, such as β -glucuronidase (GUS), green fluorescent protein (GFP), or luciferase, and then introduced into plant tissues or cells using transient expression systems. The activity of the reporter is then measured in the presence and absence of the TF to directly assess its effect on promoter activity. In addition, site-directed

mutagenesis of the identified TFBS can be used to quantitatively evaluate the contribution of specific binding sites to overall promoter activity (Yang *et al.*, 2016).

The elucidation of functional interactions between TFs and their target genes can be integrated into gene regulatory networks (GRNs), which map the regulatory relationships between TFs and their target genes in a given biological context (Springer & Grotewold, 2009). Many of the aforementioned high- and low-throughput methods help experimentally delineate GRNs but are laborious and sometimes costly, motivating advances in computational GRN inference to overcome these limitations (Kulkarni & Vandepoele, 2020). Indeed, integrating chromatin accessibility, expression data and TFBS information with machine learning has emerged as a promising approach for identifying GRNs (Manosalva Pérez *et al.*, 2023). Such computational methods have been developed, for example: MEDEA (Mariani *et al.*, 2020), CellOracle (Kamimoto *et al.*, 2023), IReNA (Jiang *et al.*, 2022), and The Inferelator 3.0 (Gibbs *et al.*, 2022). In plants, MINI-AC (motif-informed network inference based on accessible chromatin) has recently been developed and successfully applied to maize (Manosalva Pérez *et al.*, 2023). This computational method integrates TF motif information with bulk or single-cell chromatin accessibility data to perform motif enrichment and GRN inference.

Regardless of how they are identified, GRNs help explain how coordinated transcriptional regulation drives precise spatiotemporal gene expression. They are often visualized as interaction graphs, where nodes represent TFs and genes, and edges indicate regulatory interactions between them. TFs with high connectivity, meaning nodes with many outgoing or incoming edges, are often considered “master regulators,” playing central roles in controlling key developmental or physiological processes, or acting as pleiotropic factors that influence multiple pathways (Springer & Grotewold, 2009). Although elucidating such networks is a complex task, it provides valuable insights into which TF may regulate specific pathways or traits. This can be exploited to modulate specific TFs through precision breeding and genome editing approaches, thereby influencing plant traits in a targeted manner with predictable effects (Springer *et al.*, 2019).

Translating regulation diversity into crop improvement

Maize was domesticated from its wild ancestor, teosinte, approximately 10,000 years ago (Matsuoka, 2005). Over the past century, intensive selection and modern breeding have produced highly diverse inbred lines capable of

thriving in regions far from its original tropical location. Due to its agronomic importance, ease of crossing, high seed production, and genetic diversity, maize has become a powerful model for studying gene regulation and function (Richardson and Hake, 2022).

The release of multiple maize genome assemblies has revealed high levels of genetic diversity, reflected in sequence polymorphisms, large-scale structural variations, and differences in gene expression levels (Hirsch *et al.*, 2016), which contribute to the phenotypic diversity and quantitative variation in traits across lines (Springer *et al.*, 2019).

Variation in gene expression among lines offers the possibility of discovering regulatory elements underlying complex traits through genome-wide association studies (GWAS). GWAS identify correlations between genetic markers, typically single-nucleotide polymorphisms (SNPs), and phenotypic traits such as disease resistance, drought tolerance, or yield (Tibbs Cortes *et al.*, 2021). Complementing GWAS, expression quantitative trait loci (eQTL) analysis links genetic variation to changes in gene expression. In eQTL studies, gene expression levels are quantified and correlated with genotypes to identify genomic regions that contribute to variation in transcript abundance within a population (Druka *et al.*, 2010). Thus, eQTL analysis can identify *trans*-acting factors, such as TFs, when variation at their loci affects their abundance or activity, influencing the expression of target genes (Springer *et al.*, 2019). It can also reveal *cis*-regulatory variation caused by sequence changes in CREs of target genes (Druka *et al.*, 2010).

Identifying the specific DNA sequence changes underlying *cis*- and *trans*-regulatory variation is key to applying this knowledge in crop improvement by selecting favorable allele combinations or creating new ones through transgenic or genome editing approaches (Springer *et al.*, 2019).

To effectively exploit regulatory variation for trait improvement, targeting *trans*-acting factors is particularly promising. Since a TF, or a small set of TFs, can coordinate multiple steps in a pathway, modulating their activity or expression often offers broader control over metabolism than altering individual CREs (Springer *et al.*, 2019). Given the knowledge of enzymes and metabolic pathways, these GRNs are likely easier to manipulate. In contrast, complex traits like yield or plant architecture pose a bigger challenge, as they lack clearly defined pathways or enzyme targets (Wallace *et al.*, 2018). Moreover, these traits are often influenced by environmental conditions, making it more challenging to identify the critical regulatory nodes and TFs involved.

Nevertheless, the growing understanding of genetic diversity across maize offers great potential for guiding breeding strategies. Whether through traditional selection or genome editing tools like CRISPR-Cas9 (Wada *et al.*, 2020), this knowledge can accelerate the development of improved crops.

Such engineered or selected traits could contribute to global food security by enabling the development of crops that are more resilient to environmental stresses, have an optimized plant architecture for increased yield, or are adapted to new growing conditions (Wallace *et al.*, 2018).

Box 2: Transposable elements in maize

Transposable elements (TEs), also known as "jumping genes," are DNA sequences capable of moving to different positions within the genome. In maize, TEs account for approximately 85% of the genome and belong to diverse families (Schnable *et al.*, 2009). Their mobility allows them to influence genomic architecture, gene expression, and genome evolution.

TE insertions can cause variations in gene expression, with effects that can range from subtle to severe. Insertions in introns or UTRs may alter splicing by disrupting existing splice sites or creating new ones, though with generally mild effects. In contrast, insertions in exons often result in aberrant transcripts or loss-of-function alleles (Hirsch & Springer, 2017). TEs inserted upstream of genes can act as new promoters or TSSs, leading to changes in expression patterns or alternate transcripts. They may also disrupt native CREs or introduce new ones, potentially acting as enhancers or modifying existing enhancer activity. Additionally, TEs can influence gene expression by altering nearby chromatin modifications (Hirsch & Springer, 2017). Altogether, TEs play an active role in shaping gene regulation in maize, far beyond their role as passive genomic elements or simple insertional mutagens. The unique properties of TEs, especially the *Mutator* (*Mu*) system, a class of DNA TEs, provide powerful tools for altering gene expression, creating knockouts or knockdowns, and enabling functional analysis. *Mu* elements can insert into coding or regulatory regions, disrupting gene function. This has led to the creation of large, sequence-indexed insertion libraries such as Uniform*Mu* (McCarty *et al.*, 2013), China*Mu* (Liang *et al.*, 2019), and Bonn*Mu* (Marcon *et al.*, 2020). These resources support forward genetics and functional genomics by linking mutations to phenotypes. Freely accessible through platforms like MaizeGDB.com, *Mu*-tagged mutant libraries cover a large portion of the maize genome, making them invaluable for gene function studies in maize.

Although progress has been made in mapping transcriptional diversity in maize and linking it to phenotypic variation, integrating the full range of regulatory layers into coherent GRNs remains a major challenge. A step toward this goal is to dissect the transcriptional regulation of specific genes of interest. In the host lab, aquaporins (AQPs), membrane proteins involved in the diffusion of water and small solutes, are our main research focus. While

their expression profiles are well characterized in maize, the underlying regulatory mechanisms controlling their transcription remain largely unknown. This project arose from an initiative to identify both CREs and *trans*-acting factors that control *AQP* gene expression. By leveraging the natural genetic diversity in maize through eQTL mapping, regulatory components involved in *AQP* transcription were uncovered by Laurie Maistriaux and Maxime Laurent, two previous PhD fellows.

Aquaporins

AQPs are integral proteins that facilitate the passive diffusion of water and other small solutes through biological membranes in most living organisms, including plants, fungi, bacteria, and animals. Notably, plants display a much greater diversity of AQP isoforms compared to other organisms, highlighting their crucial roles in adapting to a sessile lifestyle (Laloux *et al.*, 2018). Indeed, AQP-mediated water transport in plants is essential for key physiological processes such as transpiration, cell elongation, organ development, seed germination, and osmoregulation (Chaumont and Tyerman, 2014; Maurel *et al.*, 2015).

Beyond water, some AQPs also facilitate the diffusion of a wide range of small solutes such as hydrogen peroxide, glycerol, urea, and ammonia (Gomes *et al.*, 2009; Laloux *et al.*, 2018; Ahmed and Chaumont, 2023). Given their diversity and importance in plants, understanding their activity, function, and regulatory mechanisms is essential.

Structural features of aquaporins

AQPs belong to the membrane intrinsic protein (MIP) family, which is a broader term, as not all members function as water channels. However, “aquaporin” is now widely used and will be adopted throughout this manuscript.

Plant AQPs are classified into seven subfamilies based on sequence similarity, which somehow correlate with their subcellular localization. Five of these are found in seed plants: plasma membrane intrinsic proteins (PIPs); tonoplast intrinsic proteins (TIPs); Nodulin26-like intrinsic proteins (NIPs), initially found in legume symbiosomes, but also present in the plasma membrane and the endoplasmic reticulum (ER); small basic intrinsic proteins (SIPs), localized in the ER; and X intrinsic proteins (XIPs), found in the plasma membrane (Chaumont & Tyerman, 2014). The remaining two, hybrid intrinsic proteins (HIPs) and GLpF-like intrinsic proteins (GIPs), are exclusive to early land plants such as mosses (Abascal *et al.*, 2014).

Plant AQPs are named according to their subfamily, followed by two numbers indicating their phylogenetic classification and position within the group (e.g., PIP2;5).

The AQP monomers, ranging in mass from 21 to 34 kDa, share a relatively conserved structure of six transmembrane α -helices (TM1–TM6), connected by five loops (A–E), forming a channel across the membrane (Figure 3A) (Murata *et al.*, 2000). In addition, two short α -helices from loops B and E (HB and HE) dip into the membrane from opposite sides.

The AQP pore contains two key selectivity filters: the highly conserved NPA (Asn-Pro-Ala) motif and the aromatic/arginine (ar/R) constriction (Gomes *et al.*, 2009). The NPA motifs, located in loops HB and HE, create a constriction point at the center of the pore, playing a crucial role in substrate selectivity by excluding certain molecules based on charge, polarity, and size (Sui *et al.*, 2001; Jensen *et al.*, 2003). The presence of the second filter, the ar/R constriction, at the extracellular portion of the pore prevents the passage of high molecular weight substrates. This ar/R region promotes single file transport of water molecules by creating the narrowest portion of the pore (1,5 to 2 Å) (Beitz *et al.*, 2006).

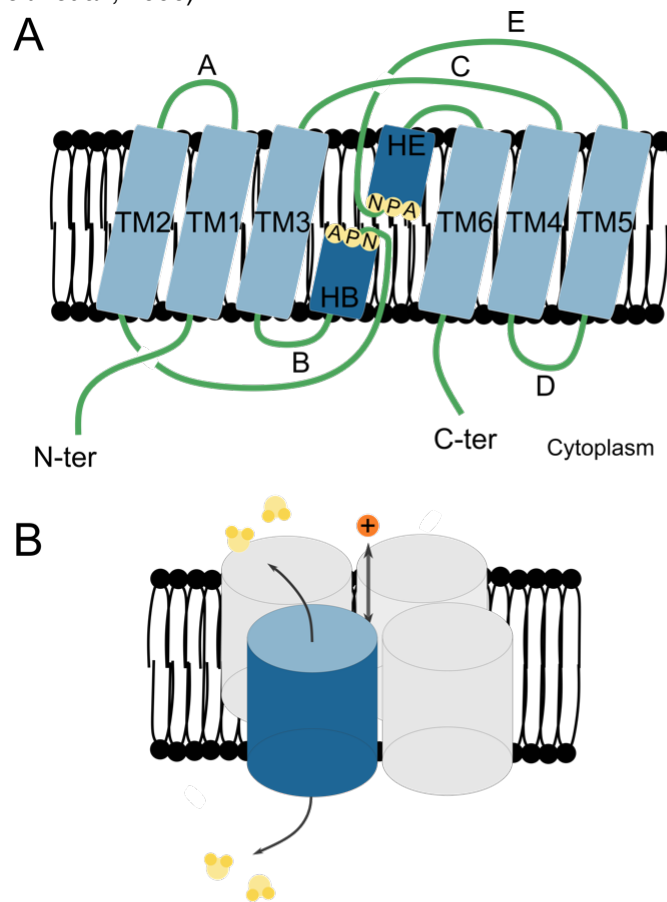


Figure 3: Representation of the aquaporin structure.

(A) AQP monomer composed of six transmembrane α -helices (TM 1–6), five connecting loops (A–E), and cytosolic N- and C-termini. Short helices HB and HE, containing the conserved NPA motifs (highlighted in yellow), meet at the center, forming a narrow constriction (adapted from Hachez *et al.*, 2006). (B) Tetrameric assembly with one monomer in blue. Water molecules (yellow spheres) move through individual monomer pores. A central fifth pore is proposed to allow diffusion of compounds such as CO_2 or cations (orange sphere).

AQPs assemble into homo- or heterotetramers stabilized by hydrogen bonds and loop interactions (Fetter *et al.*, 2004; Berny *et al.*, 2016; Jozefkiewicz *et al.*, 2017), although each monomer functions as an independent water channel (Figure 3B) (Jozefkiewicz *et al.*, 2017). It has been proposed that the tetrameric structure of AQPs forms a fifth central pore, potentially enabling the diffusion of compounds like CO₂ or cations (Yool and Weinstein, 2002). Unlike the monomer pore, which is hydrophilic and charge-repellent, the central pore is lined with hydrophobic residues, making it more suitable for this type of transport (Wang & Tajkhorshid, 2007).

Regulation of AQP channels by gating allows rapid changes in water membrane permeability. This process involves dynamic interactions between the N- and C-termini and cytosolic loops B and D, which can occlude the pore. Gating is influenced by the phosphorylation of specific residues, changes in cytosolic pH and protonation, and the presence of divalent cations, all of which altering protein conformation and channel accessibility (Törnroth-Horsefield *et al.*, 2006).

Physiological roles of aquaporins

Plants are autotrophic organisms that rely on light, carbon, water, and mineral nutrients to survive and complete their life cycle. Being sessile, they cannot escape unfavorable conditions and must efficiently absorb water and nutrients from their immediate environment. To cope with dynamic and harsh environmental conditions, such as drought, flooding, and pathogen attacks, plants constantly adjust their metabolism, growth, and development. These adaptive responses are highly dependent on the plant water status. AQPs, with their water channel activity, play a critical role in maintaining this balance and are therefore crucial in cellular and physiological processes that help plants adapt to their environment (Maurel *et al.*, 2015).

Water transport and transpiration

Plants constantly mediate a balance between gas exchange and water loss in their aerial parts. While absorbing CO₂, plants simultaneously lose water via evapotranspiration through the stomata (Steudle, 2001). This water loss is compensated by a continuous upward flow of water, from the soil to the leaf stomata where it evaporates. This long-distance water transport occurs mainly through the xylem vessels, which are composed of dead, specialized cells designed to conduct water efficiently (Steudle & Peterson, 1998). However,

the movement of water from the soil to the xylem vessels in roots and from the xylem to the stomata in leaves also requires crossing living tissues. According to the composite transport model (Steudle, 1997), water moves through plant tissues via three pathways: the apoplastic route (through cell walls and intercellular spaces), the symplastic route (cell-to-cell via plasmodesmata), and the transcellular route (across cell membranes). The latter pathway is mediated by AQPs, especially PIPs, which regulate the plasma membrane permeability to water (Steudle & Peterson, 1998).

Many AQPs, specifically PIPs and TIPs, are known to be highly expressed in roots (Sakurai *et al.*, 2005; Hachez *et al.*, 2006) supporting their role in root water transport. Although their contribution varies depending on the species and environmental conditions, experiments using AQP blockers, such as mercury, have shown that they can account for up to 70% of root water permeability measured as root hydraulic conductivity (L_{pr}) (Maggio & Joly, 1995; Javot & Maurel, 2002).

Gene expression patterns also provide insight into the role of water channels in root water uptake. In species such as Arabidopsis, rice, and maize, studies showed that PIP and TIP isoforms have distinct, cell-specific expression patterns in roots, suggesting a finely tuned regulation of water movement across different root cell layers (Javot *et al.*, 2003; Hachez *et al.*, 2006 and 2012; Sakurai *et al.*, 2008). In particular, some isoforms are predominantly expressed in barrier-forming tissues such as the endodermis and exodermis (Hachez *et al.*, 2006 and 2012). In these two cell layers, lignin deposition at Casparian strips and subsequent suberization of the apoplast create apoplastic barriers (Schreiber *et al.*, 2005), restricting water and solute flow and forcing water into the cellular pathways. The presence of such barriers explains the strong dependence of L_{pr} on PIP activity.

Reverse genetics has since confirmed the role of specific PIP isoforms in root water transport. For example, Arabidopsis mutants lacking the *AtPIP2;2* gene, expressed in different root cells, including the endodermis, showed a 14% reduction in L_{pr} compared to wild-type (WT) plants (Javot *et al.*, 2003). While reductions in L_{pr} from single gene KOs are generally modest, genetic redundancy among closely related isoforms must be considered (Maurel, 2015).

While the importance of AQPs in root water uptake has long been established, their physiological significance in leaves has also been recognized. AQPs are expressed in various leaf cell types (Hachez *et al.*, 2008; Sakurai *et al.*, 2008), suggesting roles beyond maintaining leaf hydraulic conductivity, including leaf

elongation (see 'cell elongation and development'), embolism recovery (Zwieniecki & Secchi, 2017), and stomatal regulation (Ding *et al.*, 2024). In cereals, *AQP* expression was observed in the elongation zones, in vascular bundles, especially bundle sheath cells, and to a lesser extent in the mesophyll (Hachez *et al.*, 2008; Sakurai *et al.*, 2008; Besse *et al.*, 2011). As water exits the vasculature, it must pass through the bundle sheath before reaching the mesophyll. These tightly packed cells, sometimes reinforced with lignin or suberin, act as barriers that limit apoplastic flow, similar to the root endodermis. In such cases, all water and solutes must pass through the cellular pathway, with AQPs likely facilitating and regulating water flow through these restrictive layers (Heinen *et al.*, 2009).

Stomatal aperture is a highly dynamic process, controlled by reversible changes in guard cell volume, and essential for regulating plant transpiration and gas exchange. In response to local or global signals, stomata open and close rapidly through osmosis-driven water movement between guard cells and surrounding tissues. Expression of PIPs and TIPs in guard cells has been documented in several plant species, including sunflower (Sarda *et al.*, 1997), broad bean (Sun *et al.*, 2001), and maize (Hachez *et al.*, 2008, Ding *et al.*, 2022 and 2024), supporting their involvement in stomatal gating. Furthermore, the multifunctionality of PIPs, as channels for water, CO₂, and H₂O₂, positions them as key regulators of stomatal dynamics (Ding *et al.*, 2024).

Cell elongation and development

Plants show continuous apical growth in both roots and shoots, driven by active cell division in meristems followed by cell expansion. This expansion relies on cell turgor, which provides the necessary force. Consequently, plant growth and development are closely linked to water status, with PIPs and TIPs likely contributing to transcellular water movement into expanding tissues (Fricke & Knipfer, 2017).

Enriched localization of PIPs and TIPs in the root elongation zone and root tip (Hachez *et al.*, 2006; Sakurai *et al.*, 2008; Knipfer *et al.*, 2011), along with reduced maize root growth following mercury treatment (Hukin *et al.*, 2002), supports their role in root growth. Transgenic studies confirmed these results, as *Arabidopsis* plants overexpressing the jojoba *ScPIP1* gene developed longer roots and showed improved growth compared to WT plants (Wang *et al.*, 2019). Similarly, in leaves, PIPs and TIPs are expressed in elongation zones, and their role in growth was directly demonstrated by the OE of *NtAQP1* in tobacco that enhanced leaf growth rates (Uehlein *et al.*, 2003). Conversely, antisense expression of *BnPIP1* in transgenic tobacco resulted in

deformed leaves (Yu *et al.*, 2005), providing evidence that both AQPs are involved in proper leaf development.

Beyond overall plant growth, other specific processes shaping plant morphogenesis and development depend on AQP-mediated water movements. For instance, lateral root formation and emergence was shown to depend on the precise spatiotemporal regulation of PIP and TIP expression, coordinated by auxin signaling (Péret *et al.*, 2012; Reinhardt *et al.*, 2016). This established a link between root water transport capacity and the ability of plants to grow and adapt their root architecture in response to water availability.

Flowers depend on finely regulated cell expansion for their blooming and diurnal movements. Accordingly, PIP1s and PIP2s were shown to play a role during ethylene-dependent expansion of rose petals (Chen *et al.*, 2013; Pei *et al.*, 2013). AQPs also play essential roles in the reproductive functions of flowers. For example, anther dehydration, required for dehiscence and pollen release, is impaired in tobacco plants with reduced PIP2 expression (Bots *et al.*, 2005). AQPs are also involved in seed and fruit development, processes that require rapid cell division and enlargement driven by water uptake. They also support embryonic cell elongation and early seedling growth. Although these roles are not detailed here, they are thoroughly reviewed in Wang *et al.* (2020).

Abiotic stress

Plants must adapt to changing conditions such as temperature fluctuations, water availability, nutrient limitations, and toxic environments. Most of these stresses lead, directly or indirectly, to water shortages. Therefore, AQPs play an important role in plant responses to these challenges. Studies on drought, salinity, and cold stress emphasized changes in PIP and TIP regulation and gene expression, while transgenic approaches were used to evaluate the contribution of specific isoforms to stress tolerance.

Although most stresses lead to reduced L_p , a universal expression pattern for the AQP genes cannot be observed, as these can be up- or downregulated depending on the isoform, organ or stress (Fox *et al.*, 2017).

While most PIP genes were downregulated in Arabidopsis leaves under drought stress, *AtPIP1;4* and *AtPIP2;5* were upregulated (Alexandersson *et al.*, 2005). Similarly, many PIP genes were downregulated during dehydration in tobacco (Mahdiah *et al.*, 2008). Conversely, drought stress led to the upregulation of certain PIP isoforms in rice (Guo *et al.*, 2006). These patterns reflect diverse hydraulic strategies in response to water deficit: early increases

in root hydraulic conductivity may enhance water uptake, while long-term inhibition can conserve water and prevent reverse flow into dry soil. Altogether, the specific expression and regulation of each AQP isoform allows plants to finely tune water balance, maintaining critical functions while minimizing losses (Maurel, 2015).

Salt stress typically triggers an initial downregulation of *AQP* expression, as observed in *Arabidopsis* roots following NaCl treatment (Boursiac *et al.*, 2005). In later stages, expression of stress-responsive *AQP* isoforms may recover or even increase to facilitate water uptake and cellular recovery (Banerjee & Roychoudhury, 2020).

Cold stress also leads to a reduction in L_{pr} , mainly due to a decrease in *AQP* expression. However, in some cases, cold triggers an upregulation of PIP isoforms. For example, in *Arabidopsis*, cold treatment upregulated specific isoforms, such as *AtPIP2;5*, *AtPIP1;4*, and *AtPIP2;6* (Jang *et al.*, 2004).

Transgenic approaches targeting AQP expression and function to improve stress tolerance produced contrasting effects. Ectopic expression of an AQP in a heterologous plant species can be tricky, as the precise regulatory mechanisms controlling isoform expression in the native plant may not function properly in a heterologous system (Hachez *et al.*, 2006b). Nevertheless, several successful examples exist. The OE of a *Vicia faba* PIP1 or a wheat PIP2 in *Arabidopsis* and tobacco, respectively, improved drought resistance (Cui *et al.*, 2008; Huang *et al.*, 2014). Similarly, transgenic *Arabidopsis* lines expressing PIP1 from *Panax ginseng* (Peng *et al.*, 2007), TIP1;2 from *Thellungiella salsuginea* (Wang *et al.*, 2014), and TIP2;2 from *Solanum lycopersicum* (Sade *et al.*, 2009) showed increased drought tolerance, reduced water loss, and better survival rates. Alongside these successes, there are also mitigated reports. For example, tobacco plants OE *Arabidopsis AtPIP1;2* showed improved growth under normal conditions, but became highly sensitive to drought, likely due to impaired stomatal regulation (Aharon *et al.*, 2003). Similarly, OE of *Galega orientalis* PIP1 in *Arabidopsis* increased drought sensitivity (Li *et al.*, 2015), and constitutive expression of barley PIP2;1 in rice reduced salt stress tolerance (Katsuhara *et al.*, 2003). Identifying stress-responsive AQP isoforms and modifying their expression within the same species appears to be a more reliable strategy. For example, constitutive expression of SITIP2;2 in tomato improved growth under both well-watered and drought conditions (Sade *et al.*, 2009). Similarly, OE of VvPIP2;4 in grapevine increased L_{pr} , transpiration and shoot growth under normal conditions, while maintaining typical water-saving responses during stress (Vitali *et al.*, 2016).

While AQPs are vital for many stress responses, conventional transgenic approaches relying on constitutive expression often produce inconsistent results. This emphasizes the need for deeper knowledge on regulatory mechanisms and a better understanding of possible compensation mechanisms by close homologues.

Regulation of aquaporin gene expression

The movement of water across cell membranes via AQPs is generally regulated through three main mechanisms: their expression levels, their trafficking through the secretory pathway (from the ER to the Golgi apparatus and vesicles, ultimately reaching specific cellular membranes), and their gating. Although gene regulation is the focus here, trafficking and gating mechanisms are also crucial and are discussed extensively in Chaumont & Tyerman (2014) and summarized in Figure 4.

In plants, *AQP* gene expression is subfamily- and isoform-specific expression profiles. Their precise spatiotemporal expression across tissues and developmental stages has been documented in many species, with specific isoforms localized to distinct cell types such as the exodermis and endodermis in roots or suberized bundle sheath cells in leaves, as previously discussed. *AQP* gene expression display changes, as shown in Arabidopsis and maize roots, where *PIP* transcripts peak shortly after dawn and decline toward dusk, in line with photosynthesis and transpiration cycles (Lopez *et al.*, 2003; Takase *et al.*, 2011; Caldeira *et al.*, 2014). Similar diurnal patterns have been reported in leaves (Hachez *et al.*, 2008, 2012) and stomatal complexes (Heinen *et al.*, 2014). This temporal regulation of AQPs ensures efficient water transport and hydraulic coordination throughout the day.

AQP gene expression is regulated by various plant hormones. As the main “stress hormone,” abscisic acid (ABA) influences *AQP* expression in a complex, isoform-dependent manner. In Arabidopsis, exogenous ABA treatment upregulated six of the 13 *PIP* genes in roots, while *AtPIP1;5* was notably downregulated (Jang *et al.*, 2004). In contrast, some rice isoforms such as *OsPIP1;3* and *OsPIP2;1*, though induced by drought, did not respond to ABA, suggesting the involvement of alternative regulatory pathways (Lian *et al.*, 2006). These variable responses suggest that *AQP* regulation involves both ABA-dependent and ABA-independent signaling pathways (Jang *et al.*, 2004; Alexandersson *et al.*, 2005).

Beyond ABA, several other hormones modulate *AQP* gene expression. Auxin tightly regulated *PIP* and *TIP* expression during lateral root development

(Peret *et al.*, 2012; Reinhardt *et al.*, 2016) and fruit ripening (Mut *et al.*, 2008). Gibberellin treatment increased *TIP* expression, partially rescuing the dwarf phenotype of the *ga1* mutant in Arabidopsis (Phillips & Huttly, 1994). Salicylic acid (SA) downregulated *PIP* expression (Boursiac *et al.*, 2008), whereas cytokinins promoted kiwifruit expansion by upregulating *AcPIP2;4* (Nardozza *et al.*, 2017). Ethylene also influenced *AQP* expression, notably regulating *PIP* expression in rose petals (Chen *et al.*, 2013; Pei *et al.*, 2013).

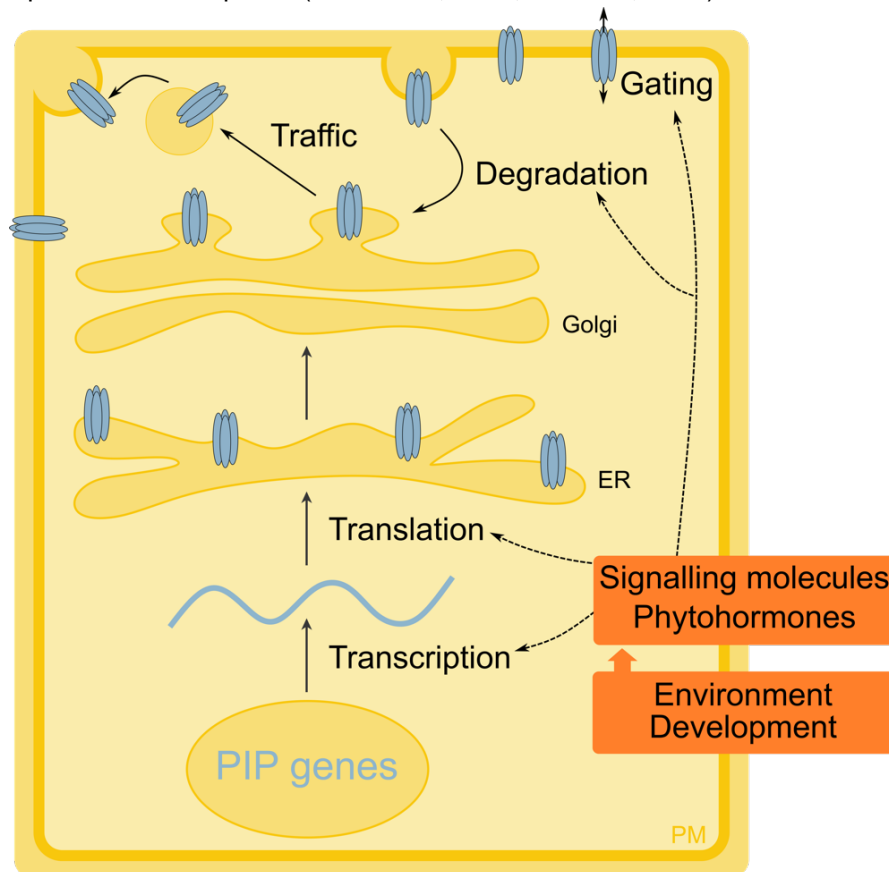


Figure 4: Schematic overview of the processes regulating PIP aquaporins. PIP genes are transcribed, and their mRNA is translated in the ER, where the proteins are synthesized. PIP tetramers transit through the Golgi apparatus, are packaged into secretory vesicles, and delivered to the plasma membrane (PM). Once at the membrane, PIPs undergo constitutive recycling through endocytosis. Internalized PIPs are either routed back to the PM via the Golgi network or directed to lytic vacuoles for degradation.

PIP gating is regulated by various mechanisms including, phosphorylation, changes in cytosolic pH and protonation, all of which alter protein conformation and channel accessibility. Overall, the transcription, translation, trafficking, and gating of PIPs are finely controlled by environmental and developmental signals, including signaling molecules, phytohormones, and the circadian clock. Adapted from Chaumont & Tyerman (2014).

Numerous studies have shown that environmental, developmental, and hormonal signals influence plant water relations by regulating specific *AQP* genes. However, the next step is to go beyond individual gene responses and identify the TFs that serve as regulators of *AQP* gene expression.

Direct and indirect regulators

AQP genes are potential downstream targets of various TFs, with numerous indirect links suggesting possible regulatory interactions. For example, analyses of promoters and *AQP* gene co-expression networks in *Vitis vinifera* revealed an enrichment of CREs associated with TF families such as AP2/ERF, DREs, MYB, and NACs (Wong *et al.*, 2018).

In Arabidopsis, transcriptome analysis of the *pip1* mutant showed significant differential expression of 150 TF genes compared to WT plants. Many of these TFs were related to developmental processes in leaves, flowers, and seeds. In particular, the expression of seven *AP2/ERF* genes was systematically upregulated in the mutant. In addition, four *NAC* genes, which were associated with leaf senescence as well as cell division and expansion, were also upregulated (Wang *et al.*, 2021). OE of *ThNAC7* from the halophyte *Tamarix hispida* resulted in the upregulation of five *AQP* genes in Arabidopsis (He *et al.*, 2019). Similarly, in tomato, OE of *SIDREB2* enhanced salt tolerance and significantly increased the expression of *SIP1;2* and *SITIP2-1-like* (Hichri *et al.*, 2016).

However, these associations should be viewed with caution, as they likely represent complex, interconnected response networks rather than direct regulatory mechanisms. However, some recent studies demonstrated direct TF binding to *AQP* gene promoters, correlating with regulatory changes *in vivo*. A summary of these known direct regulators is presented in Table 1.

Most TFs known to directly regulate *AQP* expression belong to the large AP2/ERF (APETALA2/Ethylene-Responsive Factor) family. The first identified direct regulator was TRANSLUCENT GREEN (TG), which activated specific *PIP* and *TIP* genes (Zhu *et al.*, 2014). Members of the DREB (Dehydration-Responsive Element Binding) subfamily also bound directly to DRE motifs in *AQP* promoters, playing a key role in drought stress responses. Similarly, ABIs (ABSCISIC ACID INSENSITIVE), involved in ABA signaling, regulated *AQPs* through ABA-responsive elements. In addition to AP2/ERFs, TFs from the ARF (Auxin Response Factor) family, which interact with Auxin Responsive Elements, and the plant-specific NAC family, also regulated directly *AQP* gene expression.

Table 1: Summary of confirmed TFs directly regulating AQP gene expression. This non-exhaustive list highlights selected examples to illustrate the diversity of TFs controlling AQP gene expression, organized by plant species. (Y1H = yeast one-hybrid; DLR = dual-luciferase reporter assay; GUS = β -glucuronidase reporter assay).

Aquaporin	TF	Physiological relevance	Methods	Source
AtPIP2;8 (<i>Arabidopsis thaliana</i>)	AtPLATZ4	AtPLATZ4 enhances drought tolerance and ABA sensitivity by directly binding the <i>AtPIP2;8</i> promoter suppressing its expression.	Y1H DLR ChIP EMSA	Liu <i>et al.</i> , 2023
AtTIP3;1 AtTIP3;2	AtABI3	AtABI3 binds and activates <i>AtTIP3;1</i> and <i>AtTIP3;2</i> promoters in the presence of ABA, linking TIP3s to seed longevity via the ABI3 pathway.	DLR EMSA qPCR	Mao & Sun, 2015
AtPIP2;2 AtTIP1;1 AtTIP2;3	Translucent Green (TG)	TG induces directly <i>AtTIP1;1</i> , <i>AtTIP2;3</i> , and <i>AtPIP2;2</i> , to control water balance	Micro-array GUS	Zhu <i>et al.</i> , 2014
FvPIP2;1-like (<i>Fragaria vesca</i>)	OsDRE1BI (<i>Oryza sativa</i>)	Rice <i>OsDREB1B</i> expression in strawberry improved cold/drought tolerance and upregulated four PIPs with <i>FvPIP2;1-like</i> as direct target.	RNA-seq Y1H	Gu <i>et al.</i> , 2017
MaPIP1;1 (<i>Musa acuminata</i>)	MaERF1/14 MaDREB1 MaMADS3	All cited TFs bind the <i>MaPIP1;1</i> promoter, contributing to salt and cold tolerance in banana.	Y1H DLR qPCR	Xu <i>et al.</i> , 2021
MsPIP1;3 Msγ-TIP (<i>Malus sieversii</i>)	MsDREB6.2	In apple tree, MsDREB6.2 enhances drought tolerance by directly activating <i>MdPIP1;3</i> and <i>Mdy-TIP</i> , increasing root water conductance.	qPCR EMSA	Liao <i>et al.</i> , 2017
PeTIP4;3 (<i>Phyllostachys edulis</i>)	PeMYB99 PeSAPK4	PeMYB99 and PeSAPK4 interact to directly activate <i>PeTIP4;3</i> enhancing water and salt tolerance in bamboo.	Y1H EMSA GUS	Zhu <i>et al.</i> , 2024
SIPIP1;4 (<i>Solanum lycopersicum</i>)	SIASR1	SIASR1 binds the promoter of <i>SIPIP1;4</i> to induce its expression in response to drought stress.	ChIP-seq qPCR	Ricardi <i>et al.</i> , 2014
SITIP1;1	SIERF52	SIERF52 directly binds the <i>SITIP1;1</i> promoter, enhancing H ₂ O ₂ and water influx.	EMSA Y1H GUS	Wang <i>et al.</i> , 2021
SIPIP2;4 SINIP-type like	SIARF10	SIARF10 positively regulates <i>SIPIP2;4</i> and <i>SINIP-like</i> ; its silencing reduces AQP expression and water loss.	qPCR GUS EMSA	Liu <i>et al.</i> , 2016
SITIP1;1-like	SIAB15-like	SIAB15-like binds the <i>SITIP1-1-like</i> promoter to upregulate its expression.	RNA-seq EMSA GUS	Liu <i>et al.</i> , 2016
RhPIP1;1 RhPIP2;2	RhNAC100	Ethylene-induced RhNAC100 binds <i>RhPIP1;1</i> and <i>RhPIP2;2</i>	qPCR EMSA	Pei <i>et al.</i> , 2013

(<i>Rosa hybrida</i>)		promoters, affecting rose petal cell expansion.		
ThPIP2;5 (<i>Tamarix hispida</i>)	ThNAC12	ThNAC12 directly activates <i>ThPIP2;5</i> , enhancing salt tolerance by regulating ROS scavenging.	Y1H ChIP DLR	Wang <i>et al.</i> , 2021

Maize PIP aquaporins: a case study of *PIP2;5*

This section focuses on data related to maize AQPs, with a particular emphasis on the ZmPIP2;5 isoform. ZmPIP2;5 has already been extensively characterized and serves as an ideal case study for exploring the roles and regulation of aquaporin in maize. Throughout the rest of this manuscript, the notation “PIPX;X” should be understood as referring to “ZmPIPX;X.”

Chaumont *et al.* (2001) identified at least 35 maize AQPs by screening expressed sequence tags (ESTs) libraries. Phylogenetic analysis classified these AQPs into four subfamilies: PIPs, TIPs, NIPs, and SIPs and established the nomenclature still in use today. More recently, a genome-wide *in silico* analysis by Su *et al.* (2022) and Maxime Laurent in the host laboratory (unpublished data) identified 41 and 43 complete and unique sequences, respectively.

In addition, PIP subfamily is divided into two groups PIP1 and PIP2 (Chaumont *et al.* 2001). Compared to PIP1 proteins, PIP2s have a shorter N-terminal extension and a longer C-terminal end containing putative phosphorylation sites (Chaumont *et al.*, 2000, 2001). Maize PIP1 proteins expressed in *Xenopus laevis* exhibit no water channel activity, while all PIP2 proteins show a high activity (Chaumont *et al.* 2000). Indeed, when expressed alone in *X. laevis* oocytes or plant protoplasts, PIP1s are retained in the ER, whereas PIP2s are correctly targeted to the plasma membrane (Fetter *et al.*, 2004; Zelazny *et al.*, 2007). When coexpressed, PIP1s and PIP2s form heterotetramers, allowing PIP1s to be shuttled to the plasma membrane. Notably, coexpressing PIP1;2 with PIP2;5, which contains an N-terminal diacidic motif required for plasma membrane trafficking (Zelazny *et al.*, 2009), significantly increases oocyte membrane water permeability compared to the expression of PIP2;5 alone (Fetter *et al.*, 2004). This synergistic effect was observed in other PIP1-PIP2 pairs across species (Bienert *et al.*, 2018).

Hachez *et al.* (2006, 2008) conducted comprehensive analyses of *PIP* gene expression in maize primary roots and leaves. With a few exceptions, expression of most *PIP* genes was detected in both organs, to different extents. Their expression was developmentally regulated, changed along the longitudinal axis, and exhibited diurnal variation, with a peak 2 to 4 hours after

light onset (Lopez *et al.*, 2003; Hachez *et al.*, 2006, 2008 and 2012). *PIP2;5* showed a contrasting expression pattern with high expression in roots and low expression in leaves. It was the most highly expressed PIP isoform in the primary roots, with transcript and protein abundances peaking in the mature zone, above the root hair zone, where apoplastic barriers are present and water uptake is maximum. This suggests a main role in water uptake and radial movement rather than in cell elongation, as its expression was weaker in the elongation zone (Hachez *et al.*, 2006). *In situ* immunolocalization in root tip epidermal cells revealed a polarized signal in the plasma membrane side facing the external medium, supporting its function in root water uptake (Hachez *et al.*, 2006). Additionally, *PIP2;5* showed high expression in cell types with Casparian strips, where water must enter the symplast to continue to the xylem vessels (Hachez *et al.*, 2012; Ding *et al.*, 2020).

This role was corroborated by *pip2;5* KO plants showing reduced hydraulic conductivity at both the cortex cell and whole-root levels (Ding *et al.*, 2020). In contrast, constitutive OE of *PIP2;5* increased hydraulic conductivity in cortex cells but not at the whole-root level. This apparent paradox was explained by the MECHA (Model of Explicit Cross-section Hydraulic Anatomy) hydraulic model, which computes radial water flow in root sections (Couvreur *et al.*, 2018). MECHA revealed that plasma membrane permeability is not radially uniform and suggested that *PIP2;5* may already be saturated in cell layers with apoplastic barriers, such as the endodermis and exodermis (Ding *et al.*, 2020).

In leaves, *PIP2;5* showed lower expression than in roots and compared to other isoforms, with its maximum at the end of the elongation zone and the region where the leaf emerged from the sheath (Hachez *et al.*, 2008). OE of *PIP2;5* increased leaf hydraulic conductivity, while *pip2;5* KO lines showed no difference compared to WT (Ding *et al.*, 2020). Interestingly, the leaf elongation rate was faster in *PIP2;5* OE plants under mild water stress, but not in well-watered conditions, suggesting a role in plant growth under specific conditions (Ding *et al.*, 2020). While no other obvious growth phenotypes were observed in *PIP2;5* mutant lines in the B104 background (Ding *et al.*, 2020), *PIP2;5* mutants in the ND101 background were significantly shorter than WT (He *et al.*, 2022). Additionally, overexpressing lines had significantly larger leaves than WT, whereas *pip2;5* KO plants had smaller leaves (He *et al.*, 2022).

A recent study conducted in the host laboratory established a link between *PIP2;5* and stomatal closure. In fact, *PIP2;5* gene is expressed in guard cells during the day. Under mild water deficit, transpiration and stomatal

conductance decreased to a greater extent in *PIP2;5* OE lines than in *pip2;5* KO lines compared to WT, highlighting its role in enhancing stomatal sensitivity to water deficit (Ding *et al.*, 2022). Furthermore, ABA treatment triggered faster stomatal closure in overexpressing lines than in WT, accompanied by increased H₂O₂ accumulation in their guard cells (Ding *et al.*, 2022). Since H₂O₂ is a key factor in ABA and CO₂ signaling regulating stomatal closure (Rodrigues *et al.*, 2017), and PIP2;5 has been shown to facilitate H₂O₂ diffusion in yeast (Bienert *et al.*, 2014) and plant cells (Ahmed *et al.*, 2023). This suggests that PIP2;5 functions as a water and H₂O₂ channel involved in stomatal regulation. Additional and similar data were more recently obtained using maize plants OE PIP1;6 (Lei *et al.*, 2024).

Maize PIPs, including PIP2;5, are regulated by abiotic stress and plant hormones, such as ABA. Short term (2 to 8 hours) osmotic stress induced by PEG treatment increased *PIP* gene expression in roots, with higher protein levels observed for PIP2;1/ PIP2;2 and PIP2;5 (Hachez *et al.*, 2012). The abundance of PIP2;5 protein was also enriched under hypoxia, along with PIP2;6, PIP2;4, PIP2;1, and PIP2;2, which could potentially regulate root hair development to compensate for reduced nutrient uptake. The increase in PIP2;5 levels under hypoxia aligns with its role in H₂O₂ transport, as high H₂O₂ levels were detected at the plasma membrane under hypoxic stress in maize roots (Hofman *et al.*, 2022).

Although *PIP2;5* mRNA levels were not affected in maize lines with impaired ABA biosynthesis, its protein abundance was higher in the lines with increased ABA concentration in xylem sap and lower in silenced lines, indicating post-translational regulation (Parent *et al.*, 2009). On the other hand, global upregulation of other *PIP* genes was observed in roots due to constitutive ABA production, while lines with lower endogenous ABA production showed the opposite effect (Parent *et al.*, 2009).

Furthermore, coronatine (COR), a JA analog, specifically induced *PIP2;5*, enhancing root water uptake under drought conditions. COR also directly bound to loop E of PIP2;5, activating the channel and increasing water transport activity (He *et al.*, 2022).

Interestingly, root pruning also influenced *PIP* expression, as pruned plants exhibited increased root hydraulic conductivity and upregulation of *PIP1;1*, *PIP1;5*, *PIP2;2*, and *PIP2;5*, suggesting a compensatory response to root biomass reduction (Yan *et al.*, 2023).

The expression profiles of *PIP* genes in maize have been well documented in different organs, developmental stages, and environmental conditions. However, the signaling pathways and direct regulators controlling their

expression remain unclear. The only direct evidence of a *PIP2;5* regulator comes from a study on Pb-tolerance using gene co-expression analysis and association studies (He *et al.*, 2023). A natural A/T variation in the *PIP2;5* promoter influenced its expression and Pb tolerance in maize seedlings by altering promoter activity. This variation was located in an RGRP motif (“ATTATTTTATGAA”), where an AHL TF was found to bind. Dual-luciferase assays showed that the AHL protein negatively regulated the “A” variant (Haplotype 1) but positively regulated the “T” variant (Haplotype 2), explaining expression differences between maize lines. In addition, *PIP2;5* OE enhanced Arabidopsis growth under Pb stress, increasing root length and fresh weight, likely by maintaining water balance (He *et al.*, 2023).

A recent eQTL study conducted in our laboratory examined the variability in expression of four *PIP* genes in maize leaves across 252 hybrids, identifying multiple local and distant eQTLs (Maistriaux *et al.*, 2024). This highlighted the natural diversity of *PIP* gene expression and the potential for discovering regulatory mechanisms. A local eQTL linked to *PIP2;5* expression variation was functionally characterized, revealing that a MITE transposon insertion in the *PIP2;5* promoter region was associated with lower expression. The negative impact of this MITE-containing indel on promoter activity was confirmed through genomic sequence comparison and *in vivo* reporter assays using confocal microscopy (Maistriaux *et al.*, 2024).

A distal eQTL linked to *PIP2;5* expression was identified, containing the NAC24 TF. This is a strong candidate regulator, given the known interaction between NACs and *PIP* genes (see Table 1).

While the first two chapters provided a general overview of gene transcriptional regulation and aquaporins, this chapter offers a more detailed and exhaustive review on NAC transcription factors, the central focus of this project.

NAC transcription factors

NACs constitute one of the largest plant-specific TF families, as illustrated by the identification of ~150 different isoforms in maize (Fan *et al.*, 2014; Shiriga *et al.*, 2014) and 109 isoforms in Arabidopsis (Jensen *et al.*, 2010). Their nominative acronym stands for NAM (no apical meristem), ATAF1/2 (Arabidopsis transcription activation factor) and CUC2 (cup-shaped cotyledon), the names of the first genes initially discovered, whose mutations induced defects in the shoot apical meristem and flower formation (Souer *et al.*, 1996; Aida *et al.*, 1997). NAC TFs are involved in numerous developmental processes, including seed, embryo and flower development, lateral root formation, and leaf senescence (Puranik *et al.*, 2012; Nuruzzaman *et al.*, 2013). More recently, they have gained popularity due to their association to plant immunity and stress responses, positioning them as promising targets for breeding strategies aimed at improving stress tolerance and enhancing crop yields.

Structural organization of NAC TFs

The main shared feature of NAC TFs is the NAC domain, located at the N-terminus and composed of five conserved subdomains (A-E) (Ooka *et al.*, 2003). This domain is involved in DNA-binding activity, nuclear localization, and homo/heterodimer formation (Olsen *et al.*, 2005). Conversely, the C-terminal domain (CTD), responsible for transcriptional regulation, is longer and more variable, reflecting the various regulatory roles of different NAC isoforms (Figure 5A).

The C-terminal domain (CTD)

Compared to the NAC domain, the CTD is less amenable to structural studies, but some information is nevertheless available. Although the C-terminal is highly variable between NAC members, it generally has a high content of low-complexity amino acid repeats (Jensen *et al.*, 2010). *In silico* analyses of Arabidopsis NAC proteins revealed intrinsic disorder in their C-termini (Jensen *et al.*, 2010). This has since been confirmed by biophysical analysis for the CTDs of two barley NAC proteins, HvNAC005 and HvNAC013, which do not form fixed tertiary structures (Kjaersgaard *et al.*, 2011). No catalytic domain is

found in the C-terminal, but it contains the transcription regulatory domain (TRD), functioning either as a transcriptional activator or repressor (Jensen & Skriver, 2014). This was demonstrated through a systematic analysis of ten NAC members from different clades, where all but one of the NAC proteins depended on the CTD for transcriptional activity in a *transactivation* assay in yeast (Jensen *et al.*, 2010). Truncation of the CTD revealed that activity was dependent on short, group-specific sequence motifs such as WQ and LP motifs (Kjaersgaard *et al.*, 2011, Taoka *et al.*, 2004). CTDs were also found to interact with other proteins, even though most protein-protein interactions are attributed to the NAC domain. HvNAC013, for example, interacted with barley RADICAL-INDUCED CELL DEATH (RCD1) via the very C-terminal part of its CTD (Kjaersgaard *et al.*, 2011). Finally, some NAC proteins possess a potent α -helical transmembrane motif in their CTD and are translocated to the nucleus upon stress-induced proteolytic cleavage (Kim *et al.*, 2007). Undoubtedly, the goal is to improve our understanding of the structure of NAC CTDs, in order to gain a better understanding of these powerful NAC TRDs.

The NAC domain

The NAC TFs are named after their conserved N-terminal NAC domain, for which different crystallographic structures are available. The first reported structure, the NAC domain of the *Arabidopsis* ANAC019 (168 AA), revealed a dimeric fold primarily composed of a β -sheet (Ernst *et al.*, 2004). Later the structure was studied in interaction with oligonucleotides (Welner *et al.*, 2012). In addition, two NAC structures were obtained: the rice SNAC1 protein (Figure 5C) (Chen *et al.*, 2011) and the *Arabidopsis* ORE1 (ANAC092) protein (Chun *et al.*, 2023). The folds of the three characterized domains are very similar, providing a good representation of the NAC domain structure. The fold consists of seven antiparallel twisted β -strands (β 1-7) flanked by an α -helical element on each side (Figure 5B). The structure of the N-terminal region of the domain is poorly defined, except when involved in protein-protein interaction.

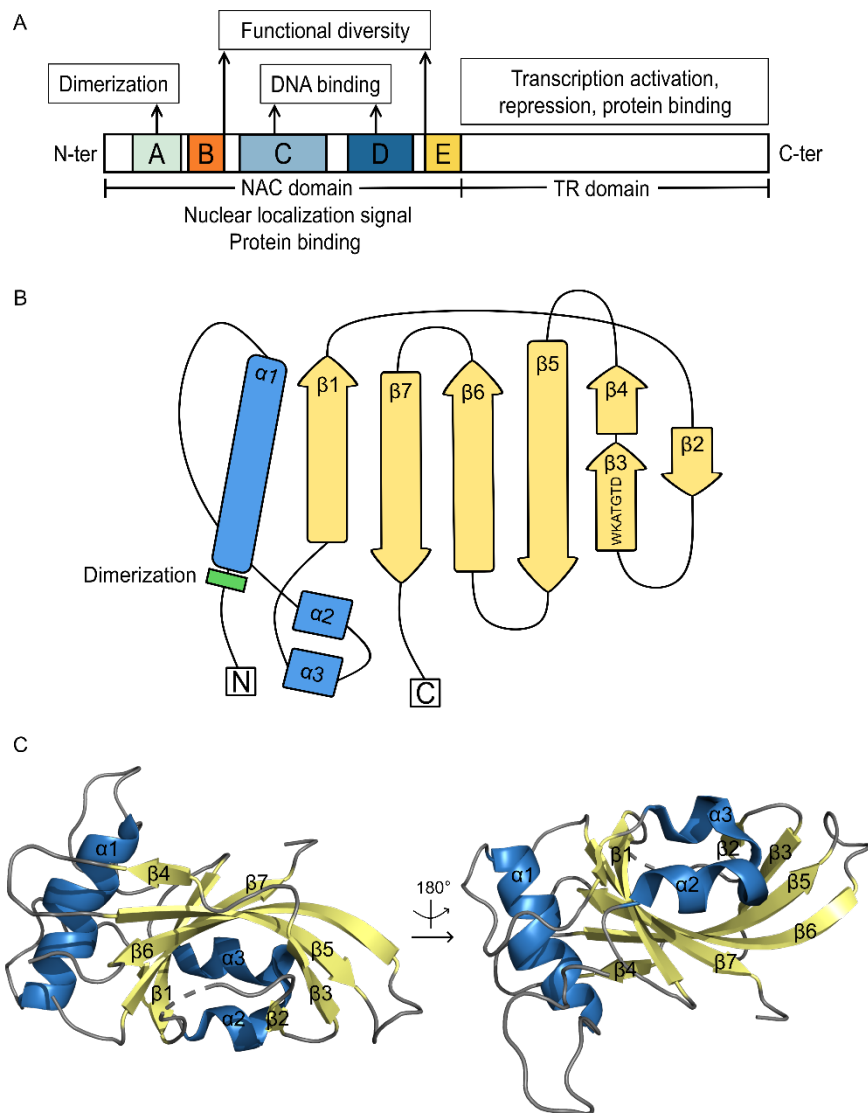


Figure 5: Structural representations of the NAC TFs.

(A) Typical structure of NAC proteins. The conserved N-terminal NAC domain, divided in five subdomains (A to E), is essential for DNA-binding, nuclear localization, and the formation of dimers. The C-terminal region consists of simple amino acid repeats and serves as a transcriptional regulatory region (TRD).

(B) Topology diagram of the NAC domain representing the seven conserved antiparallel β -sheets (yellow) flanked by three α -helices (blue) (based on the SNAC1 structure, Chen et al., 2011). The NAC dimerization domain and conserved “WKATGTD” domain are annotated.

(C) Crystal structure of the SNAC1 NAC domain (PDB: 3ULX). The domain is colored according to its secondary structure with helices and strands colored blue and yellow respectively (Figure generated with Pymol Version 2.2.5, Schrödinger).

Some structural similarities between the twisted β -sheet of NAC domains and the WRKY TFs suggest that NAC proteins evolved outside of their family (Yamasaki, 2016). The four central β -strands of the NAC domain align with the four-stranded β -sheet of the WRKY DNA-binding domain, resulting in a similar orientation (Welner *et al.*, 2012; Yamasaki *et al.*, 2008). In the WRKY-DNA complex, a β -strand, corresponding to the β 3 strand of NAC, inserts perpendicularly into the DNA major groove, similar to the DNA-binding mechanism observed in NAC proteins (Yamasaki *et al.*, 2012). The DNA-binding β 3-strand of NAC domains also contains the highly conserved “WKATGTD” sequence, similar to the “WRKYGQK” family-defining motif of WRKY TFs. The conserved glycine residue induces a curvature in the strand, allowing deep penetration into the DNA groove. The importance of this conserved glycine was demonstrated in *Arabidopsis*, where a mutant encoding a defective SOG1 (suppressor of gamma response) (NAC008) with a glycine-to-arginine substitution exhibited pleiotropic phenotypes and abnormal genomic stress responses (Yoshiyama *et al.*, 2014).

In a broader evolutionary context, NAC and WRKY TFs have been linked to the mammalian glial cells missing (GCM) TFs. GCM proteins share the same core fold and conserved glycine in their DNA-binding region, allowing them to bind the DNA in a similar way to NAC and WRKY. However, there are notable differences: WRKY and GCM contain a Zn-binding motif, which is absent in NAC proteins, and while NAC domains can dimerize, WRKY and GCM function as monomers. Despite these differences, the structural similarities and common DNA-binding mechanisms suggest an evolutionary connection between NAC, WRKY, and GCM (Welner *et al.*, 2016).

The NAC dimer

The three different crystal structures were found to form functional homodimers, capable of binding DNA (Chun *et al.*, 2022). The dimer interface, formed by the LPPGFRFYPTDEEL sequence, is highly conserved among members of the NAC family (Ooka *et al.*, 2003), with the arginine residue being conserved in 81% of them (Jensen *et al.*, 2010). Among the dimer interactions, two salt bridges form between the arginine residue of one monomer and the glutamic acid of the other. In addition, many hydrophobic interactions also occur at the monomer–monomer interface, mainly involving residues within this conserved N-terminal block (Olsen *et al.*, 2005).

Dimerization is necessary for high affinity *in vitro* binding to DNA-, as the disruption of the dimerization interface led to decreased affinity for DNA, as shown for both ANAC019 and TaNAC69 (Olsen *et al.*, 2005; Xue *et al.*, 2006). Chun *et al.* (2022) showed that the dimer interface of ORE1 is flexible around

the dimerization arm, allowing for different dimer conformations. The flexibility of ORE1 was tested using DNA-binding assays using palindromic oligonucleotides of NAC binding sites (NACBS) with different linker lengths. ORE1 showed the highest affinity for the NACBS palindromic sequences separated by a 5-bp linker. Reducing the linker by 1 bp altered the binding more than increasing it by 1 bp, indicating that the ORE1 dimer has a limited angle of closure but more flexible opening angles. The angle during DNA binding may be determined by the spacing between the two halves of the palindromic NACBS.

NACs are known to bind DNA as homodimers, but they can also form heterodimers. Olsen *et al.* (2005) demonstrated that ANAC019 and ANAC092 can bind DNA both as homodimers and heterodimers. This is not surprising, given that the residues forming the dimerization interface are highly conserved within the NAC protein family. Furthermore, ONAC127 can interact with ONAC129 in yeast, although neither can form homodimers under the same conditions (Ren *et al.*, 2021).

The complex with DNA

The initial crystallographic structures of the ANAC019 and SNAC1 domains did not allow the determination of DNA binding modes. However, one side of the structure was positively charged, making it highly suited for DNA recognition (Chen *et al.*, 2011, Ernst *et al.*, 2004). Later, Welner *et al.* (2012) confirmed that this side interacts with DNA by obtaining the ANAC019 structure interacting with oligonucleotides. This structure revealed that the β 3 strand of the NAC domain inserts into the major groove of the DNA, almost perpendicular to its helical axis, a mechanism that is thought to be shared by all NAC proteins (Welner *et al.*, 2016). The β -strand that protrudes into the major groove contains the highly conserved WKATGTDK motif, which is positioned appropriately to mediate specific base recognition. However, the resolution of the ANAC019/DNA complex was still limited, so the exact nucleotide-interacting residues could not be probed (Welner *et al.*, 2012).

In 2022, a new crystal structure of a NAC TF bound to the promoter sequence of *BIFUNCTIONAL NUCLEASE1 (BFN1)* was obtained: ORE1-NAC:DNA (Chun *et al.*, 2022). The structure of the complex unambiguously revealed a DNA-binding mode by providing the molecular details of the protein-DNA interactions for the first time. In this structure the primary interactions with DNA are mediated by residues 85-93, part of the β 2- β 3 loop, and residues 125-137, located at the edge of the β 6 strand. The residues 85-93 are positioned within the minor groove of the DNA, where the DNA contact region consists mainly of positively charged residues. Meanwhile, the residues 125-137 sit on

the major groove of the DNA. Thus, the two strands participate in grabbing the DNA strand from opposite sides (Figure 6). Interestingly, the 85-93 loop is disordered in the absence of DNA (Chun *et al.*, 2022).

The DNA-interacting residues are classified into two groups: residues interacting with DNA nucleobases (sequence-specific recognition) and residues interacting with the DNA phosphate backbone (overall affinity). Arg91, Lys102, and Thr104 are responsible for sequence-specific recognition. Thr104 and Lys102, interact with the imine groups through hydrogen bonds and are part of the highly conserved WRKY motif (WKATGKD), which is characteristic of NAC proteins. Lys85, Thr92, Arg94, Ala95, Arg129, Lys135, and His141 contribute to the overall affinity of the protein-DNA interaction by binding to the phosphate backbone of the DNA. Substitution by alanine of any of these residues in ORE1 resulted in a reduction in DNA binding compared to the WT (shown by surface plasmon resonance (SPR) experiments) (Chun *et al.*, 2022).

Previous mutagenesis studies on ANAC019 also identified residues Lys79, Arg85, and Arg88 (corresponds to Lys85, Arg91, and Arg94 of ORE1) as essential for DNA binding, as their mutation into alanine impaired DNA binding (Olsen *et al.*, 2005; Welner *et al.*, 2012). Arg88 appears to be particularly important for DNA binding, as it is conserved in all NAC proteins (Puranik *et al.*, 2012).

Other positively charged residues surround the DNA-binding region of ORE1–NAC. Although these residues do not interact directly with DNA in the crystal structure, they contribute to a large positively charged surface. Mutation of any of these residues significantly reduced DNA binding affinity, suggesting that they likely interact with DNA, even transiently, in solution (Chun *et al.*, 2022).

It should be noted that, while the DNA-binding capability of NACs is generally attributed to isolated NAC domains, some research suggests that the CTD may also influence binding as well (Hegedus *et al.*, 2003; Jeong *et al.*, 2009). For example, Lindemose *et al.* (2014) found that the full-length ANAC092 exhibited higher DNA affinity than the NAC domain alone. This suggests that the CTD assists DNA binding, potentially by modulating conformation, flexibility, or spacing within the DNA-protein complex.

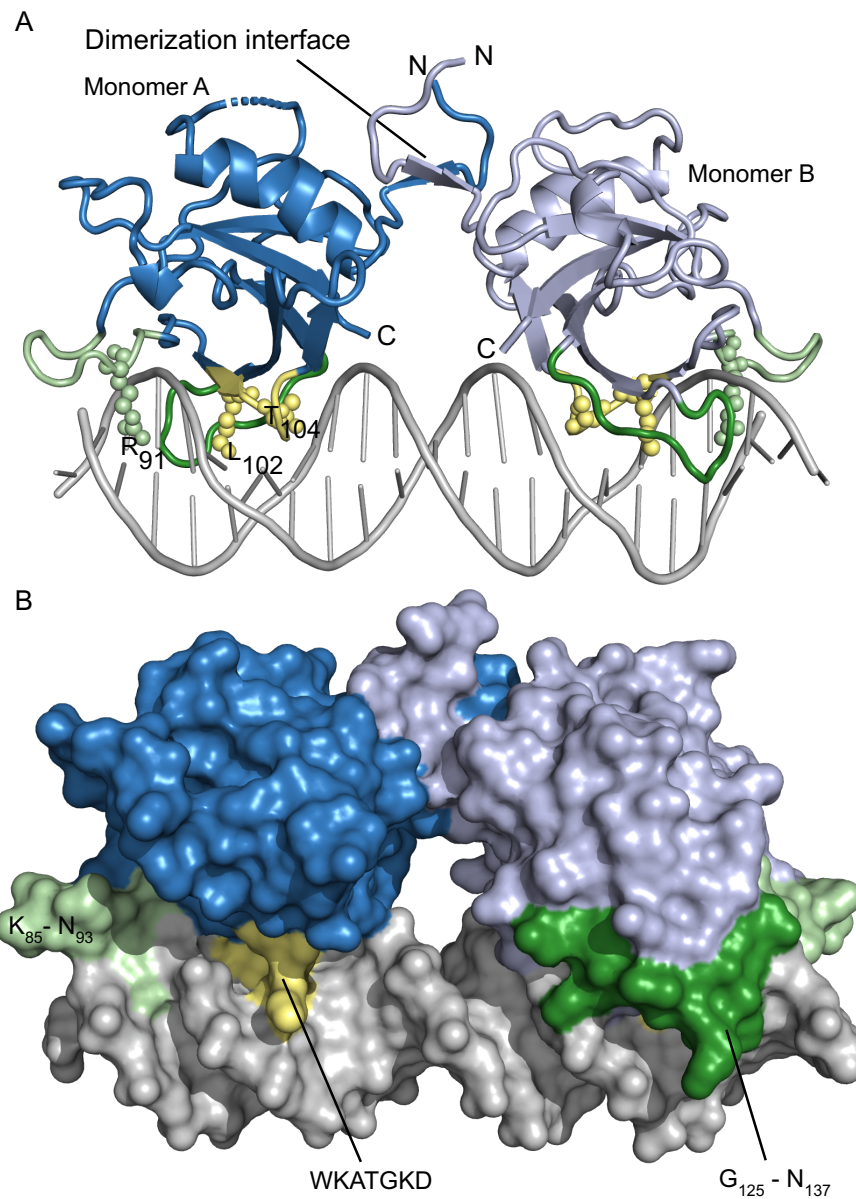


Figure 6: Crystal structure of ORE1-NAC:DNA (PDB: 7xp3) (Chun et al., 2022). (A) Cartoon representation of the ORE1 NAC dimer, with the dimer interface, interacting with DNA (gray). Monomer A is shown in blue and monomer B in light blue. The residues K85-N93 of both monomers, colored in light green, are positioned within the minor groove of the DNA, while the residues G125-N137, colored in forest green, sit on the major groove of the DNA. The conserved WRKY motif (WKATGKD) is shown in yellow. The residues R91, K102, and T104, implicated in DNA binding, are depicted as spheres (Figure generated with Pymol Version 2.2.5, Schrödinger). (B) Surface representation of ORE1-NAC:DNA complex using the same color scheme as panel A (Figure generated with Pymol Version 2.2.5, Schrödinger).

Box 3: Nuclear localization of NAC TFs

Entering the nucleus is essential for TFs to function as transcriptional regulators. This occurs via the nuclear pore complex, which penetrates the nuclear envelope, and is directed by nuclear localization signals (NLS). NLS are short peptide sequences recognized by importin proteins, enabling TFs to cross the nuclear envelope. In the nucleus, the importin-TF complex dissociates, and the TF can bind DNA and regulate gene expression (Marathe *et al.*, 2024). Unlike signal peptides of proteins destined for the ER or mitochondria, NLS motifs remain intact, enabling multiple cycles of nucleocytoplasmic shuttling (Lu *et al.*, 2021). NLS motifs are generally rich in positively charged residues, such as lysine and arginine, and are classified as monopartite or bipartite. The monopartite NLS contains a single cluster of positively charged amino acids, and the bipartite motifs contain two clusters separated by a 10–12 amino acid linker sequence. However, many nuclear proteins lack classical NLS motifs and must rely on alternative nuclear entry mechanisms. These include non-classical NLS motifs, which are longer and more variable, or piggybacking on other proteins that contain a classical NLS (Marfori *et al.*, 2011; Lu *et al.*, 2021).

NAC proteins are directed to the nucleus by classical NLS, but a single conserved NLS motif has not been identified. NAC TFs exhibit diverse NLS motifs in either the N-terminal or C-terminal regions, although the N-terminal region is more commonly associated with nuclear localization (Greve *et al.*, 2003; Le *et al.*, 2011; Mohanta *et al.*, 2020). Some NAC proteins, including ANAC019, have a NLS within their D subdomain, which is characterized by clusters of basic amino acids (Olsen *et al.*, 2005; Tran *et al.*, 2009). Interestingly, some NAC TFs are anchored in the cytoplasm by a membrane-associated domain at their C-terminus, functioning as latent TFs. These proteins are sequestered in an inactive form in cellular membranes and are released in response to environmental stress or specific cues. For example, ANAC013 is released during hypoxic stress, ANAC053 is released in response to heat stress, and ANAC062 in response to low temperatures (Marathe *et al.*, 2024). This release is mediated by intramembrane proteases or the ubiquitin-proteasome pathway, allowing the TFs to translocate to the nucleus and activate target genes (Shu *et al.*, 2024). Proteins that shuttle between the nucleus and cytoplasm also contain a nuclear export signal (NES), which is essential for their export and subsequent proteasomal degradation in the cytoplasm. This process is facilitated by the binding of NES motifs by a Ran-GTP complex (Marathe *et al.*, 2024). In NAC TFs, a conserved hydrophobic L-V-F-Y motif, found in most members, has been confirmed to function as a NES (Kosugi *et al.*, 2008; Hao *et al.*, 2010).

DNA recognition and binding of NAC TFs

DNA sequence specificity of NAC proteins

The specificity and affinity of the NAC domain for target DNA sequences, along with its interactions with other TFs, contribute to the diverse functions of the different NAC isoforms (Welner *et al.*, 2016). Thus, many research efforts have focused on the interactions between NAC TFs and DNA, leading to progress in the elucidation of DNA sequence preference of NAC proteins. Target NACBS of NAC proteins from distant clades have already been identified in natural promoters or by *in vitro* selection techniques (Huh *et al.*, 2012; Matallana-Ramirez *et al.*, 2013; Olsen *et al.*, 2005; Tran *et al.*, 2004). The first NACBS, identified using SELEX on random oligonucleotides, contained the core motif CGT[AG] palindromically repeated (Olsen *et al.*, 2005). This core consensus sequence was later confirmed for various NAC proteins, supporting the existence of a conserved NAC DNA-target site, albeit with considerable sequence differences in the bases flanking the core NACBS. Thus, the bases surrounding the CGT[AG] motif in promoters could influence binding specificities and fine-tuning of NAC-DNA interactions *in vivo* (Lindemose *et al.*, 2012). The optimal spacing between the palindromic repeats can also slightly vary, conferring additional DNA-binding specificities (Jensen and Skriver, 2014). For example, wheat NAC69 shows maximal binding activity with a 6-bp spacer between CGT and ACG, reduced activity with a 7 bp spacer, and no activity with shorter or longer spacers (Xue *et al.*, 2006). ANAC019 shows optimal DNA binding to two 7-bp NACBS sequences separated by an 8-bp spacer, while ANAC092 prefers a 7-bp spacer (Chun *et al.*, 2021; Jensen *et al.*, 2010; Matallana-Ramirez *et al.*, 2013). A DAP-seq experiment involving 529 TFs from Arabidopsis further validated the NACBS and identified C₁ and T₃ as the most conserved nucleotides in the NAC consensus motif (O'Malley *et al.*, 2016).

Interestingly, several functional promoters targeted by NAC TFs only contain one consensus NACBS (non-palindromic repeats) (Jensen and Skriver, 2014), while some NAC members completely deviate from the consensus core motif. For example, CASTing with the barley IDEF2 and the Arabidopsis CBNAC selected NACBS with a GCTT core sequence (Kim *et al.*, 2007; Ogo *et al.*, 2008). ATAF2, a NAC TF involved in viral defense, also deviates from the consensus binding site and recognizes AT-rich sequences in the nitrilase2 (*NIT2*) promoter (Huh *et al.*, 2012). Another NAC protein, the turnip crinkle virus interacting protein (TIP), has been reported to bind DNA in a non-sequence-specific manner (Ren *et al.*, 2005).

Furthermore, Lindemose *et al.* (2014) investigated any potential correlation between the amino acid sequence of the NAC domain and preferred recognized DNA sequences but found very little correlation. This suggests that, while individual residues may influence DNA-binding specificity, factors such as conformational changes in DNA-contacting loops could also dictate specific recognition (Lindemose *et al.*, 2014).

Another element to consider is heterodimerization and other protein-protein interactions that could alter the affinity and specificity of NAC proteins for DNA.

Protein-protein interactions involving NAC proteins

The most important protein-protein interaction involving NAC proteins is certainly dimerization, where NAC monomers form either a homodimer or a heterodimer, resulting in a functional DNA-binding TF. In rice, OsNAC5 forms both homo- and heterodimers with other stress-associated NAC proteins, such as OsNAC6, to regulate the transcription of stress-inducible genes (Jeong *et al.*, 2009; Takasaki *et al.*, 2010). The peach BLOOD NAC TF forms a heterodimer with PpNAC1, binding to the promoter region of *PpMYB10.1* and inducing its transcription, which leads to anthocyanin accumulation in peach fruits (Zhou *et al.*, 2015).

In addition, the regulation of essential plant cell functions by NAC proteins involves a variety of other protein–protein interactions. However, relatively few studies have documented direct physical interactions with other proteins, and in-depth analyses are often lacking.

NAC proteins have been found to physically interact with TFs belonging to other classes. For example, ANAC096 improves Arabidopsis survival under dehydration and osmotic stress by interacting with ABA-responsive element (ABRE) binding factors ABF2 and ABF4 to activate ABA-inducible genes (Xu *et al.*, 2013). In red-fleshed apples, MdNAC1 interacts with MdbZIP23 to activate *MdMYB10* and *MdUFGT*, promoting anthocyanin synthesis (Liu *et al.*, 2023). ANAC092 interacts with G2-like TFs GLK1 and GLK2, which regulate chloroplast maintenance, to fine-tune leaf aging (Rauf *et al.*, 2013).

Studies on the interactions between NAC and viral proteins have emerged, revealing their roles in the ongoing battle between plants and viral pathogens. These interactions can either enhance plant defense responses or help viruses circumvent them (Alves *et al.*, 2014; Welner *et al.*, 2016). The first report on the interaction between a NAC protein and a viral protein involved Geminivirus RepA-binding (GRAB) proteins, GRAB1 and GRAB2, which specifically interact with the wheat dwarf geminivirus RepA protein. GRAB proteins inhibit geminivirus DNA replication in cultured cells (Xie *et al.*, 1999). ATAF2 was found to interact with the tobacco mosaic virus (TMV) replicase,

leading to ATAF2 degradation, presumably as a viral strategy to suppress plant defenses (Wang *et al.*, 2009). The NAC protein TCV-INTERACTING PROTEIN (TIP) in Arabidopsis interacts specifically with the capsid protein of the turnip crinkle virus (TCV). TIP promotes basal resistance through transcriptional activation, but the viral CP acts as a virulence factor by binding to TIP to suppress this resistance (Ren *et al.*, 2005).

Specific NAC target genes

In theory, genes with a NACBS in their promoter regions are potential targets for NAC proteins, but confirmation of the direct regulation requires further experimental validation. While numerous target genes have been identified, many remain to be discovered, highlighting the need to further explore and expand the regulatory network of NAC TFs (Diao *et al.*, 2020).

Table 2 presents a non-exhaustive list of promoters recognized by NAC TFs, along with their corresponding NACBS sequences and the methods used to identify the binding. The list is limited to Arabidopsis NAC TFs as maize NAC TFs will be discussed in more detail below. It shows that NAC proteins bind to promoter regions containing the NAC core motif, CGT[AG]. NACBS with less stringent motifs or lacking the classical NAC core motif have also been identified in the promoters of target genes. For example, ANAC019/055/072 and VNI2 preferentially bind to a CATGT sequence in their respective target promoters.

The diversity of NAC target genes, including genes involved in dehydration responses, ABA biosynthesis, and pathogen defense, highlights their immense potential in plant engineering. A thorough understanding of NAC-DNA interactions will be essential for developing stress-resilient crops capable of withstanding biotic and abiotic challenges.

Table 2: A representative overview of promoters targeted by Arabidopsis NAC TFs. The specific sequences bound by NAC TFs within the promoters are presented in the 5' to 3' orientation, with the NAC core motif CGT(A/G) (or CACG) underlined. The methods used to identify these binding interactions are also provided.

NAC TF	Target gene	NACBS	Method	Source
ANAC019/055/072	<i>early responsive to dehydration stress 1 (ERD1)</i> : encodes a subunit of the caseinolytic ATP-dependent protease	CATGTG motif	Y1H	Tran <i>et al.</i> , 2004
ANAC019	<i>vegetative storage protein1 (VSP1)</i> : JA-induced storage protein accumulating in vegetative organs	CATCTCCACG	EMSA	Bu <i>et al.</i> , 2008
ANAC017	<i>xyloglucan endotrans hydrolase (XTH31)</i> : encodes an enzyme modifying xyloglucan for detoxification	Unknown	Y1H ChIP	Tao <i>et al.</i> , 2022
ATAF1	<i>9-cis-epoxycarotenoid dioxygenase-3 (NCED3)</i> : ABA phytohormone biosynthetic gene	TTGCGTA	ChIP	Jensen <i>et al.</i> , 2013
ATAF2	<i>nitrilase2 (NIT2)</i> : encodes an enzyme converting indole-3-acetonitril into auxin	AT-rich sequence	EMSA	Huh <i>et al.</i> , 2012
AtNAP	<i>senescence-associated gene113 (SAG113)</i> : encodes a Golgi-localized protein phosphatase	CACGTAAGT	Y1H EMSA	Zhang & Gan, 2012
JUB1	<i>dehydration-responsive element-binding protein2a (DREB2A)</i> : encodes an AP2/EREBP TF	GATGCCGTTA GAGACACG	EMSA ChIP	Wu <i>et al.</i> , 2012
NAC016 NAP	<i>abscisic acid-responsive element binding protein1 (AREB1)</i> : encodes a TF in the ABA signaling pathway	TTGGATCTA (NAC016) CACGTAAGT (NAP)	Y1H ChIP	Sakuraba <i>et al.</i> , 2015
NTL4	<i>Respiratory Burst Oxidase Homologs (Atrboh A, C, and E)</i> : encode ROS biosynthetic proteins	TGTCGTA	ChIP	Lee <i>et al.</i> , 2015
NTL6	<i>Pathogenesis-related1 (PR1)</i> : encodes antimicrobial proteins	TTGCGTGTTG GAACACGCAT	EMSA	Seo <i>et al.</i> , 2010
ORE1	<i>Bifunctional nuclease1 (BFN1)</i> : encodes a senescence-associated type I nuclease	ACGTANNNNN CGTG	EMSA ChIP	Matallana-Ramirez <i>et al.</i> , 2013
VNI2	<i>cold-regulated (COR15)</i> and <i>responsive to dehydration (RD29)</i> encode proteins involved in leaf aging	AACATGTAA (COR15) ATCATGTAA (RD29)	EMSA ChIP	Yang <i>et al.</i> , 2011

Regulation of NAC proteins

The versatility in NAC protein functions stems from their finely tuned regulation at multiple levels, including transcriptional, post-transcriptional, and post-translational mechanisms. These regulatory controls have evolved to ensure the proper activity of NAC TFs, thereby enhancing their functional versatility in plants (Puranik *et al.*, 2012).

Transcriptional regulation

The presence of diverse *cis*-elements in *NAC* promoters suggests potential regulation by TFs from various families, including the NAC family itself (Diao *et al.*, 2020).

The expression of stress-related NAC TFs is regulated by stress-responsive CREs in their promoter regions, such as ABA-responsive elements (ABREs), drought-responsive elements (DREs), and MYB/MYC binding sites (Nakashima *et al.*, 2012). For example, in rice, the promoter activities of *OsNAC5* and *OsNAC6* are strongly induced by ABA through ABREs in their promoter regions. Similarly, the expression of *OsNAC3* and *SNAC1* is upregulated by dehydration via DREs (Takasaki *et al.*, 2010) and the expression of *ONAC045* is linked to MYB and MYC binding sites (Zheng *et al.*, 2009). Although the specific TF binding to these *NAC* promoters remains unidentified, some upstream regulators of stress-responsive NAC TFs have been characterized. For example, the bZIP TFs ABF3 and ABF4, key regulators of ABA signaling, directly bind to the promoters of *ANAC019*, *ANAC055*, and *ANAC072*. The *ANAC072* promoter also contains a DRE motif, which is bound by DRE-binding TFs (CBF1-CBF4). In addition, one MYC and five MYB members are known to be upstream regulators of these three *NAC* genes (Hickman *et al.*, 2013). In banana, cold stress enhances MalCE1(inducer of C-repeat binding factor expression 1) binding to the *MaNAC1* promoter, contributing to cold tolerance in banana fruit (Chan *et al.*, 2014).

Several *NAC* genes associated with developmental processes are well-established downstream targets of diverse TF families. For example, the litchi fruit *LcNAC1* is targeted by LcMYC2 to regulate fruit senescence (Jiang *et al.*, 2017). Similarly, *EIN3* (*ETHYLENE-INSENSITIVE3*) binds to the promoters of *ORE1* and *AtNAP*, inducing their transcription and positively regulating leaf senescence (Kim *et al.*, 2014).

VND7 (*VASCULAR-RELATED NAC-DOMAIN 7*), which plays a role in secondary wall biosynthesis, is regulated by E2Fc in a dose-dependent

manner. Extremely high or low levels of E2Fc repress *VND7* expression, while moderate E2Fc levels activate it (Taylor-Teeples *et al.*, 2015). In black cottonwood (*Populus trichocarpa*), PtrMYB161 mediates feedback regulation of *PtrSND1* (*Secondary wall-associated NAC-Domain 1*), playing a crucial role in wood formation (Wang *et al.*, 2020).

NAC TFs are also regulated by proteins belonging to their own family. For example, all seven VNDs can induce the expression of *VND7* by binding directly to its promoter (Nakano *et al.*, 2015). In addition, NAC TFs may exhibit self-regulation, as seen in *SND1*, which is self-activated by binding to its own promoter (Wang *et al.*, 2011).

While some upstream regulators of NAC genes have been identified, they are largely limited to specific stress conditions and signaling pathways. These interactions between upstream regulators and NAC TFs ensure the production of an optimal amount of NAC transcripts necessary for their downstream functions. However, additional post-transcriptional regulatory mechanisms are also in place to maintain appropriate levels of NACs.

Post-transcriptional regulation

The post-transcriptional regulation of NAC genes largely involves the cleavage of mRNA by microRNAs (miRNAs) (Mathew & Agarwal, 2018). The most striking example is the miR164 family, which acts as key regulators of NAC genes involved in various developmental processes, including organ boundary formation, lateral root development, and flower development (Dong *et al.*, 2022). The miR164 targets the transcripts of the two well-known NAC TFs, *CUC1* and *CUC2*, which are involved in the regulation of organ architecture (Hernández & Sanan-Mishra). In tomato, miR164 OE reduces the accumulation of GOB, SINAC1, SINAM2, and SINAM3, with GOB and SINAM2 being involved in flower boundary formation (Hendelman *et al.*, 2013). In *Rosa hybrida*, an increase in ethylene production affects miR164 abundance, leading to the accumulation of RhNAC100, which limits cell expansion and slows petal growth (Pei *et al.*, 2013). *ZmNAC1* in maize, along with *NAC1* in *Arabidopsis*, both involved in auxin-dependent lateral root development, are also targeted by miR164 (Guo *et al.*, 2005; Li *et al.*, 2012). This miRNA164-mediated regulation of root development may improve tolerance to salt and drought stress (Hernández & Sanan-Mishra). For example, in *Arabidopsis*, ectopic expression of the stress-related *PeSNAC1* from *Phyllostachys edulis* (tortoise-shell bamboo), along with Pe-miR164b, improves tolerance to salinity and drought by regulating root development (Wang *et al.*, 2016).

Beyond miR164, other miRNA-mediated regulatory pathways also contribute to abiotic stress adaptation. For example, OE of the rice miR319a in *Agrostis stolonifera* (creeping bentgrass) improves salt and drought tolerance in transgenic plants by significantly down regulating its target genes, including *AsNAC60* (Zhou *et al.*, 2013).

In conclusion, miRNAs are essential for fine-tuning *NAC* expression, ensuring both normal growth and an adaptive response to environmental stresses.

NAC TFs can also be regulated by long non-coding RNAs, such as *cis*-natural antisense transcripts (*cis*-NATs). These RNAs are transcribed from the same genomic locus as their target genes, but from the opposite DNA strand, allowing them to form perfect complementary pairs. This pair of sense-antisense transcripts form self-regulatory circuits, providing increased responsiveness of gene expression and cell to cell variability (Reis & Poirier *et al.*, 2022). In *Gramineae*, 51 out of 521 *NAC* genes are associated with a corresponding *cis*-NAT. One example is *ZmNAC48* in maize, which is regulated by its antisense transcript, *cis-NATZmNAC48*. Both transcripts co-localize in the nucleus and are responsive to drought stress. Overexpression of *cis-NATZmNAC48* suppresses *ZmNAC48* expression, resulting in increased stomatal opening, greater water loss, and increased leaf senescence (Mao *et al.*, 2021).

Alternative splicing can also play a role in the complex regulation of *NAC* TFs. In *Populus trichocarpa*, *PtrWND1* is regulated through intron-mediated alternative splicing, producing two isoforms that act antagonistically to control cell wall thickening during fiber cell differentiation (Zhao *et al.*, 2014). Furthermore, a splicing variant of *PtrSND1*, which lacks DNA-binding and *transactivation* abilities, forms a dimer with the functional *PtrSND1*, inactivating it and affecting secondary cell wall biosynthesis in *Arabidopsis* and poplar species (Li *et al.*, 2012).

Post-translational regulation

The post-translational regulation of *NAC* proteins includes protein-protein interactions that result in covalent modifications, such as ubiquitination. For example, *NAC1*, which regulates auxin-dependent lateral root formation, interacts with the RING-type E3 ligase *SINAT5*, leading to its ubiquitination and degradation, thereby reducing the auxin signal (Xie *et al.*, 2002). Similarly, other *NAC* TFs, including *ANAC055*, *ANAC019*, and tomato *NAC1*, interact with RING ubiquitin ligases and are targeted for degradation via the ubiquitin–proteasome system (Greve *et al.*, 2003; Bu *et al.*, 2009; Miao *et al.*, 2016). These interactions highlight how ubiquitin ligases regulate *NAC* protein levels through polyubiquitination.

Another major mechanism controlling the activity of NAC TFs is their sequestration in cellular compartments outside the nucleus, followed by their controlled release into the nucleus. Nuclear localization can be triggered through heteromerization or phosphorylation. For example, ONAC023 localized in the cytoplasm and ONAC020, localized in the ER, translocate to the nucleus when they interact with ONAC026 (Mathew *et al.*, 2016). Similarly, phosphorylation is required for the nuclear localization of OsNAC4, ensuring its presence in the nucleus only under favorable conditions (Kaneda *et al.*, 2009).

Overall, *NAC* gene regulation is a highly coordinated process, summarized in Figure 7. While transcriptional control dictates *NAC* gene expression, post-transcriptional regulation fine-tunes the pool of functional transcripts, and post-translational modifications adjust protein stability, activity, or localization.

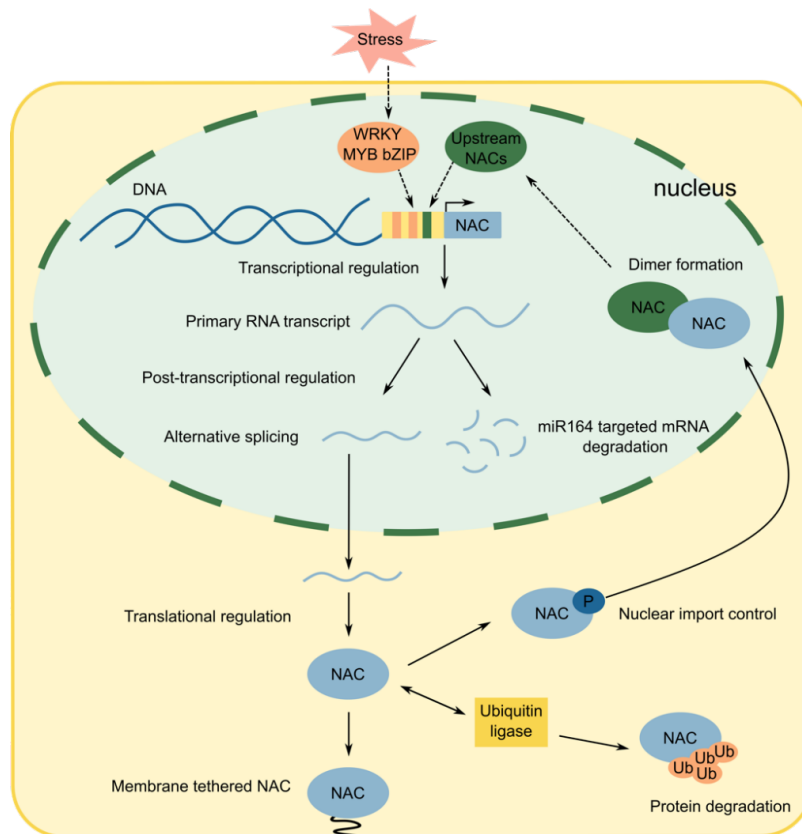


Figure 7. Multi-level regulation of NAC TFs. Regulation occurs at the transcriptional level via upstream TF binding (including NACs to NACBSs) leading to the production of pre-mRNA. Post-transcriptional mechanisms include miRNA-mediated degradation and alternative splicing. Post-translational control includes cytoplasmic sequestration and regulated nuclear import via cleavage or modification, as well as proteasomal degradation and dimerization.

Evolution of NAC proteins

The folding of the NAC domain shares structural similarities with the DNA-binding domain of WRKY and GCM proteins, suggesting a common evolutionary origin for these families. Only WRKY proteins are present in primitive eukaryotes, whereas the others have evolved later. NAC TFs are plant-specific, while GCM proteins are restricted to metazoans. Thus, the current NAC and GCM TFs may have evolved from ancestral WRKY proteins (Yamasaki *et al.*, 2016).

Within the plant kingdom, the first phylogenetic analyses of NAC proteins, facilitated by the availability of complete plant genomes, made two major observations (Shen *et al.*, 2009; Zhu *et al.*, 2012; Nagata *et al.*, 2015). First, NAC proteins were found exclusively in land plants, but the lack of genomic data for streptophytic green algae, proposed as the closest relatives of land plants (Wickett *et al.*, 2014), did not allow to determine whether NAC proteins are strictly specific to land plants or whether they appeared earlier. Second, mosses and lycophytes had fewer predicted members (<30) compared to angiosperms (>60), suggesting that an expansion of NAC proteins took place after the evolution of vascular plants (Shen *et al.*, 2009).

The large-scale gene sequencing data from phylogenetically diverse species, generated by the 1000 Plants Project (www.onekp.com), provided new insights into the evolutionary origin of the NAC proteins (Wickett *et al.*, 2014; Maugarny-Calès *et al.*, 2016). This analysis revealed that NAC TFs arose before the evolution of land plants, as homologs were identified in several groups of streptophytic green algae, including Klebsormidiales.

This study also uncovered a stepwise expansion of the NAC gene family, rather than a continuous expansion. The first expansion occurred in land plants, particularly in bryophytes, after their evolution from the streptophytes. The NAC genes that underwent a major expansion included those involved in water conduction and mechanical strength, highlighting their significance in the evolution of prevascular plants. The second expansion is thought to have occurred in flowering plants after their divergence from other vascular plants (Maugarny-Calès *et al.*, 2016). These findings emphasize the critical role of NAC TFs in plant evolution, particularly in adaptations related to terrestrial life (Mathew & Agarwal, 2018).

To better understand the evolutionary relationships within the NAC family, several phylogenetic classifications have been proposed (Shen *et al.*, 2009; Zhu *et al.*, 2012; Pereira-Santana *et al.*, 2015; Jin *et al.*, 2017; Kim *et al.*, 2024). A previous classification divided the NAC superfamily from nine species into 21 groups based on protein alignments (Zhu *et al.*, 2012).

However, this analysis was limited by its small number of species, primarily flowering plants, and lacked representation from evolutionarily diverse groups. An updated study analyzed 26 plant species covering all major plant lineages, redefining and expanding the NAC subfamilies while maintaining consistency with previous classifications (Kim *et al.*, 2024). In this updated study, 23 NAC subfamilies were identified, including 10 specific to seed plants and one specific to monocots. In general, the number of NAC TFs expanded significantly from green algae to modern cultivated crops, which can be explained by multiple whole genome duplication events (Kim *et al.*, 2024). The subfamilies were renamed using Roman numerals (I to X) combined with lowercase letters (*e.g.*, Va) (Kim *et al.*, 2024). NAC genes with similar functions were grouped in specific subfamilies, showing functional divergence between subfamilies. For example, genes in clades Ia and Ic act as developmental regulators. Clade Ia includes the genes *CUC1* and *CUC2*, which play critical roles in shoot apical meristem formation and cotyledon separation during Arabidopsis embryogenesis (Hibara *et al.*, 2003). Clade Ic comprises genes like *VND7*, associated with flowering time (Fujiwara & Mitsuda, 2016), as well as *VND4* and *VND5*, which activate secondary wall biosynthesis genes in vessel cells (Zhou *et al.*, 2014). Some clades, such as IVd, include genes involved in stress responses, such as *JUB1* and *ZmNAC55*, which contribute to drought tolerance (Wu *et al.*, 2012; Mao *et al.*, 2016). As additional NAC protein functions are characterized, other functional grouping patterns may emerge.

While phylogenetic analysis could help select candidate genes, it cannot alone determine their function conclusively. Combining phylogenetic clustering with motif identification and coexpression analysis could already improve candidate identification, but functional studies remain essential to confirm the roles of specific NAC proteins.

NACs modulate developmental processes

As a large and functionally diverse TF family in plants, NAC proteins play a role in almost all stages of plant growth and development. They are involved in processes ranging from cell division and secondary wall formation to shoot apical meristem maintenance, flower organ development, fruit ripening, leaf senescence, and root development (Shao *et al.*, 2015). Although many NAC functions have already been mentioned above, the following section provides an overview with some relevant examples. Examples from maize are excluded here, as maize NACs will be discussed separately in a later section.

Early embryogenesis and flower development

The earliest known function of NAC TFs was their role in plant development, from early embryogenesis to flowering. The first NAC gene identified, *NAM* in *Petunia*, is essential for the formation of shoot apical meristem (SAM). The SAM gives rise to all above-ground plant organs (Souer *et al.*, 1996). The *nam* mutants have fused cotyledons and floral organs, as well as an inability to form apical shoots, highlighting its role in SAM formation (Souer *et al.*, 1996). Similarly, the functionally redundant *CUC1* and *CUC2* genes in *Arabidopsis* are essential for boundary formation and meristem maintenance, as *cuc1/cuc2* double mutants exhibit fused cotyledons and floral organs, along with defective shoot apical meristem development (Aida *et al.*, 1999; Takada *et al.*, 2001). This shows that NAC TFs involved in meristem formation and maintenance contribute to organ separation, thereby ensuring proper floral organ morphogenesis.

Beyond their roles in floral organ boundary formation and maintenance, NAC proteins regulate the timing of flowering, as shown with *OsNAC2* in rice and *BnNAC485* in *Brassica napus* (Ying *et al.*, 2014; Chen *et al.*, 2015). *OsNAC2* affects plant height and flowering time by regulating the gibberellic acid (GA) signaling pathway (Chen *et al.*, 2015). In *Rosa hybrida*, *RhNAC2* contributes to petal expansion by modulating dehydration tolerance (Dai *et al.*, 2012). Overall, NAC genes can play key roles in meristem formation, floral organ separation, and flowering time regulation.

Root development

Recent research has highlighted the role of NAC TFs in various aspects of root development, including lateral root formation, root cap formation, root tip growth, root ground tissue patterning, and root responses to abiotic stress (Xiong *et al.*, 2025).

Lateral roots are essential components of the plant root system, increasing the surface area for water and nutrient absorption. Numerous studies have shown that overexpression of NAC members enhances lateral root formation by modulating auxin signaling, cell division, and differentiation. In *Arabidopsis*, for example, *NAC1* promotes auxin-dependent lateral root development by activating downstream auxin-responsive genes such as *DBP* and *AIR3* (Xie *et al.*, 2000). In soybean, *GmNAC109* and *GmNAC20* stimulate lateral root formation in transgenic *Arabidopsis* and rice by upregulating auxin-related genes (Yang *et al.*, 2019; Yarra & Wei, 2021). Similarly, in rice, *OsNAC2* shapes root system architecture, including lateral root growth, by regulating genes involved in cytokinin and auxin signaling and metabolism (Mao *et al.*, 2020).

The root cap, a protective layer covering the root apical meristem, plays important roles in shielding the meristem, secreting mucilage to facilitate root penetration through soil, and sensing environmental cues (Xiong *et al.*, 2025). In *Arabidopsis*, the NAC TF *SOMBRERO* (SMB), along with its homologs *BEARSKIN1* and *BEARSKIN2*, regulates the maturation and detachment of root cap cells (Bennett *et al.*, 2010). In addition, SMB is implicated in root halotropism, steering root growth away from high-salt zones by directing the asymmetric distribution of auxin in the lateral root cap (Zheng *et al.*, 2024). Beyond the root cap, NAC TFs also regulate root tip growth and root tissue patterning by influencing cell cycle progression and differentiation through interaction with other regulators. For example, in *Arabidopsis*, NAC1 maintains root meristem activity and root elongation by repressing *E2Fa*, a core TF involved in cell cycle control (Xie & Ding, 2022).

Seed development

The regulatory roles of NAC TFs throughout seed development have been well established, impacting traits such as seed size, nutrient accumulation, dormancy, and germination.

NACs affect seed size and morphology by modulating cell elongation, cell proliferation, and endosperm development. For example, *OsNAC24* and *OsNAC25* interact with *OsMED15a* in rice to influence grain size and weight (Dwivedi *et al.*, 2019). In addition, *OsNAC120* directly activates *BIG GRAIN1*, encoding a major regulator of grain development that controls grain size (Xie *et al.*, 2024). NACs also regulate seed composition by affecting starch synthesis and nutrient transport. In wheat, *TaNAC019* binds to the promoters of genes involved in starch metabolism, thereby impacting grain quality and yield (Gao *et al.*, 2021). NAC TFs are also involved in controlling seed dormancy and germination. In *Arabidopsis*, *ANAC060* and *ANAC040* are membrane-associated TFs that redundantly inhibit seed dormancy (Song *et al.*, 2022), while *OsNAC3* enhances germination in rice, by activating an ABA degradation gene and a cell expansion gene (Huang *et al.*, 2024).

Cell wall biosynthesis

NAC proteins have been shown to coordinate many aspects of secondary cell wall (SCW) biosynthesis in various plant species. SCWs are specialized structures that form after cell growth, composed mainly of cellulose, lignin, and hemicellulose, and are essential for mechanical support and water transport (Xiong *et al.*, 2024). In vascular plants, xylem differentiation involves SCW formation and programmed cell death (PCD), which are both regulated by NAC TFs.

In *Arabidopsis* and poplar, VND6 and VND7 regulate xylem vessel differentiation, acting as transcriptional switches regulating genes related to SCW formation (Yamaguchi *et al.*, 2010). VND1, VND2, and VND3 activate genes involved in SCW formation and PCW, which are essential for xylem vessel development in cotyledons (Tan *et al.*, 2018).

Similarly, SNDs are secondary wall associated NACs, particularly involved in cell-wall biosynthesis process in xylem fiber cells. SND1 and NST1 redundantly control SCW formation, with SND1 regulating fiber thickness through its downstream targets, SND2 and SND3 (Zhong *et al.*, 2007).

NAC TFs also control cell wall biosynthesis in woody trees. *PtWND* in poplar and *EgWND* in *Eucalyptus* activate secondary wall biosynthetic genes by binding to NAC-binding elements during wood formation (Jiang *et al.*, 2021).

Leaf senescence

Leaf senescence marks the final stage of leaf development, culminating in leaf death and abscission through a coordinated series of physiological and biochemical changes. This process, which is essential for nutrient relocation from leaves to reproducing seeds, is tightly regulated at multiple levels by a complex genetic program and involves numerous TFs (Lim *et al.*, 2007). Plant hormones also play a key role in leaf senescence, often in coordination with NAC TFs. ABA, for example, is a key hormone promoting senescence. In rice, OsNAC2 upregulates ABA biosynthesis genes and downregulates ABA catabolism genes, resulting in elevated ABA levels and enhanced leaf senescence (Mao *et al.*, 2017).

In the JA pathway, OsNAP promotes senescence by increasing JA levels and activating biosynthesis genes (Zhou *et al.*, 2013). Ethylene signaling also integrates with NAC activity; in *Arabidopsis*, ORE1 is regulated by EIN2, in the ethylene signaling pathway, promoting senescence (Kim *et al.*, 2014).

In addition to their established role in leaf senescence and longevity, NAC proteins are also involved in petal and fruit senescence. *EPH1* promotes ethylene-driven petal senescence in Japanese morning glory, while SINAC1 accumulates in immature green tomatoes and contributes to fruit softening during ripening (Shibuya *et al.*, 2014; Meng *et al.*, 2016). Thus, NAC proteins are not only involved in flower and fruit formation, but also their senescence.

Stress-related NAC TFs

Recently, NAC proteins have received much attention as regulators of stress signaling pathways, often involving interplay with phytohormones. Many NAC TFs play roles in plant responses to abiotic stresses such as drought, salinity, cold, heat, oxidative stress, and nutrient deficiencies, as well as biotic stresses, by directly or indirectly regulating downstream stress-related genes. Some NACs regulate different stress signaling pathways, enabling plants to respond and cope with multiple stresses.

Abiotic stress

Countless studies have demonstrated that NACs play key roles as regulators in plant drought and salt responses. These involve complex mechanisms, including integration of hormone signaling, regulation of reactive oxygen species (ROS) homeostasis, and gene expression control.

For instance, NAC TFs participate in the ABA signaling pathway, acting as both positive and negative regulators of drought and salt tolerance. In *Arabidopsis*, ANAC096 enhances ABA signaling by directly interacting with ABF2 and ABF4 to synergistically activate the expression of the stress-responsive gene *RD29A*, thereby contributing to dehydration and osmotic stress tolerance (Xu *et al.*, 2013). On the other hand, NAC016 and NAP have a negative impact on drought tolerance by inhibiting *AREB1* expression (Sakuraba *et al.*, 2015). In sweet potato, IbNAC3 promotes the transcription of *ERA1*, a negative regulator of ABA signaling, thereby reducing ABA sensitivity and improving salt tolerance (Meng *et al.*, 2023).

Drought and salt stress lead to excessive accumulation of ROS in plants, which can cause oxidative damage. NACs help mitigate this damage by regulating ROS-scavenging systems. For example, in rice, SNAC3 enhances drought tolerance by upregulating genes encoding ROS-scavenging enzymes and downregulating those involved in ROS production (Fang *et al.*, 2015). OsNAC2 promotes salt stress-induced PCD by modulating the expression of the ROS-scavenging gene *OsCOX11* and the caspase-like protease gene *OsAP37* (Mao *et al.*, 2018).

NAC TFs may also promote plant adaptation to salt and drought stress by regulating the expression of general stress-responsive genes. For example, GmNAC109 in soybean improves salt tolerance by regulating key stress-

related genes such as *DREB1A*, *DREB2A*, *AREB1*, *AREB2*, *RD29A*, and *COR15A* (Yang *et al.*, 2019). In rice, OsNAC5 interacts with OsNAC6 and SNAC1 to upregulate genes like *OsLEA3*, thereby enhancing salt tolerance (Takasaki *et al.*, 2010). Similarly, *MusaNAC68* OE in transgenic banana leads to increased expression of stress-responsive genes, including *DREB* and *LEA*, improving drought and salinity tolerance (Negi *et al.*, 2016).

While most NAC genes are known to confer salt and drought stress tolerance, some also contribute to high and low temperature stress tolerance. For example, OE of wheat *TaNAC2L* in *Arabidopsis* improves heat tolerance by activating heat-related genes, such as *AtHsfA3*, *AtDREB2A*, and *LEA*, during heat stress (Guo *et al.*, 2015). In tomato, OE of *SINAM3* improves cold tolerance by promoting ethylene production (Dong *et al.*, 2022). Some NACs, such as *LINAC014* in lily and *OsNTL3* in rice, contain transmembrane domains that enable them to sense heat signals; under heat stress, they move from the membrane to the nucleus to activate heat-responsive genes (Liu *et al.*, 2020; Wu *et al.*, 2023).

Some NAC proteins are known to regulate multiple stress responses. For example, ONAC023 in rice enhances both heat and drought tolerance at the seedling and reproductive stages (Chang *et al.*, 2024). Similarly, TaNAC2 in wheat responds to drought, salt, cold, and ABA by upregulating stress-responsive genes, thereby increasing tolerance to drought, salinity, and freezing in transgenic *Arabidopsis* (Mao *et al.*, 2012).

Biotic stress

The involvement of NACs in the response to biotic stress has been repeatedly demonstrated in relation to various pathogens (viruses, bacteria, and fungi) and in different crop species. They often regulate hormone-mediated defense pathways. For example, SINAP1 in tomato boosts resistance by activating salicylic acid (SA) biosynthesis genes (Wang *et al.*, 2020), while ANAC019 and ANAC055 enhance defense by inducing jasmonic acid (JA)-responsive genes (Hickman *et al.*, 2017).

Some NACs undergo subcellular delocalization upon infection. For example, Rph7 in barley moves from the membrane to the nucleus after a pathogen attack to activate defense gene expression and strengthen resistance to leaf rust (Chen *et al.*, 2023). While many NACs promote immunity, others are known to suppress it; ONAC083 in rice, *ScATAF1* in sugarcane, and *ATAF2* in *Arabidopsis* are induced by pathogens, but negatively regulate immune responses (Xiong *et al.*, 2025).

Of course, NACs sometimes confer stress resistance through developmental processes, such as promoting lateral root formation, which is important for water uptake and thus confers drought tolerance. However, NAC TFs also actively coordinate plant development and stress responses by participating in different hormone signaling pathways. For example, OsNAC120 in rice modulates the balance between growth and drought resistance by enhancing GA signaling while suppressing ABA signaling (Xie *et al.*, 2024). Similarly, OsNAC016 promotes BR signaling to support growth, while simultaneously repressing ABA-dependent drought-responsive genes, thereby balancing hormonal growth and stress tolerance (Wu *et al.*, 2022). A notable example is OsNAC2, which negatively regulates root development by integrating auxin and cytokinin (CK) signaling (Mao *et al.*, 2020), while also promoting leaf senescence via the ABA signaling pathway (Mao *et al.*, 2017). Under abiotic stress conditions, OsNAC2 contributes to the regulation of ROS accumulation, which can lead to cell death, a potential mechanism for eliminating damaged cells during salt stress (Mao *et al.*, 2018). Collectively, these examples highlight the crucial role of NACs in integrating growth and stress responses, enabling plants to adapt to changing environmental conditions.

Box 4: Case Study of Arabidopsis SOG1

SOG1 (SUPPRESSOR OF GAMMA RESPONSE 1) is one of the best characterized NAC TFs in Arabidopsis, making it an excellent case study, integrating all aspects of NAC TFs: function, target genes, post-translational regulation, and, more recently, protein structure.

In plants, chromosomal DNA is constantly damaged by environmental stresses, such as UV radiation and soil chemicals, as well as by ROS produced both by environmental stress and during photosynthesis (Yoshiyama *et al.*, 2014). DNA damage can be deleterious, preventing DNA replication and transcription, resulting in mutation and chromosomal aberrations, disrupting the transmission of genetic information across generations. To cope with this, plants have evolved a DNA damage response (DDR) to maintain genomic integrity. DDR senses DNA damage and activates signaling pathways that regulate DNA repair, cell cycle arrest, and PCD. This signaling pathway is precisely regulated (Ciccica & Elledge, 2010; Pedroza-Garcia *et al.*, 2022). SOG1 governs this DDR in Arabidopsis. It is phosphorylated by the ATM kinase in response to double-strand DNA breaks, a post-translational modification critical for activating downstream genes involved in DNA repair and transient cell cycle arrest (Yoshiyama *et al.*, 2013). The target genes of SOG1 have been identified through transcriptomics, microarray, and ChIP-seq (Bourbousse *et al.*, 2018; Ogita *et al.*, 2018). These studies revealed that SOG1 directly activates ~300 genes, including many TFs and known DNA repair genes, such as *GMI1*, *BRCA1*, and *PARG2*, as well as early cell cycle regulators. This placed SOG1 at the top of a complex GRN, where it acts as a major activator (Bourbousse *et al.*, 2018). Defense-related genes were also enriched among SOG1 targets, including stress-responsive genes such as *DREB19*, *NTL4/ANAC053*, and *ERD14*, suggesting that at least some of the SOG1 targets respond to abiotic stresses (Ogita *et al.*, 2018). Additionally, ChIP-seq data identified the palindromic SOG1 binding motif CTT(N)₇AAG, highlighting the role of the specific CTT and AAG sequences and their spacing (Ogita *et al.*, 2018). Although SOG1 and p53, a key tumor suppressor in mammals, show no sequence similarity, they play analogous roles in DDR, including ATM-dependent activation, and regulation of cell cycle arrest, cell death, and genome stability. These similarities have led to the functional assimilation of SOG1 to p53, as they both act as transcriptional activators, target similar biological processes, and share orthologous target genes (Ogita *et al.*, 2018). Recently, recombinant SOG1 protein has been purified and preliminary characterization has shown that, like other NAC proteins, SOG1 forms a dimer in solution. This enables future structural and biochemical studies of SOG1, such as X-ray crystallography (Mignon *et al.*, 2025).

Maize NAC members

At the start of this project in 2020, about 12 maize NAC TFs had been characterized. By 2025, 22 additional NAC TFs had been specifically studied, leading to a better understanding of their functions. This demonstrates the growing interest in these transcriptional regulators. In the rest of this thesis, the notation “NACxx” should be understood as referring to “ZmNACxx.”

Although multiple studies were identified members of the NAC family and performed phylogenetic analyses (Shiriga *et al.*, 2014; Fan *et al.*, 2014), the present study follows the most recent classification by Wang *et al.* (2020). A total of 147 NAC genes were identified in maize, 87 of which show differential expression in response to drought stress and rewatering. The NAC members were classified into 12 subgroups (NAM, OsNAC7, TIP, ONAC022, ATAF, SEN5, ONAC003, NAC1, ANAC011, NAC2, ANAC104, and AtNAC3), based on NAC domain alignments and C-terminal motifs, and each subgroup was named after its homolog in Arabidopsis or rice (Wang *et al.*, 2020). The structure of NAC genes in maize varies in terms of the number of introns and exons, although members of the same subgroup tend to share similar gene structures (Wang *et al.*, 2020).

Different naming conventions were used for NAC proteins across different organisms by different annotation teams, making discussions about the whole family of NAC proteins difficult. For NACs from other species, we used the names reported in their respective publications, while for NACs from maize, we followed the names from the relevant study along with their *MaizeGDB* annotation. Table 3 summarizes the known functions of specifically characterized maize NAC members in 2025, with their *maizeGDB* annotations.

Several maize NAC TFs were functionally linked to stress responses through overexpression studies in transgenic Arabidopsis or maize (Table 3). In addition to these specifically characterized NACs, other members were identified as responsive to various abiotic stresses without detailed functional analysis. For example, Lu *et al.* (2015) reported that seven NAC TFs (*NAC4*, *NAC5*, *NAC20*, *NAC23*, *NAC60*, *NAC70*, and *NAC109*) showed different responses to drought, salt, and cold stress, although not all were responsive to all conditions. Some membrane-bound TFs (NTL1-7, NAC66, NAC12, NAC26, NAC79, NAC97, NAC117, NAC61) were upregulated in the root and stem, but down-regulated in the leaf when plants were exposed to ABA or H₂O₂ treatment (Wang *et al.*, 2016).

Maize NACs involved in developmental processes regulate key traits such as seedling growth, kernel size, starch synthesis, senescence, and fertility. For

example, NAC126 promotes leaf senescence by upregulating ethylene pathway-related genes (Yang *et al.*, 2020). NAC128 and NAC130 regulate starch synthesis in maize kernels (Zhang *et al.*, 2019). KIRA1-LIKE1 (KIL1) promotes senescence and PCD at the base of the silk, limiting the fertilization window and potentially affecting grain yield (Šimášková *et al.*, 2022). NAC11 and NAC29 activate the expression of *EXPB15* to increase kernel size and weight (Sun *et al.*, 2022). Notably, NAC29 also influences total root length under salt stress (Zhang *et al.*, 2023), confirming that NACs are involved in multiple processes, a characteristic that is likely to be common to most NAC proteins.

Table 3: Summary of maize NAC TFs involved in abiotic stress responses and developmental processes. The table lists the NAC TFs, with their MaizeGDB annotations provided in parentheses, their known functions or phenotypes, and relevant sources.

NAC TF	Function / phenotypes	Source
Maize NAC TFs involved in abiotic stress response		
NAC2	OE of <i>NAC2</i> in Arabidopsis improves seed germination, cotyledon greening, stomatal closure, drought tolerance, and lateral root development under stress.	Chen <i>et al.</i> , 2023
NAC84 (NAC16)	OE of <i>NAC84</i> in tobacco improves drought tolerance and alleviates drought-induced oxidative damage of transgenic plants.	Zhu <i>et al.</i> , 2016
NAC20	OE of <i>NAC20</i> enhances drought resistance in maize by promoting ABA-induced stomatal closure.	Liu <i>et al.</i> , 2023
SNAC13 (NAC23)	OE of <i>SNAC13</i> in Arabidopsis enhances drought and salt tolerance. It functions in the ABA-dependent stress response, promoting genes like <i>PYL9</i> and <i>DREB3</i> to boost abiotic stress tolerance.	Luo <i>et al.</i> , 2022
NAC111 (NAC25)	OE of <i>NAC111</i> in maize enhances seedling drought tolerance by activating drought-responsive genes. Its expression is repressed by a MITE insertion in the promoter. Co-expression with <i>VPP1</i> (encoding a vacuolar-type H ⁺ pyrophosphatase) further improves drought resistance, boosting photosynthesis, antioxidant activity, and grain yield under water stress.	Mao <i>et al.</i> , 2015 Liu <i>et al.</i> , 2023
NAC087 (NAC29)	Natural variations in <i>NAC087</i> promoter are responsible for differential <i>NAC087</i> expression and total root length in maize inbred lines under salt stress.	Zhang <i>et al.</i> , 2023
NAC48	OE of <i>NAC48</i> in Arabidopsis improves drought tolerance by enhancing stomatal closure.	Mao <i>et al.</i> , 2021
SNAC1 (NAC49)	OE of <i>SNAC1</i> in Arabidopsis increases sensitivity to ABA and osmotic stress during germination but improves dehydration tolerance in seedlings. In maize, OE of <i>SNAC1</i> enhances drought tolerance by reducing transpiration, stomatal conductance, and density. This effect is linked to the repression of the stomatal development gene <i>MUTE</i> .	Lu <i>et al.</i> , 2012 Xiang <i>et al.</i> , 2021
NAC55 (NAC60)	OE of <i>NAC55</i> in Arabidopsis leads to hypersensitivity to ABA at the germination stage but enhances drought tolerance.	Mao <i>et al.</i> , 2016

NAC074 (NAC66)	OE of <i>NAC074</i> in Arabidopsis confers heat stress tolerance by modulating the accumulation of various stress-related metabolites, including ROS.	Xi <i>et al.</i> , 2022
NAC70	OE of <i>NAC70</i> in Arabidopsis enhances drought tolerance, while natural variations in its 5'-UTR are associated with increased gene expression and improved grain yield under drought conditions.	Wang <i>et al.</i> , 2021
NAC78	NAC78 enhances Fe concentrations in maize kernels by modulating the abundance of Fe transporters, improving tolerance to Fe deficiency.	Yan <i>et al.</i> , 2023
NAC55 (NAC118)	OE of <i>NAC55</i> in Arabidopsis causes drought sensitivity, while KD in maize enhances drought tolerance, with reduced water loss and membrane leakage. NAC55 activates <i>HOP3</i> expression by binding to the CACG motif in its promoter.	Fan <i>et al.</i> , 2024
NAC89 (NAC122)	OE of <i>NAC89</i> gene in Arabidopsis and maize enhances salt tolerance at the seedling stage.	Hu <i>et al.</i> , 2023
SNAC06 (NAC125)	OE of <i>SNAC06</i> in Arabidopsis increases ABA sensitivity during germination and enhances drought tolerance.	Wang <i>et al.</i> , 2024
NAC071 (NAC135)	OE of <i>NAC071</i> in Arabidopsis leads to elevated ROS and increased water loss, increasing sensitivity to ABA and osmotic stress. NAC071 significantly represses ABA- and stress-related genes such as <i>ABIs</i> , <i>RD29A</i> , <i>DREBs</i> , and <i>LEAs</i> .	He <i>et al.</i> , 2019
Maize NAC TFs involved in developmental processes		
NAC1	OE of <i>NAC1</i> in Arabidopsis enhances lateral root development. miR164, which negatively regulates <i>NAC1</i> , leads to distinct lateral root phenotypes by altering <i>NAC1</i> expression patterns.	Li <i>et al.</i> , 2012
NAC11 NAC29	NAC11 and NAC29 promote kernel size and weight by activating the expression of <i>EXPB15</i> , which encodes a β -expansin protein.	Sun <i>et al.</i> , 2022
NTL2 (NAC12)	A deletion in the transmembrane NTL2, referred to as NTL2 Δ , causes a miniature maize mutant. This mutation eliminates the transmembrane domain, localizing the NTL2 Δ protein to the nucleus. The deletion potentially affects hormone homeostasis (auxin and cytokinin).	Li <i>et al.</i> , 2024
NAC19	NAC19 is involved in seed development. OE of <i>NAC19</i> in Arabidopsis significantly increases seed size and seed yield and leads to increased transcription levels of two embryo development-related genes <i>YUC1</i> and <i>RGE1</i> .	Li <i>et al.</i> , 2024
NRP1 (NAC34)	<i>NAC34</i> , specifically expressed in maize endosperm, functions as a negative regulator of starch synthesis. OE of <i>NAC34</i> in rice decreases total starch accumulation, while increasing amylose fractions. The transgenic seeds exhibited altered starch structure and abnormal morphology.	Peng <i>et al.</i> , 2019
KIL1 (NAC36)	KIRA1-LIKE1 (KIL1) promotes senescence and PCD at the base of silk, limiting the fertilization window. Loss-of-function extends silk receptivity and increases grain yield following late pollination.	Šimáško vá <i>et al.</i> , 2022
NAC126 (NAC38)	NAC126 acts in starch synthesis by transcriptionally regulating starch synthesis-related genes in maize kernels.	Xiao <i>et al.</i> , 2021
NAC84	NAC84 negatively regulates pollen development in maize by directly repressing <i>RbohH</i> , highly expressed in pollen.	Yang <i>et al.</i> , 2018
NAC126	NAC126 regulates ethylene-triggered leaf senescence in maize. OE in Arabidopsis and maize accelerates chlorophyll degradation	Yang <i>et al.</i> , 2020

	and senescence by activating chlorophyll catabolic genes (<i>NYE1</i> , <i>NYC1</i> , <i>PAO</i> , <i>RCCR</i>).	
NAC128 (NRP1) NAC130	NAC128 and NAC130 regulate starch biosynthesis by controlling the expression of <i>Bt2</i> . KD of <i>NAC128</i> and <i>NAC130</i> results in shrunken kernels, accompanied by a reduction in starch and protein content.	Zang <i>et al.</i> , 2019
NAC132	NAC132 regulates leaf senescence and male fertility. KO of <i>NAC132</i> delays chlorophyll degradation and senescence, while OE accelerates these processes. NAC132 binds to the <i>NYE1</i> promoter to promote chlorophyll breakdown. It also affects male fertility by upregulating <i>EXPB1</i> , a gene linked to sexual reproduction.	Yuan <i>et al.</i> , 2023
Various SWNs	Secondary wall-associated NACs (SWNs), including SWN1(NAC86), SWN3(NAC62), SWN6(NAC131), SWN7 (NAC46), regulate secondary wall biosynthesis. OE in Arabidopsis activates secondary wall biosynthetic genes, resulting in the deposition of cellulose, xylan and lignin.	Zhong <i>et al.</i> , 2011
Various NACs	<i>NAC</i> genes (<i>NAC30</i> , <i>NAC32</i> , <i>NAC40</i> , <i>NAC108</i> , <i>NAC113</i>) contribute to internode elongation and affect both ear and plant height through regulation by <i>miR164</i> .	Yang <i>et al.</i> , 2022

CONTEXT & RESEARCH OBJECTIVES

The case for studying NAC24 in maize

This study focuses on the NAC24 TF in maize, building on a previous expression quantitative trait loci (eQTL) study carried out in our laboratory. This work investigated the expression variability of four *PIP* genes in maize leaves among a diversity panel of 252-hybrids, leading to the detection and mapping of numerous eQTLs, both local and distant (Maistriaux *et al.*, 2024). One of the distant eQTL associated with variation in *PIP2;5* expression in the leaf mature zone attracted our interest. This eQTL was located on chromosome 2, distant and independent from the *PIP2;5* gene. A closer examination revealed the presence of three annotated genes within this eQTL boundary, including *NAC24* (GRMZM2G008374) encoding a NAC TF. The significant single nucleotide polymorphism (SNP) was located within the first 100 bp of the *NAC24* gene promoter (Figure 8A). Furthermore, the similar expression profiles for *NAC24* and *PIP2;5* (low expression in leaf, high expression in roots) (Figure 8B), their reported drought responsiveness (Hachez *et al.*, 2006; Hachez *et al.*, 2008; Wang *et al.*, 2020), and the presence of putative NAC binding sites within the *PIP2;5* promoter (PlantRegMap, Tian *et al.*, 2020) (Figure 8C) provided additional convincing arguments for investigating NAC24 as a putative regulator of the *PIP2;5* expression.

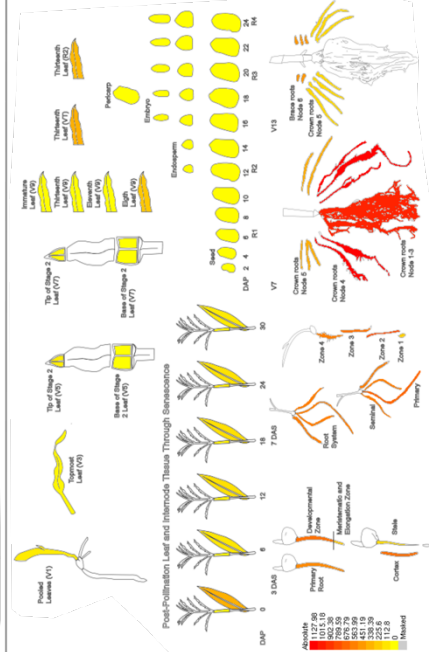
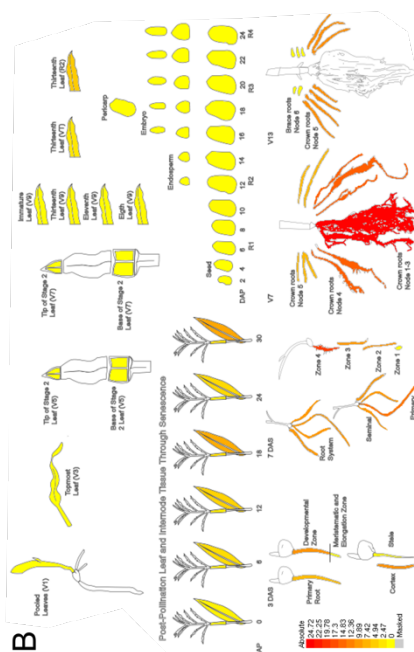
An increasing number of studies on NAC TFs and their roles in maize are appearing, making them a hot research topic. Understanding the function of NAC24 is therefore interesting due to its potential involvement in maize responses to biotic and abiotic stresses. In addition, NAC TFs have been associated with key processes such as grain filling, root development and nutrient use efficiency – traits that are linked to improved maize productivity under adverse environmental conditions. Studying NAC24 is very promising for advancing knowledge in crop science and breeding programs focused on improving stress tolerance and increasing yield.

Figure 8: The case for studying the NAC24 and PIP2;5 transcriptional relationship.

(A) eQTL analysis for PIP2;5 gene expression. A Manhattan plot of $-\log(p\text{values})$ for the association between individual SNPs and expression of PIP2;5. Each data point represents a single SNP and the x-axis indicates the location of the SNPs in the maize genome. The blue horizontal line marks the threshold of significance, all SNPs above this line are significantly associated with the PIP2;5 expression. The black arrow indicates a SNP present in the promoter of a the NAC24 gene (Maistriaux et al., 2024).

(B) eFP Browser of tissue-specific gene expression for NAC24 (top panel) and PIP2;5 (bottom panel). Both PIP2;5 and NAC24 are preferentially expressed in the roots (shown by red areas) compared to leaves. Expression data is derived from Hoopes et al. (2018) and Stelpflug et al. (2016). Adapted from figures from the maizeGDB browser (Winter et al., 2007).

(C) NAC transcription factor binding sites (TFBS) predicted in the PIP2;5 promoter sequence (1800 bp upstream of the TSS). Three NAC TFBS were identified in the PIP2;5 promoter, highlighted in yellow. Each contains the CACG sequence, recognized as a conserved NAC TFBS. The prediction was performed with the PlantRegMap prediction tool (Tian et al., 2020), using the databases available for *Z. mays*.



Research objectives

The first objective of this project is to explore the regulatory relationship between NAC24 TF and *PIP2;5* AQP in maize. AQPs, like *PIP2;5*, play important roles such as the hydraulic regulation of roots and leaves, cell expansion, organ development and stress responses. Their major contribution to the sessile autotrophic lifestyle is highlighted by their large diversity in plants. Over the years, research has revealed the complex regulation of plant AQPs at multiple levels; however, the molecular actors involved in regulating their expression are still largely unknown. Although the expression patterns of aquaporin genes have been well documented in various organs, developmental stages, and environmental conditions, the specific signaling pathways and specific players that regulate their expression remain unclear. To characterize the NAC24-*PIP2;5* relationship, this study will use techniques such as a *transactivation* assay in maize epidermal cells, the Dual Luciferase® Reporter (DLR) Assay in maize protoplasts and the Electrophoretic Mobility Shift Assay (EMSA) to determine whether NAC24 binds directly to the *PIP2;5* promoter sequence *in vitro*. This work aims to lay the foundations for identifying a regulatory element and the transcriptional players influencing the expression of *PIP2;5* gene, and to integrate it into broader regulatory networks linked to key physiological processes and plant adaptation strategies.

In the second part of the project, the focus is on the broader role(s) of NAC24 in maize, a TF that, to our knowledge, has not yet been specifically studied. The direct targets of NAC24 will be identified using DNA Affinity Purification sequencing (DAP-seq), which allows for uncovering binding sites across the maize genome. Identified target genes will then be validated through EMSA and DLR assays to assess the impact of NAC24 on their transcriptional activity. In addition, the project includes phenotyping *nac24* mutant maize plants, analyzing the potential correlation between observed phenotypes and the expression of the newly identified target genes.

By combining these approaches, this research aims to provide a comprehensive understanding of the role of NAC24 at the molecular level, as well as its integration at the organism level. This research is particularly interesting given the broader role of NAC TFs as key integrators of stress and developmental signaling networks in plants. Understanding the specific contributions of NAC24, like other members of the NAC family, could ultimately contribute to understanding and improving crop resilience and adaptability to global environmental challenges.

RESULTS

PART I: NAC24-*PIP2;5* relationship

Subcellular localization of NAC24

To determine the subcellular localization of NAC24, we prepared genetic constructs to express NAC24 fused to YFP in maize leaf protoplasts. The *NAC24* coding sequence (CDS) was PCR-amplified from B73 cDNA prepared from leaf samples, cloned, sequenced, and fused with the *yellow fluorescent protein* (YFP) CDS in 5' or 3' end under the control of the *35S* promoter from the Cauliflower Mosaic Virus (*p35S*). Transfection of the construct in maize leaf protoplasts showed that YFP-NAC24 and NAC24-YFP were localized in the nucleus (Figure 9), indicating that the position of the YFP did not alter the nuclear localization. Verifying the nuclear localization of NAC24 was an important preliminary step, as some NAC members are known to be anchored to the plasma membrane or ER and released by proteolytic cleavage under specific conditions (Shu *et al.*, 2024), which could have complicated the subsequent experiments.

The effect of NAC24 on the *PIP2;5* promoter

Transactivation assay in maize epidermal cells

To determine whether *PIP2;5* expression was regulated by NAC24, we compared the activity of the *PIP2;5* promoter in the presence and absence of NAC24 using a reporter assay. The promoter region of *PIP2;5* (B73 inbred line) was defined as the sequence spanning from the boundary of the first upstream annotated gene to the translation initiation codon (ATG, excluded) of *PIP2;5* for a total length of 1,884 bp. Maize epidermal cells were transiently transfected by biolistic particle delivery with a plasmid containing the *PIP2;5* promoter driving *mVenus* expression, and the *NAC24* cDNA controlled either the constitutive *p35S* (test condition) or no promoter (negative control). A double *35S* minimal promoter driving *mCherry* expression, present on the same plasmid, served as transfection normalizer. Fluorescence intensities were recorded using confocal microscopy, and the mVenus/mCherry ratios were analyzed.

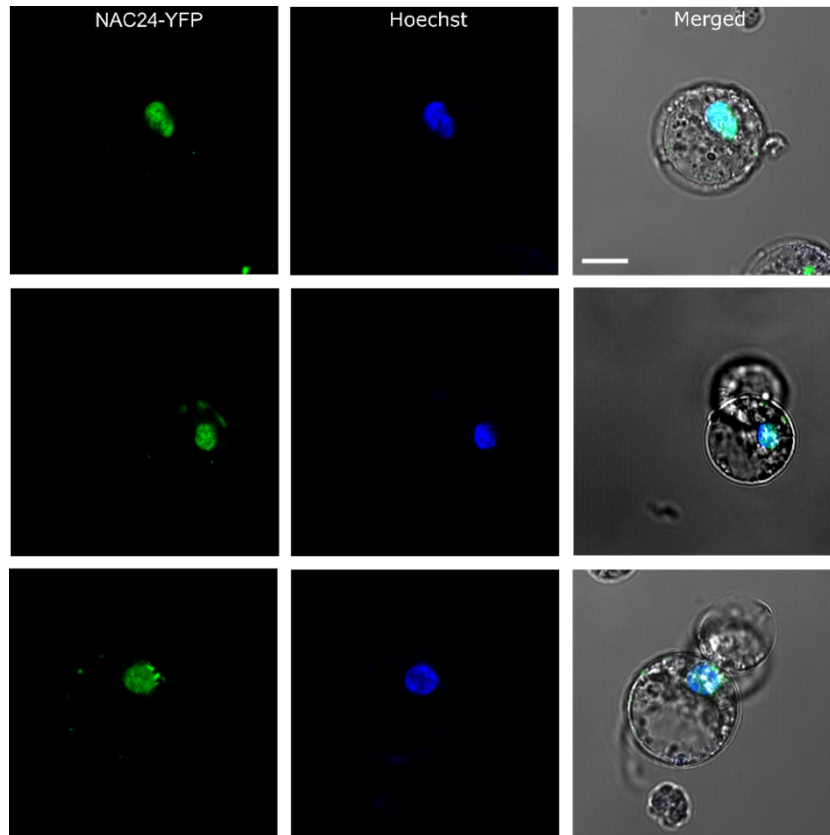


Figure 9: Nuclear subcellular localization of NAC24. The NAC24-YFP and YFP-NAC24 fusion proteins were expressed in maize leaf protoplasts (left panels), with Hoechst staining used as a nuclear marker (middle panels). The merged images (right panels) indicate colocalization of both fluorophores in the nucleus. The first two cells (top panels) expressed YFP-NAC24, while the last cell (bottom panel) expressed NAC24-YFP. Scale bar = 10 μ m.

As whole young plants (12-day-old) were used for the bombardments and maintained intact under normal growth conditions until the signal analysis, this enabled us to stay as close as possible to the physiological reality. Since *PIP* gene expression varies according to environmental factors such as the diurnal cycle, light, and humidity, it was essential to perform each biolistic bombardment at the same time, maintain the plants under identical conditions, and observe them consistently at the same intervals. Additionally, limiting all transient assays to the same leaf stage helped minimize potential variations in cellular development that could affect responsiveness to bombardment. As observed in Figure 4, a significantly higher mVenus/mCherry fluorescence signal ratio was observed in the presence of NAC24 (p-value = 0.0025 **), suggesting a *transactivation* of the *PIP2;5* promoter by NAC24 (Figure 10).

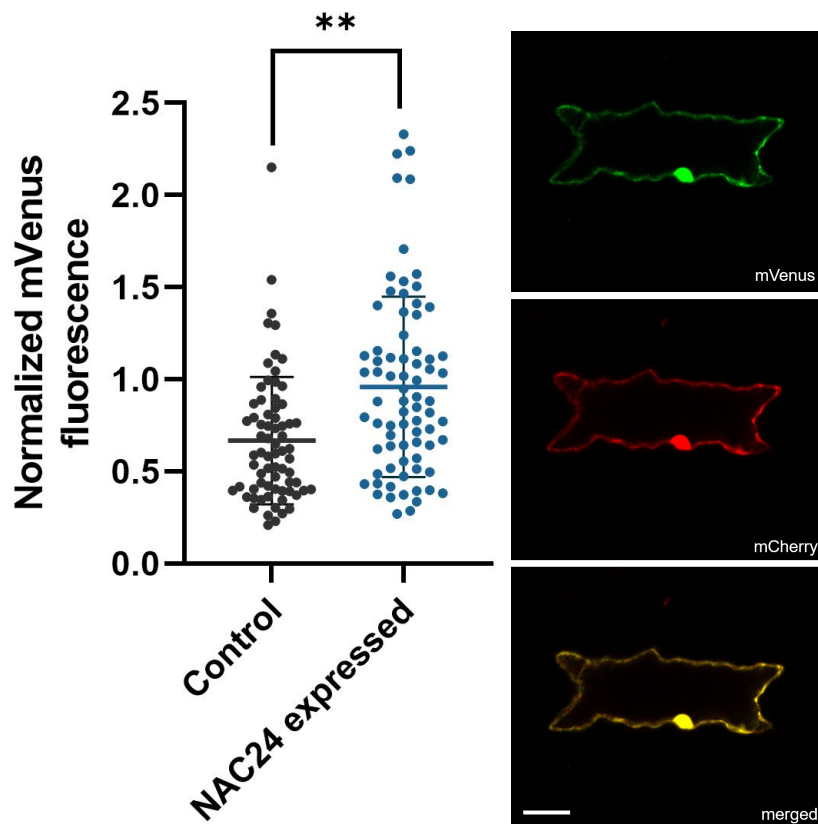


Figure 10: NAC24 affects the PIP2;5 promoter activity in transient transactivation assay performed in intact maize leaf epidermal cells. The PIP2;5 promoter drives the expression of mVenus, while mCherry, controlled by a double minimal 35S promoter on the same vector, serves as the normalizer. An increased mVenus/mCherry ratio indicates the transactivation of the PIP2;5 promoter by the NAC24 TF. Each dot represents an individual cell transfected by biolistics. Data from 76 cells across four independent blind experiments were analyzed using a linear mixed model, accounting for the experiment's dependency with a random intercept (p -value = 0.0025, **, lmer function in R). Representative images of transformed epidermal cells are provided. Scale bar = 20 μ m.

Dual-Luciferase® Reporter Assay in maize mesophyll protoplasts

To confirm the *PIP2;5* transactivation by NAC24 observed in maize epidermal cells, a Dual Luciferase® Reporter (DLR) Assay was conducted in maize mesophyll protoplasts. This assay can easily assess changes in promoter activity caused by the presence of a TF. The term “dual reporter” indicates the simultaneous expression of a firefly luciferase (*Photinus pyralis*), acting as an experimental reporter, and a Renilla luciferase (*Renilla reniformis*, sea pansy), acting as an internal control (Sherf *et al.*, 1996). Firefly luciferase (FLuc) and

Renilla luciferase (RLuc) display different enzyme structures and substrate requirements, making it possible to distinguish their respective bioluminescent reactions. Normalizing the activity of FLuc, the experimental reporter, to the RLuc activity, the internal control, reduces experimental variability generated by differences in cell viability, transfection efficiency, and cell lysis rate. Such assay has also the advantage to be highly sensitive and displaying concentration-signal linearity over a wide range, enabling a quantitative assay (Sherf *et al.*, 1996). In general, FLuc and RLuc cassettes are expressed from separate vectors that are co-transfected. However, we found that this approach affected the interpretation of results, as differences in ratios were primarily due to variations in the RLuc activity (data not shown). Previous studies have reported potential detrimental effects when co-transfecting plasmids (Farr & Roman, 1992; reviewed by Stepanenko & Heng, 2017). Experimental data indicate that co-transfection of different plasmids can influence the expression of each plasmid, which may limit the validation of the transfection experiment results (Huszár *et al.*, 2001). However, conducting the assay without an internal control plasmid would be time-consuming and the results would be difficult to analyze.

As a trial, we decided to express the reporter (FLuc and RLuc) and effector (NAC24) cassettes from the same plasmid to overcome the problems we encountered with co-transfection. Accordingly, the vectors to carry out the DLR experiment were designed using the Golden Gate modular cloning toolbox for plants (Engler *et al.*, 2014). We obtained genetic constructs in which the promoter activity of *PIP2;5* was directly correlated with the FLuc-produced bioluminescence, and in which the RLuc cassette served as a normalizer. We could therefore assess the changes in promoter activity due to the presence of the NAC24 effector, which was expressed from the same plasmid under the control of the *p35S*. Two control plasmids were constructed as well: one lacking a promoter for *NAC24* and the other expressing only the NAC domain of *NAC24* (NAC24₁₋₁₇₇) (Figure 11A). Since the NAC domain generally lacks a transcriptional regulatory domain (TRD) (Jensen & Skriver, 2014), this plasmid should serve as a negative control.

The preparation and transfection of maize mesophyll protoplasts were optimized for this assay (see Materials and Methods). Protoplasts were transfected with each construct. Following protoplast lysis, the FLuc reporter was first measured by adding Luciferase Assay Reagent II (LAR II) (Promega) to generate a luminescent signal. After quantifying the FLuc, this reaction was quenched, and the RLuc reaction was initiated simultaneously by adding Stop and Glo Reagent (Promega) to the same sample. Successive measurement

of FLuc and RLuc activities allowed for assessing the *PIP2;5* promoter activity of protoplasts transfected in the presence (or not) of NAC24 (Figure 11B).

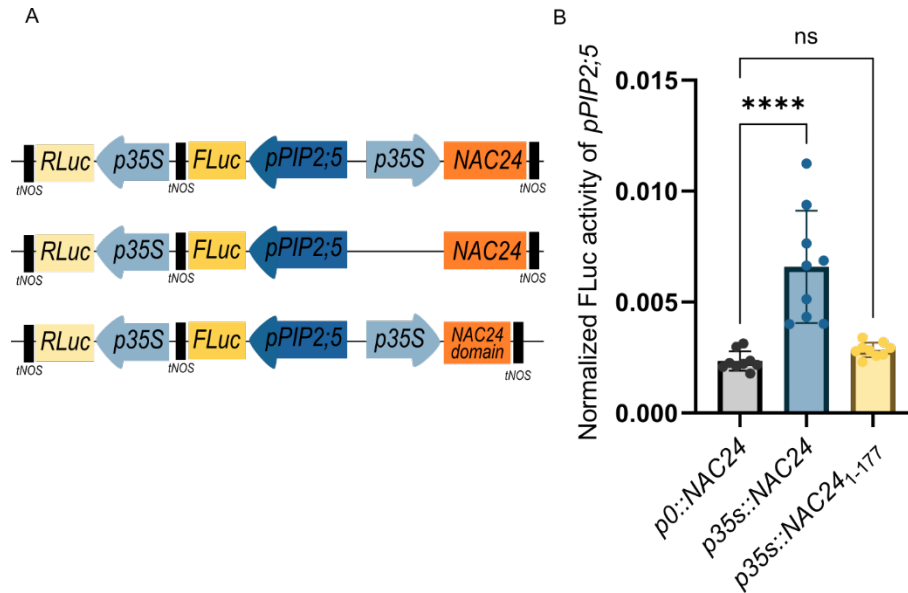


Figure 11: Dual luciferase reporter (DLR) assay conducted in maize mesophyll protoplasts for the activation of the *PIP2;5* promoter (*pPIP2;5*) by NAC24.

(A) Schematic representation of the polycistronic constructs used for the DLR assay (not drawn to scale). Final genetic constructs were obtained using the Golden Gate modular cloning toolbox for plants (Engler et al., 2014). Three constructs were assembled in which the reporters FLuc and RLuc are driven by the *PIP2;5* and 35S promoters, respectively. The NAC24 effector cassette was expressed on the same plasmid either under the control of the 35S promoter (*p35s::NAC24*) or without a promoter (*p0::NAC24*). A truncated version containing only the NAC domain (*p35s::NAC24₁₋₁₇₇*) was also used as a control.

(B) Results of the DLR assay. The three constructs were transfected in maize mesophyll protoplasts, and FLuc and RLuc activities were measured with the Glomax® 20/20 luminometer (Promega). The normalized FLuc activity (FLuc/RLuc) of *pPIP2;5* significantly increased when NAC24 was expressed (*p35s::NAC24*) compared to the control (*p0::NAC24*) (p -value = 1.15×10^{-6} , ****). This significant increase disappeared when a truncated version of NAC24 is expressed (*p35s::NAC24₁₋₁₇₇*) (p -value = 0.4402, ns). The data represents the mean ($\pm$ SE) of four independent experiments, each with three technical replicates. A linear mixed model was used to test the NAC24 presence effect, incorporating a random intercept taking the experience replication dependency into account (lmer function in R).

Firstly, expressing FLuc and RLuc reporters and the NAC24 effector from the same plasmid solved the issue met when co-transfecting two plasmids (not shown). As observed in Figure 11, the normalized FLuc activity, which is the ratio between the FLuc and the RLuc activity, was significantly higher when NAC24 was expressed compared with the control plasmid (NAC24 lacking a promoter). This confirmed that NAC24 activates the *PIP2;5* promoter in a DLR

assay performed in maize mesophyll protoplasts, in agreement with the *transactivation* results observed in maize epidermal cells.

However, when only the NAC domain of NAC24 was expressed, this significant increase disappeared, indicating that the TRD was located in the C-terminal of NAC24, and that the NAC domain alone could not activate the *PIP2;5* promoter.

***PIP2;5* expression in NAC24 mutants**

***PIP2;5* expression in Black Mexican sweet (BMS) lines expressing NAC24-FLAG**

Maize Black Mexican Sweet (BMS) suspension-cultured cells provide a convenient model for studying *AQP* gene expression and activity (Moshelion *et al.*, 2009; Cavez *et al.*, 2009). Unlike whole plants, which are complex multicellular organisms composed of various cell types with distinct gene expression profiles, the BMS single-cell system offers a simpler, low-complexity model with a homogenous population derived from a single cell type (Sheridan, 1975). This simplicity can be advantageous for studying the regulatory effects of TFs on gene expression. In maize, transgenic BMS lines are easier and faster to generate than fully transformed plants, a process that requires specialized infrastructure and expertise. Therefore, we developed a transgenic BMS line overexpressing a tagged version of NAC24 in a constitutive manner to investigate its impact on the *PIP2;5* expression.

The complete CDS of *NAC24* was amplified and the *FLAG-tag* sequence was added in 3' to follow its expression in transgenic BMS lines. Using the Golden Gate modular cloning toolbox for plants (Engler *et al.*, 2014), we created a final vector containing *NAC24-FLAG* under the control of the *35S* promoter, along with an *mCherry* cassette also driven by the *35S* promoter (Figure 12A). In addition, the vector contained a kanamycin resistance gene to select transformed BMS cells on kanamycin-containing medium. BMS cells were transformed with this vector via biolistic particle delivery, which enables DNA to be integrated into the cell genome. Only four kanamycin-resistant and fluorescent calli expressing mCherry were obtained and transferred to 5 ml liquid medium in a 6-well plate. After 14 days, nuclear and soluble fractions were analyzed by western blot using anti-FLAG and anti-mCherry antibodies (Figure 12B).

The NAC24-FLAG protein (~44 kDa) was detected in the nuclear fraction of the four independent cell lines (NAC24.1 – NAC24.4), while the mCherry signal was found in the soluble fraction. Although, the different lines showed

different levels of NAC24-FLAG protein, the quantity of mCherry protein appeared to be more uniform. An additional band was detected by the anti-FLAG antibody in the supernatant and was also observed in the WT. This band likely corresponds to an endogenous peroxidase responsible for the signal; however, this was not confirmed experimentally.

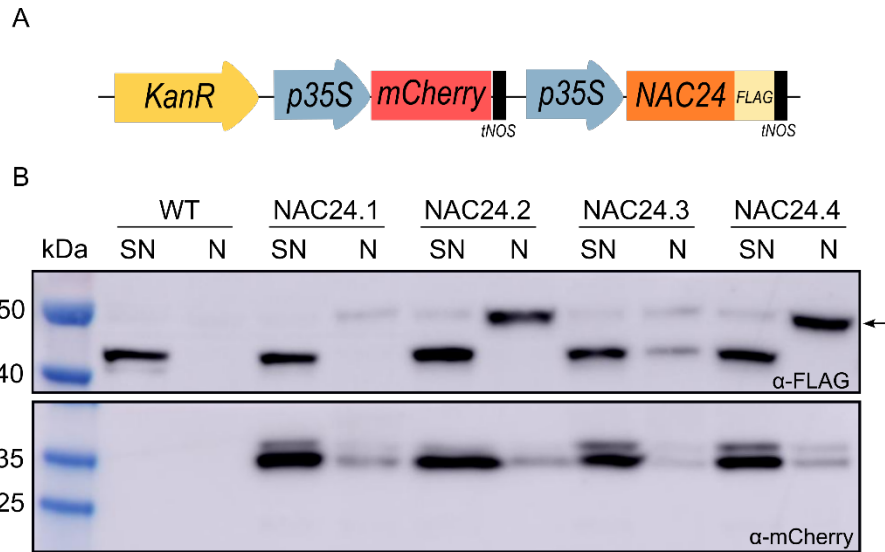


Figure 12: Generation and screening of stable BMS cell lines expressing NAC24-FLAG and mCherry.

(A) Schematic representation of the construct used for BMS transformation (not drawn to scale). The genetic construct was obtained using the Golden Gate modular cloning toolbox for plants (Engler et al., 2014). NAC24-FLAG and mCherry cassettes are driven by the 35S promoter. Additionally, the vector contains a kanamycin resistance cassette (The BMS lines overexpressing NAC24 were obtained by Jean Fontaine during his supervised master thesis).

(B) Screening of the stably transformed BMS cell lines for the presence of NAC24-FLAG and mCherry. After transferring fluorescent and kanamycin resistant calli into liquid medium (5 ml), nuclear (N) and soluble (SN) fractions were extracted from 14-day-old cultures. Each fraction (40 µg) was analyzed by western blot and compared to WT fractions using FLAG (Sigma-Aldrich) and mCherry (Rockland Immunochemicals) antibodies. The FLAG antibodies detected NAC24-FLAG (~44 kDa) in the nuclear fraction (black arrow), along with an off-target band also present in the WT fraction. The mCherry protein is observed in the soluble fraction of the four selected lines.

Since the liquid cultures of the different lines grew at different rates, we standardized the subculture process using plant cell volume (PCV). Fourteen-day-old BMS cells were systematically diluted to 5% PCV, and samples for RNA extraction were collected at 15% PCV during the log phase (around day 7 post-subculture). This method was chosen, instead of sampling at a fixed time point, because of the growth phase-dependent mRNA expression of several *PIPs* (Moshelion *et al.*, 2009). The extracted RNA was then used to

synthesize cDNA to compare *NAC24* and *PIP2;5* expression in the transgenic lines versus the WT using Real-Time quantitative PCR (RT-qPCR). For the RT-qPCR, we designed specific sets of primers to distinguish the transgene from the total *NAC24* mRNA. To target the *NAC24* transgene, the reverse primer was designed in the *FLAG* sequence, while for the total *NAC24* expression, primers located in the exons were used. For *PIP2;5* expression analysis, we used well-established primers previously validated in the laboratory, as well as *PIP1;1* primers as a control (Hachez *et al.*, 2006).

To assess transgene expression levels in the different lines, RT-qPCR data obtained with *NAC24*-*FLAG*-specific primers was normalized against two housekeeping genes (*EF1a* and *actin*) using the $2^{-\Delta C_t}$ method. This method is similar to the $2^{-\Delta\Delta C_t}$ method, except that the expression is not compared to a calibrator, as the transgene was not detectable in WT BMS cells (Schmittgen & Livak, 2008). Transgene expression was detected in the *NAC24.3* and *NAC24.4* lines, while the *NAC24.1* line showed no expression and the *NAC24.2* line failed to grow in liquid medium (Figure 13A). The expression levels of endogenous and total *NAC24*, along with *PIP2;5* and *PIP1;1*, were analyzed in the *NAC24.3* and *NAC24.4* lines (Figure 13B). Gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method, normalized to the endogenous reference genes *EF1a* and *actin*, which exhibited stable expression across samples (data not shown). Expression data are presented as fold changes relative to WT cells. Therefore, it is not possible to directly compare the expression levels of *PIP2;5*, *NAC24*, and *PIP2;1* genes.

A significant increase in total *NAC24* expression was observed, with a twofold increase in line 3 and a fourfold increase in line 4 compared with WT cells. This increase in *NAC24* expression was accompanied by a significant upregulation of *PIP2;5* expression, following the trend in *NAC24* expression. In contrast, *PIP1;1* expression, used as a negative control, remained unchanged. These results suggest that *NAC24* transcription factor activates *PIP2;5* expression when overexpressed in BMS cells, reinforcing its role as a transcriptional regulator of *PIP2;5*.

However, no *PIP2;5* protein signal was detected in western blot analysis of the microsomal fraction extracted from BMS cells using the *PIP2;5* antibodies (data not shown). This suggested that despite the increase in transcript levels, *PIP2;5* protein was still present at very low levels in BMS cells.

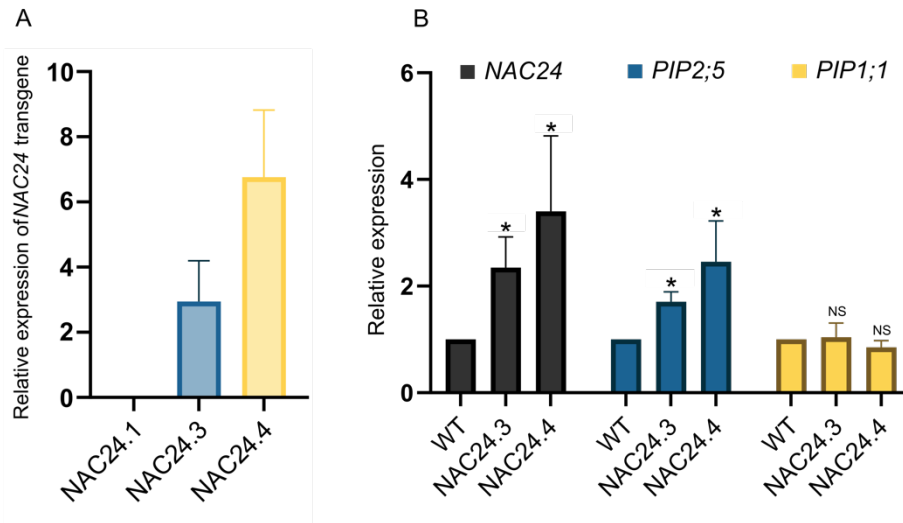


Figure 13: Expression of the transgene and endogenous NAC24, PIP2;5 and PIP1;1 in NAC24 overexpressing BMS lines determined by qPCR.

(A) NAC24 transgene expression levels in BMS cell lines. The transcript levels of NAC24-FLAG were calculated, using RT-qPCR, relative to that of endogenous genes EF1 α and actin. No transgenic NAC24 transcripts were detected in WT cell line. Lines 3 and 4 showed detectable NAC24 transcript levels, while line 1 did not express the transgene. Data represent the mean ($\pm$ SE) of two independent RT-qPCR experiments, each performed with two technical replicates per sample and primer set.

(B) Relative expression of NAC24, PIP2;5 and PIP1;1 in BMS lines overexpressing NAC24. The expression levels of NAC24 (endogenous and transgene), PIP2;5, and PIP1;1 were analyzed in two independent NAC24 overexpressing BMS lines using RT-qPCR. Gene expression data were calculated using the $2^{-\Delta\Delta Ct}$ method, with expression normalized to the endogenous genes EF1 α and actin and presented as fold changes relative to the WT line, whose expression levels were set to 1. A significant increase in PIP2;5 expression (p -value = 0,0137 *) was observed in NAC24 overexpressing lines compared with WT, while PIP1;1 expression remained unchanged. Data represent the results of two independent RT-qPCR experiments, each performed with two technical replicates per sample and primer set. Statistical significance was assessed using t -tests (GraphPad Prism).

Transposon-induced mutation in maize: Mu insertion in NAC24

To date, three public libraries of transposon-tagged maize mutants have been developed as resources for conducting functional genomics studies through loss-of-function mutations on virtually all maize genes (UniformMu: McCarty *et al.*, 2013; ChinaMu: Liang *et al.*, 2019; BonnMu: Marcon *et al.*, 2020). One Mu transposon allele was identified for NAC24 from publicly available F₂-stocks, which was designated as BonnMu0061727 (Marcon *et al.*, 2020). This *nac24* mutant, derived from a B73 and Co125 BonnMu line, has a transposon insertion in the first intron of the NAC24 gene, along with additional insertions

in other unknown genes. We received ten F₂ seeds, which required propagation and validation of *Mu* insertion in the *NAC24* intron by PCR-based genotyping. This method used gene-specific primers and *MuTIR6* primers to amplify DNA between the conserved terminal inverted repeats (TIR) flanking *Mu* elements and the adjacent genome. Leaf tissue samples from the third leaf were collected for gDNA extraction, followed by PCR with *NAC24*-specific and *TIR6* primers to confirm insertion presence (Figure 14).

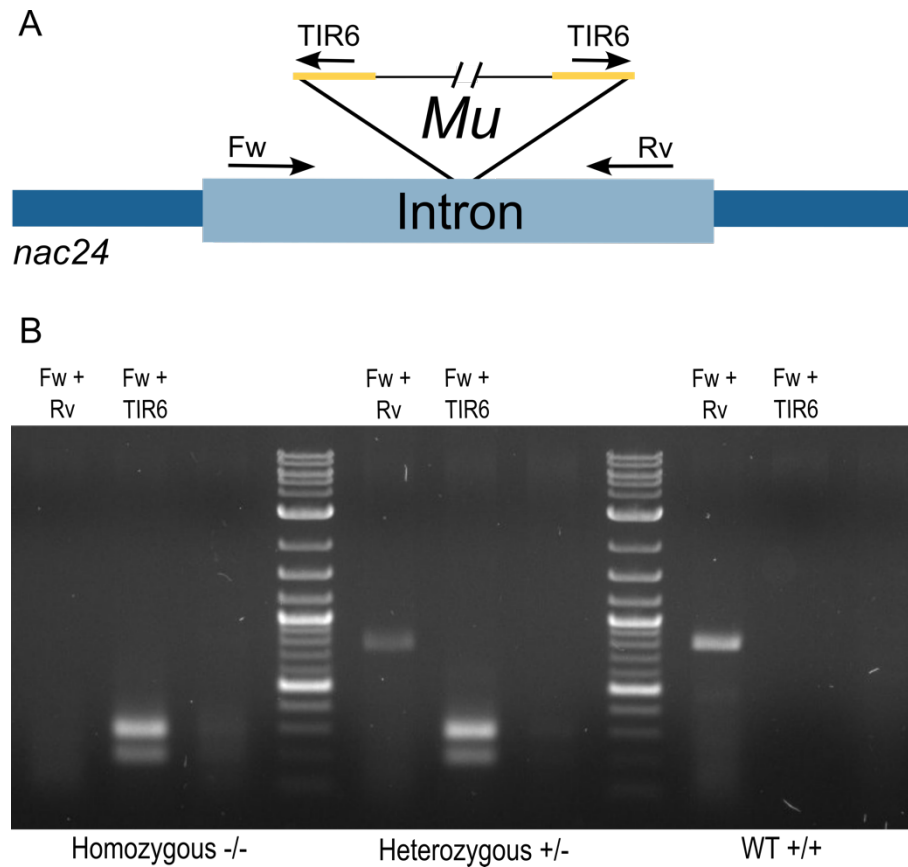


Figure 14: PCR Genotyping of F₂ plants carrying a *Mu* insertion in *NAC24*.

(A) Schematic representation of the *NAC24* gene disrupted by the *Mu* insertion in the first intron, with terminal inverted repeats (TIRs) highlighted in yellow at both ends. Yellow arrows indicate the outward-facing TIR-specific primers (*TIR6*) for the *Mu* transposon, while black arrows represent the position of the *NAC24*-specific forward (*Fw*) and reverse (*Rv*) primers flanking the insertion site.

(B) PCR genotyping of *nac24* mutants and WT plants using genomic DNA as a template. Gene-specific *Fw* and *Rv* primers generated an expected 800 bp product in WT and heterozygous plants, with no amplification observed in homozygous mutants. In contrast, a 300 bp product was amplified from *nac24* mutants when gene-specific primers were combined with the *MuTIR6* primer.

Gene-specific forward and reverse primers amplified the expected 800 bp product in WT and heterozygous plants, while no amplification was detected in homozygous mutants. A 300 bp product was specifically amplified in *nac24* mutants when gene-specific forward primer was paired with the MuTIR6 primer.

Sequencing of the PCR products verified both the Mu insertion and its exact location, allowing identification of the F₂ plants as WT², homozygous or heterozygous for the *NAC24* insertion.

After confirming the insertion site by sequencing and the genotypes of the F₂ plants, these had to be multiplied. As Bonn*Mu* lines typically contain several *Mu* insertions, we backcrossed them with B73 to help separate these insertions, enabling them to sort independently in future generations. This approach clarified whether the desired *Mu* insertion correlated with the initial observed phenotype. Notably, pollen production and ear development were affected in some F₂ plants, often making pollination less effective or even impossible. Figure 15 illustrates the crosses performed. Briefly, a homozygous F₂ plant was backcrossed with a B73 plant, and another homozygous F₂ plant was crossed with a heterozygous plant. Two heterozygous maize plants from the F₃ generation were then crossed to produce ears with WT, homozygous, and heterozygous seeds, which served as siblings for comparison in subsequent experiments.

RNA and gDNA were extracted from the primary roots of WT, homozygous, and heterozygous F₄ plants. The gDNA was used for PCR genotyping, while RNA was synthesized to cDNA to assess *NAC24* and *PIP2;5* expression across genotypes using RT-qPCR (Figure 16). Gene expression was quantified by the 2^{-ΔΔCt} method, normalized to the endogenous reference genes *actin* and *GADPH*, and presented as fold changes relative to a WT plant. A significant decrease in *NAC24* expression was detected in the primary roots of plants carrying the transposon in *NAC24*, with the reduction more pronounced in homozygous compared to heterozygous plants. Initially we were unsure whether the Mu insertion into the intron would result in a complete knockout (KO) or a knockdown (KD), but the observed decrease in *NAC24* expression, rather than a complete loss, confirmed that it was a knockdown. The reduction in *NAC24* expression was accompanied by a significant down regulation of *PIP2;5*, this decrease being more pronounced in homozygous than in heterozygous plants. This pattern confirmed the role of *NAC24* as a

² What is referred to as WT here does not represent true wild type; instead, these plants share the same genetic background (B73 and Co125 Bonn*Mu* line) but lack the transposon insertion in the *NAC24* gene.

transcriptional activator of *PIP2;5* expression and underlined its importance in maintaining normal *PIP2;5* expression levels in maize plants.

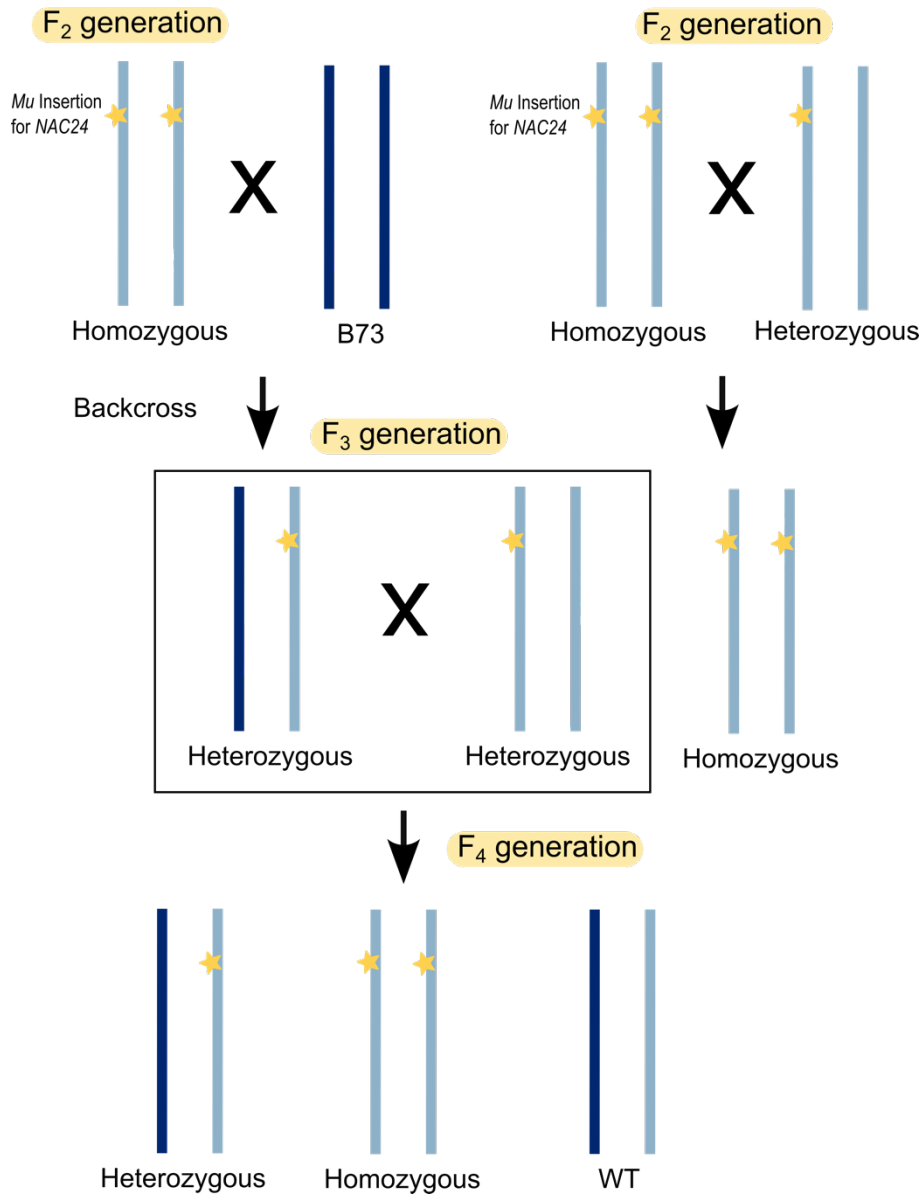


Figure 15: Schematic diagram of the crossing workflow to produce WT, homozygous, and heterozygous maize seeds. A homozygous F₂ plant for the transposon insertion was backcrossed with a B73 plant, while another homozygous F₂ plant was crossed with a heterozygous plant. Two heterozygous maize plants from the F₃ generation were then crossed to generate F₄ ears containing WT, homozygous, and heterozygous seeds, for the use in subsequent experiments.

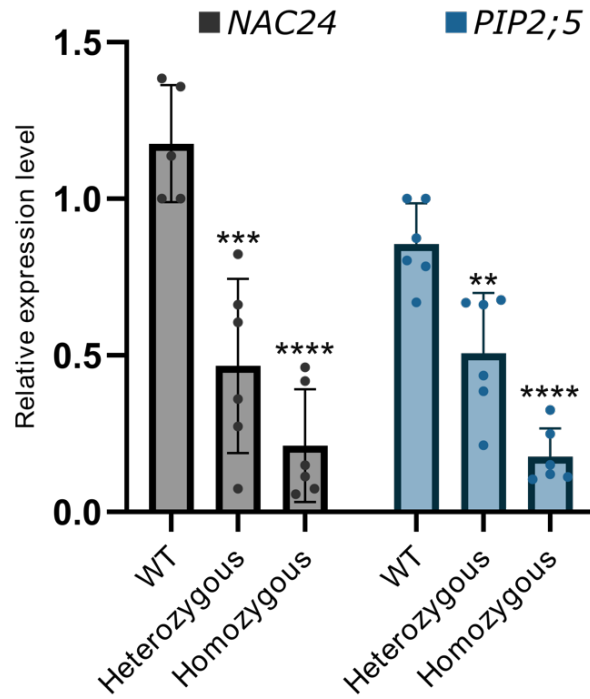


Figure 16: RT-qPCR analysis of NAC24 and PIP2;5 expression in BonnMu *nac24* mutants.

Relative expression levels of NAC24 and PIP2;5 were measured in six plants per genotype (WT, heterozygous, and homozygous) from two F_4 -generation ears, using cDNA from primary root tissue. Expression was calculated using the $2^{-\Delta\Delta C_t}$ method, normalized to the endogenous genes GADPH and actin and presented as fold changes relative to a WT plant, whose expression levels were set to 1. Significant reductions in mRNA levels were observed for NAC24 (p -value = 0,0009 and <0,0001, *** and ****) and PIP2;5 (p -value = 0,0043 and <0,0001, ** and ****) in mutants compared to WT, with the decrease more pronounced in homozygous plants. Data represent the results of two independent RT-qPCR experiments, each performed with two technical replicates per plant and primer set. Statistical significance was assessed using t -tests (GraphPad Prism).

The observed phenotypes of the *nac24* mutants, along with the potential physiological role of NAC24, will be discussed in Part II of the results.

Part I: Take-home messages

- The NAC24 TF, localized in the nucleus, *transactivates* the *PIP2;5* promoter in maize epidermal cells.
- A successful DLR assay was established in maize mesophyll protoplasts, using a single plasmid containing both Firefly and Renilla luciferase reporters along with the effector cassette.
- NAC24 *transactivates* the *PIP2;5* promoter in this DLR assay, with its transcriptional regulatory domain being located in the C-terminal region.
- OE of *NAC24* in BMS cells increases *PIP2;5* expression levels while transposon-induced *nac24* mutated plants exhibit reduced expression levels of both *NAC24* and *PIP2;5*.
- Although the experiments carried out do not definitively establish the direct or indirect nature of *PIP2;5* regulation by NAC24, NAC24 unquestionably activates the *PIP2;5* expression in maize.

Part II: NAC24 characterization in maize

What was already known about NAC24?

Even though no study had ever been published about NAC24, literature mining combined with bioinformatic tools provided useful information about this TF. The *NAC24* gene (GRMZM2G008374) is 1814 bp long, including two introns, and codes for a protein of 388 amino acid residues, with a calculated molecular mass of 42,5 kDa (Wang *et al.*, 2020). Multiple sequence alignment with other members of the NAC family confirmed that NAC24 contains the conserved NAC domain in its N-terminal region, divided into five subdomains (A–E) (Figure 17). NAC24 contains the conserved dimerization interface LPPGFRFHPTDEEL, where arginine and glutamic acid residues form salt bridges involved in dimerization. NAC24 also possesses the conserved DNA-binding motif WKATGTD, albeit with a unique substitution of alanine for threonine (WKTGTD) not observed in the other analyzed NAC TFs. Despite this variation, NAC24 maintains the conserved glycine residue, which induces strand curvature to facilitate entry of the DNA groove (Yoshiyama *et al.*, 2014) (Figure 17). Since NAC24 localizes in the nucleus, its nuclear import pathway may be linked to the presence of a nuclear localization signal (NLS) in its sequence. Using the NLS mapper prediction tool, NLSExplorer, an NLS was identified: the sequence VGMKKTLV (Figure 17). This NLS contains two basic lysine residues and is located in the D subdomain. Although this sequence resembles a monopartite NLS (a single cluster of basic residues), other basic residues, such as arginine and lysine, are present further down the sequence of the D domain. This suggests the possibility of a bipartite NLS, characterized by two clusters of positively charged amino acids. Notably, the D domain of NAC TFs has been shown to contain a bipartite NLS, defined by an abundance of basic amino acids, particularly lysine, which confer nuclear import capacity (Greve *et al.*, 2003; Welner *et al.*, 2016).

NAC24 has been annotated under different names across maize genome-wide studies, including ZmNAC8 (Fan *et al.*, 2014), ZmNAC38 (Shiriga *et al.*, 2014) and ZmNAC29 (Peng *et al.*, 2015), although it is referred to as NAC24 in MaizeGDB and by Wang *et al.* (2020). Despite the variation in nomenclature, phylogenetic analyses consistently place NAC24 within the NAM subfamily, one of the twelve NAC subfamilies (Fan *et al.*, 2014; Shiriga *et al.*, 2014; Wang *et al.*, 2020). Members of the NAM subfamily are primarily associated with apical shoot meristem formation, flower development and senescence (Souer *et al.*, 1996; Aida *et al.*, 1997; Pei *et al.*, 2013; Shibuya *et al.*, 2014), although some are also involved in responses to abiotic stress (Tweneboah & Oh, 2017). The members of the NAM subfamily are thus

predominantly expressed in tissues involved in flower development, which is not the case with NAC24.

NAC24 has a preferential, but low, expression in roots, according to the GENEVESTIGATOR database and the eFP Atlas browser (Hoopes *et al.*, 2019; Hruz *et al.*, 2008; Stelpflug *et al.*, 2016; Winter *et al.*, 2007) (Figure 8B). NAC24 transcripts were also found at moderate levels at all developmental stages with a peak at the seedling stage. Wang *et al.* (2020) reported that the NAC24 expression was also stimulated after 96 h of drought and repressed after rewatering. Additional data from the eFP stress browser showed an increase in NAC24 expression after imposing water stress (24 h) (Makarevitch *et al.*, 2015; Opitz *et al.*, 2014).

Using PlantRegMap (Tian *et al.*, 2020), several *cis*-regulatory elements were identified within the 2-kb promoter region of NAC24. These include binding sites for TFs from various families, such as BES1 (BRI1-EMS-Suppressor1), ERF (Ethylene-Responsive Factors), HSF (Heat Shock Factors), MADS-box, MYB (Myeloblastosis), NAC, and WRKY. BES1 TFs regulate brassinosteroid(BR)-responsive genes, playing crucial roles in plant growth and adaptation to environmental challenges (Shi *et al.*, 2022). ERF proteins contribute to growth, development, and stress responses by binding to ethylene-responsive elements, which are essential for activating genes involved in both abiotic stress responses and disease resistance (Zhang *et al.*, 2022). Similarly, MYB and WRKY TFs are key players in regulating plant stress responses and development (Farooqi *et al.*, 2023; Leng & Zhao, 2020; Ma *et al.*, 2023). This suggests that NAC24 might play a role in ethylene-responsive pathways and BR signaling, potentially involved in abiotic stress responses and developmental adaptation. This is consistent with the well-established roles of NAC TFs in broad regulatory functions in maize.

Figure 17: Multiple sequence alignment of different NAC domains. NAC domain sequences from ZmNAC24 and selected NAC members from maize (ZmNAC60, ZmSNAC1, ZmSNAC13) and Arabidopsis (ATAF1, ATAF2, AtNAM, ANAC019, CUC1, CUC2, ANAC092) were aligned with ClustalW (msa function in R). The position of the five highly conserved motifs (A-E) are indicated by red lines. Conserved residues involved in dimerization (Arg and Glu) are highlighted in orange, the WKTTGKD DNA-binding motif is highlighted in green, and the predicted NLS of NAC24 (identified with NLSExplorer) is highlighted in red (Li *et al.*, 2024). Identical amino acids are denoted with an exclamation mark, and similar amino acids are marked with an asterisk. The Uniprot accession numbers of the selected NAC proteins: the Arabidopsis ATAF1 (Q39013); ATAF2 (Q9C598); AtNAM (Q9ZNU2); ANAC019 (Q9C932); CUC1 (Q9FRV4); CUC2 (O04017); ANAC092 (Q9FKA0); the maize ZmNAC60 (A0A1D6GER6); ZmSNAC1 (B5A4B4); ZmSNAC13 (A0A804QVK5); ZmNAC24 (A0A1D6ESJ5).

Phenotyping of *nac24* transposon induced mutants

When propagating the *BonnMu nac24* mutant maize plants (Mu insertion in the first intron), we initially observed strong phenotypes in heterozygous and homozygous individuals. However, to link these phenotypes to the insertion, we analyzed segregating F₄ siblings coming from F₃ ears producing seeds having all three genotypes: WT, heterozygous, and homozygous (see Figure 15, Part I). In the F₄ generation, *nac24* KD plants exhibited phenotypes strongly correlated with the genotype: homozygous plants displayed more severe traits than heterozygous plants, compared to their WT siblings.

Effect of nac24 mutation on maize growth and leaf morphology

The *nac24* KD significantly affects plant and leaf morphology, producing a variety of phenotypes. Mutant plants displayed stunted growth (Figure 18A) and distinct leaf abnormalities, including yellow dots and lines (Figure 18B), reduced leaf size, and fusion of leaf blades. In some homozygous plants, the leaf blades were fused with each other or with themselves; they appeared to be rolled into each other (Figure 18C). The severity of the leaf phenotype varied among plants, with the most severe cases displaying extremely small leaves compared to the WT (Figure 18D). The height of the F₄ plants grown under normal conditions was measured from base to tassel and *nac24* KD plants were significantly shorter than their WT siblings (Figure 19A). The length of the internodes, measured between the third and fourth leaves at the 6th-leaf stage, was also reduced in the *nac24* KD plants (Figure 19B). Furthermore, mutant plants required more days to reach the same developmental stages as their WT counterparts. For example, at 25 days after germination (DAG), most WT plants had eight leaves, whereas homozygous plants typically had seven leaves, with some having only six (data not shown).

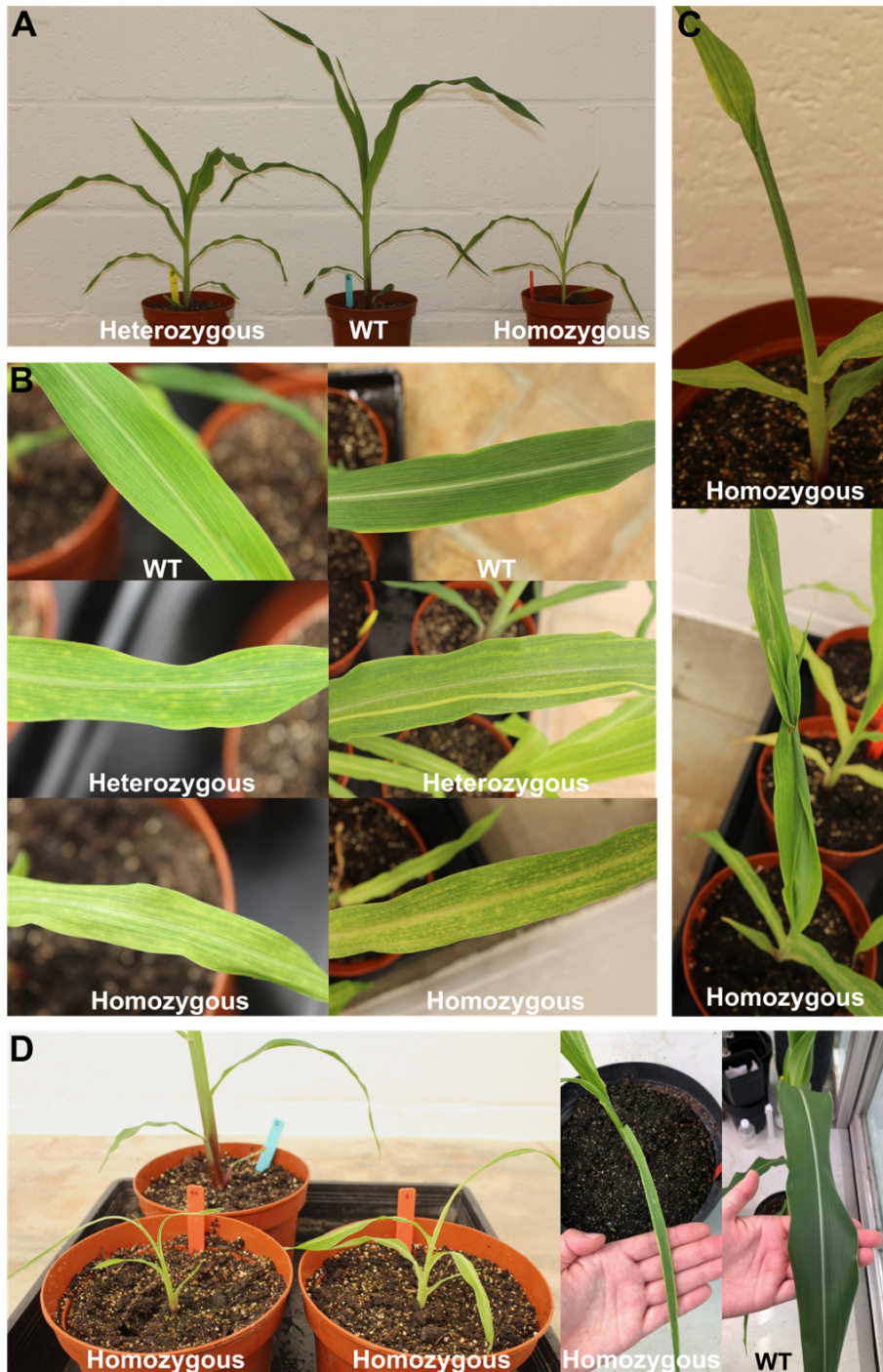
Figure 18: Leaf and plant morphology of *BonnMu nac24* mutant maize plants.

(A) Size comparison of WT, heterozygous, and homozygous plants at 23 DAG (Days after germination). Homozygous plants are noticeably smaller than their heterozygous and WT siblings.

(B) Mutant leaves display distinctive yellow dot patterns and yellow stripes. The first image for each genotype shows plants at 15 DAG, and the second shows plants at 26 DAG.

(C) Fused leaf blades in homozygous plants, appearing rolled into each other. The upper image shows a plant at 18 DAG, while the lower image shows a plant at 26 DAG.

(D) The most severe *nac24* mutants display extremely narrow leaves paired with a highly stunted growth. Two homozygous plants (26 DAG) are shown on the left, compared with a WT plant of the same age in the background. On the right, the leaf area of a 2-month-old homozygous plant is compared with a WT plant (hand for scale). All plants were grown in a phytotron under identical conditions: well-watered with a 16/8 h light/dark cycle (25°C/18°C) and ~70% humidity.



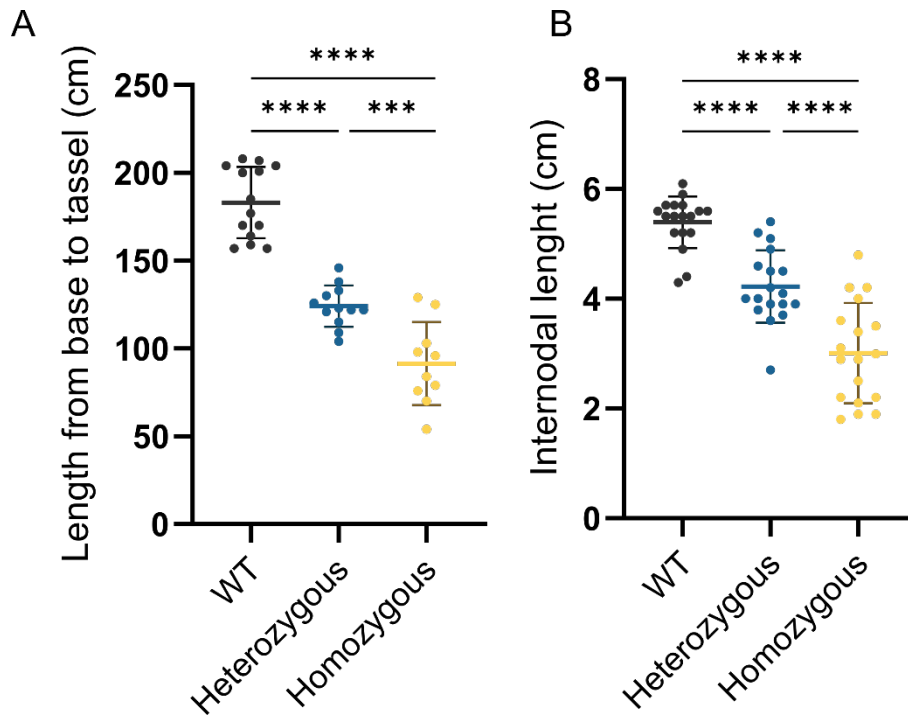


Figure 19: Plant and internodal length of BonnMu *nac24* mutants.

(A) Size comparison of WT, heterozygous, and homozygous plants. Plant height was measured from the base to the first branch of the tassel. Homozygous and heterozygous plants show significantly reduced height compared to WT plants (p-value <0.0001, ****). Data represents measurements from 15 plants per genotype; however, some homozygous plants failed to develop tassels.

(B) Internodal length measured between the third and fourth leaves at the 6th-leaf stage. Internodal lengths are significantly shorter in homozygous and heterozygous plants compared with their WT counterparts (p-value <0.0001, ****). Data represents measurements from 15 plants per genotype.

Data is represented as mean $\pm$ SD and statistical significance was determined using t-tests (GraphPad Prism).

*Effects of *nac24* mutation on maize fertility*

The *nac24* mutation appears to have an impact on fertility, as shown by the poor fertilization by the mutant pollen or the reduced fertility of female ears. Crosses involving mutant plants consistently produced ears with fewer grains. Flowering was irregular, with some plants not developing male or female flowers and others producing fewer anthers and silk (not quantified). The morphology of the tassels was also altered: unlike the typical long branches surrounding a central spike, the mutant tassels were reduced to a single central spike without lateral branches (Figure 20A).

Pollen viability was assessed with the fluorescein diacetate (FDA) test, also known as the fluorochromatic reaction test (Heslop-Harrison *et al.*, 1984),

evaluating plasma membrane integrity and esterase activity. Non-polar FDA enters the cytoplasm of viable pollen through intact membranes, where esterases hydrolyze it into fluorescein, a polar and fluorescent compound that accumulates, causing bright green fluorescence. If the plasma membrane is damaged or esterases are inactive, fluorescein leaks out, and the pollen fluorescence decreases. This rapid and reliable method has been widely used to assess pollen viability across species and correlates well with *in vitro* germination tests (Pinillos & Cuevas, 2008; Impe *et al.*, 2020). However, consistent results required optimization of the reaction time, FDA concentration, and ensuring measurements are taken within the first hour after staining, as fluorescein fades over time. After staining fresh pollen with FDA, we captured images using an epifluorescence microscope (Figure 20B) and quantified the fluorescence in pollen grains from six plants per genotype across two independent experiments (30 pollen grains per plant). *nac24* KD pollen showed a significant reduction in fluorescence intensity, indicating lower enzymatic activity or plasma membrane damage (~65% in homozygous and ~59% in heterozygous plants) (Figure 20C). The reduced fluorescence indicates lower enzymatic activity or damage to the plasma membrane in *nac24* mutant pollen, resulting in reduced viability.

Seeds from crosses with heterozygous or homozygous pollen/silk were significantly smaller and lighter and had lower germination rates than WT seeds (70% vs. 90%, respectively) (Figure 20D).

Although we did not directly phenotype the roots of these plants, it is highly likely that a root phenotype exists, given that *NAC24* is mainly expressed in the roots.

Overall, the *nac24* KD mutants exhibited reduced fertility and germination success, as well as altered leaf morphology and overall growth retardation, highlighting the role of *NAC24* in regulating multiple aspects of plant development and reproduction.

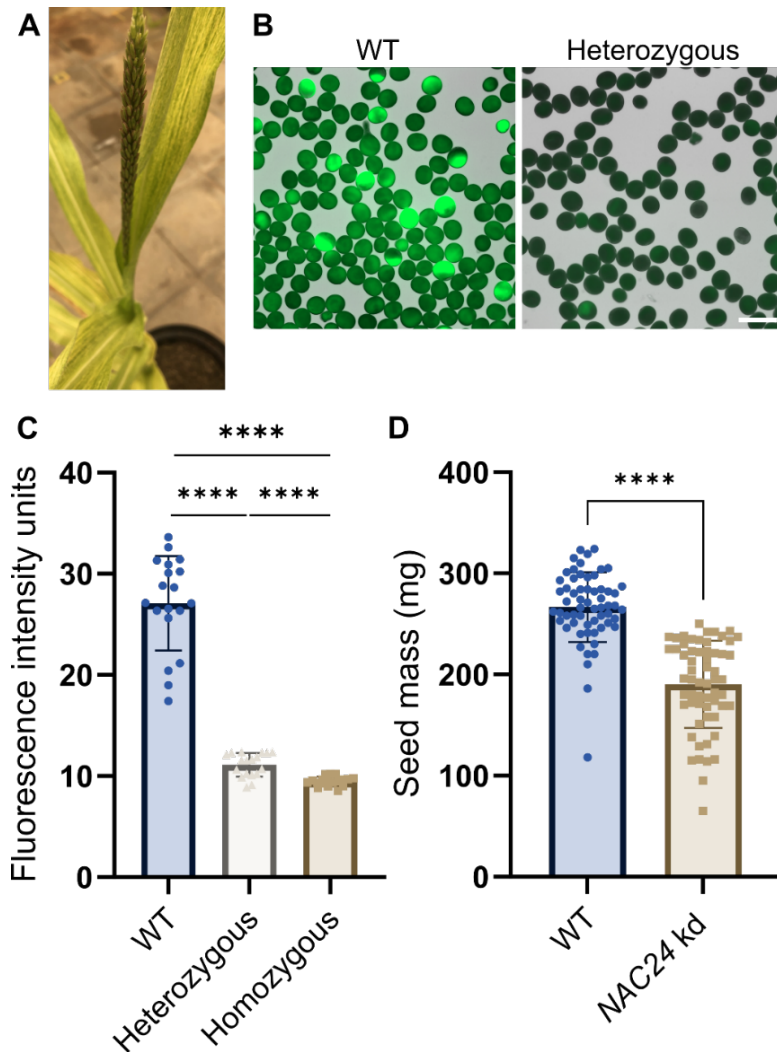


Figure 20: Impact of *nac24* mutation on maize fertility.

(A) Tassel morphology of *nac24* homozygous mutants. Mutant tassels are reduced to a single central spike with no lateral branches.

(B) Representative images of FDA-stained pollen from WT and *nac24* homozygous plants, captured using epifluorescence microscopy to assess pollen viability. Scale bar = 200 μ m.

(C) Quantification of FDA fluorescence reporting the enzymatic activity in mature pollen from WT, heterozygous, and homozygous plants. Pollen from homozygous and heterozygous plants show significantly reduced fluorescence compared to WT ($p < 0.0001$, ****). Data represent measurements from 6 plants per genotype, analyzing 30 pollen grains per plant. Each point on the graph represents the mean fluorescence of 10 pollen grains. Results are shown as mean $\pm$ SD.

(D) Seed mass comparison between ears from crosses involving heterozygous or homozygous pollen/silk and crosses with only WT plants. Seeds from *nac24* knockdown plants are significantly lighter than those from WT plants ($p < 0.0001$, ****). Data are based on measurements of 100 seeds from two different ears per genotype.

Identification of NAC24 target genes using DAP-seq

The pleiotropic phenotypes of *nac24* KD plants suggested that this TF acts on several pathways controlling development and physiology. To uncover NAC24 binding sites in the maize genome, we conducted DNA affinity purification sequencing (DAP-seq), which uses exogenously expressed TFs to probe genomic DNA (gDNA), avoiding the need for transgenic lines or gene-specific antibodies (O'Malley *et al.*, 2016).

DAP-seq workflow for NAC24

In accordance with the protocol of Bartlett *et al.* (2017) (Figure 21), DAP-seq libraries were generated using gDNA extracted from primary roots - where NAC24 is highly expressed - and from the tenth leaves at the 14-leaf stage (B73 accession) in order to compare NAC24 target genes between roots and leaves. The pFN19K vector (Promega) was used to fuse NAC24 to the HaloTag, and the NAC24-Halo fusion protein was expressed using the TNT SP6 Wheat Germ Protein Expression System (Promega). This fusion protein was then co-incubated with Magne® HaloTag® Beads (Promega), which covalently capture HaloTag fusion proteins. Following incubation with 1000 ng of library DNA and multiple washes, the DNA fragments bound to NAC24 were eluted by heating at 98°C. The eluted DNA was PCR-amplified to add Illumina-compatible indexes. For negative controls, mock DAP-seq libraries were prepared similarly, but using GFP-HaloTag fusion proteins instead.

Both NAC24 and GFP DAP-seq libraries were sequenced using the Illumina platform (Macrogen), generating approximately 30 million paired-end reads of 150-bp per biological replicate (three biological replicates for both NAC24 and GFP in roots and leaves, for a total of 12 replicates). After being trimmed to remove ambiguous nucleotides, adapters, and polymer tails, the reads were uniquely mapped to the maize B73_V4 reference genome using CLC Genomics (QIAGEN). The average mapping ratio was 88% for root samples and 99% for leaf samples; however, reads with multiple mapping locations in the genome were discarded. The ChIP-seq analysis tool of CLC Genomics Workbench was used as a peak caller to identify regions with enriched read coverage, pinpointing NAC24 binding sites.

In both DAP-seq and ChIP-seq analyses, saturation refers to the point at which increasing sequencing depth no longer results in the identification of new binding sites or peaks. Determining the sequencing depth required to reach saturation is essential for accurate and reliable peak detection (Pepke *et al.*, 2009). This depth depends largely on the size of the genome and the number and size of the TF binding sites. A common approach to assess

saturation involves subsample the data and evaluate the reproducibility of peaks at different sequencing depths. In addition, guidelines such as those from the ENCODE consortium provide recommended minimum read counts based on the target type.

For ChIP-seq experiments in maize, a minimum of 25–50 million read-pairs is typically recommended (Tu *et al.*, 2020). In our study, the lowest read count for all sample was 27 million reads, which is at the lower end of this range. However, the CLC Genomics peak caller incorporates a built-in saturation analysis, which did not indicate insufficient sequencing depth, suggesting that our analysis reached saturation.

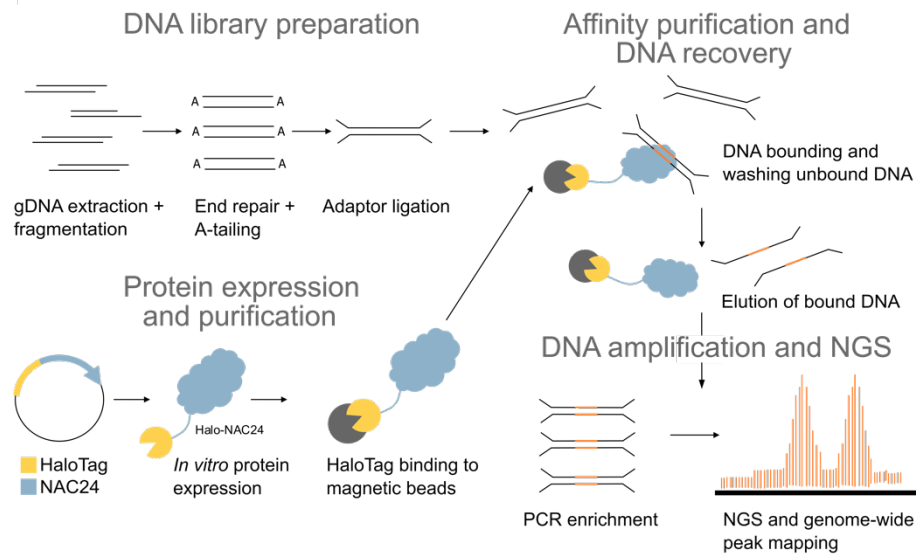


Figure 21: DNA affinity purification sequencing workflow. Genomic DNA was extracted from maize roots and leaves, fragmented into ~200 bp pieces, and adapters were ligated to repaired ends. NAC24 was cloned with a HaloTag, expressed in vitro, and bound to magnetic beads to capture the protein fusion specifically. After washing away non-specific proteins, the HaloTag-NAC24-bound beads were incubated with the gDNA library. Unbound DNA was removed, and DNA bound by NAC24 was eluted by heating and then PCR-amplified to add indexed sequencing primers. The final PCR products were purified and sequenced on an Illumina platform, with mapped reads used to identify NAC24 binding sites across the genome (adapted from Bartlett et al., 2017).

For the primary root gDNA, a total of 1119 peaks across the maize V4 genome showed significant read enrichment. Approximately 38% of these (420 peaks) were located within genic regions, including the distal promoter (-3 kb to -1 kb), the proximal promoter region (-1 kb to the transcriptional start site (TSS)), the transcribed region (5' UTR, 3' UTR, introns, and exons), and downstream regions (+1 kb after the stop codon) (Figure 22A). Among these, NAC24

probably bound in the promoter regions of 176 genes. For the leaf samples, 2040 peaks were identified, with 531 located in genic regions (26%). In these, NAC24 binding was observed in the promoter regions of 206 genes. To compare peak data between roots and leaves, gene information was extracted, and duplicate genes were removed. A Venn diagram revealed 176 common genes shared between the root and leaf datasets (Figure 22B). This initial dataset was too large to decide which genes to prioritize for follow-up experiments. To narrow down the list of potential NAC24 target genes, we used MEME analysis to identify NAC binding sites and GO (Gene ontology) analysis to classify genes by biological functions. This approach helped us differentiate likely positive hits from contaminants and select candidates with productive interactions for experimental validation.

Motif discovery for NAC24 binding sites

One of the strengths of DAP-seq is the ability to identify genome-wide DNA binding motifs for the target TF. To uncover conserved motifs in NAC24-bound regions, we analyzed sequences using MEME (Multiple EM for Motif Elicitation), a widely used tool for detecting recurring, ungapped sequence patterns in DAP-seq and ChIP-seq data (<https://meme-suite.org>; Bailey & Elkan, 1994; Bailey *et al.*, 2006). In the root dataset, MEME analysis identified two consensus motifs (Figure 22C). Motif 1 is a 21-bp palindromic sequence with two 4-bp C [G/T] T [G/A]³ sites separated by a 5-bp linker, found in 196 of the 420 peak sequences. Motif 2 is a 12-bp sequence containing a single CACG without palindromic repetition, detected in 128 sequences. This suggests that NAC24 preferentially binds to CGTG or CACG motifs, either in a palindromic arrangement or not. Motif 1 was compared to known TF binding sites (TFBS) from the JASPAR database (<https://jaspar.elixir.no>) (Sandelin *et al.*, 2004) using the Tomtom motif comparison tool (Gupta *et al.*, 2007). Tomtom identified 95 matches in the ArabidopsisDAPV1 database (O'Malley *et al.*, 2016) and 16 matches in the PBM (Protein binding microarray) database (Franco-Zorilla *et al.*, 2014). These matches are exclusively binding sites from Arabidopsis NAC TFs (e.g.: NAM, NAP, CUC1, ANAC038, ANAC057, ANAC087, ...). The MAST (Motif alignment & search tool) web tool was used to identify sequences in the *Z. mays* B73RefGen v4 (upstream genes) database that matched motif 1.

³ Throughout the rest of the manuscript, this C[G/T]T[G/A] sequence will be referred to as 'CGTG' representing the most common nucleotides within this motif.

A total of 55 upstream sequences matched the motif (p-value < 0.0001). The three top-scoring sequences were located in genomic regions upstream of genes targeted by NAC24 in the DAP-seq data:

- *cis-zeatin O-glucosyltransferase 1* (Zm00001d002707), which codes for a glucosyltransferase, a candidate gene for germination and seedling traits under stress (Rida *et al.*, 2021).
- *receptor-like kinase (TMK2)* (Zm00001d013415), likely an ortholog of *AtTMK1*, a transmembrane auxin receptor significantly upregulated in the preligule band (Neher *et al.*, 2023).
- *xyloglucan galactosyltransferase (MUR3)* (Zm00001d048387).

A GO analysis using GOMo for *A. thaliana* found no significant GO terms associated with motif 1. The same analyses were performed with motif 2, which yielded similar results, as motif 1 comprises motif 2.

To confirm NAC24 binding to motif 1, it was used to design a synthetic oligonucleotide for an electrophoretic mobility shift assay (EMSA) (see below ‘Electrophoretic mobility shift assays with NAC24’).

In the leaf dataset, the same motifs were detected but in a smaller fraction of the peaks: motif 1 appeared in 98 out of 531 peaks, while motif 2 was present in 67 peaks. Interestingly, these motifs were only found in peaks shared between the root and leaf datasets. Approximately 70% of the peaks in the leaf dataset lacked a motif, which raises the question of whether the DAP-seq was more effective in roots than in leaves, or if this reflects a biological difference between the tissues. In view of this, we focused the follow-up experiments on the genes identified in the root dataset, or the genes common to both datasets. Table S1 presents the final list of genes identified through DAP-seq performed on root gDNA, with their peak sequences containing motif 1 or 2.

We performed a GO enrichment analysis for biological processes and molecular functions using the PANTHER (Protein ANalysis THrough Evolutionary Relationships) statistical overrepresentation test (<https://www.pantherdb.org/>; Thomas *et al.*, 2022). Among the 420 genes located near the NAC24 binding sites, no enriched biological processes were identified. However, when focusing on the genes located near the peaks containing one or other of the two motifs, we observed a significant enrichment of biological processes within this gene subset compared to the whole *Zea mays* gene list (Figure 22D). The enriched biological processes included jasmonic acid signaling (GO:0009864, p = 0.0146), positive regulation of organ growth (GO:0046622, p = 0.0338), seed maturation (GO:0010431, p = 0.0338), and regulation of stress response (GO:0080134, p = 0.00648), among others. The enriched molecular functions identified in the same dataset

are shown in Figure S1, included DNA demethylase activity (GO:0032291, $p = 0.0328$), voltage-gated channel activity (GO:0022843, $p = 0.0423$), and alpha-amylase activity (GO:0004556, $p = 0.038$).

The GO analysis identified enriched biological processes related to the binding and regulation of NAC24. By combining GO and motif discovery, we selected candidate genes for follow-up experiments to investigate potential NAC24 targets. We focused on genes with peaks in the promoter region, as these are suitable for DLR assays to confirm the regulatory effect of NAC24. Although NAC24 may also influence gene expression with binding peaks in intronic or UTR regions, we chose not to pursue these genes. We also prioritized genes that could help explain the phenotypes of *nac24* mutants and contained motifs 1 or 2 in their promoter regions. To validate the binding of NAC24 to these promoters, we set up an electrophoretic mobility shift assay (EMSA) using a purified recombinant NAC24 protein. Additionally, DLR assays were performed to assess NAC24 regulatory effect on these target genes.

Our DAP-seq analysis did not identify *PIP2;5* or other *AQPs* as targets of NAC24. The absence of mapped reads in the *PIP2;5* promoter region suggests it is not a direct NAC24 target. However, the *PIP2;5* promoter contains two sequences (highlighted in Figure 8C) that closely resemble the NAC24 binding motif 2, sharing the conserved ACACGnnA motif. This raises the possibility that NAC24 could bind to the *PIP2;5* promoter, even though this interaction was not detected during the DAP-seq assay. To investigate further, an EMSA was set-up to test whether NAC24 is able to bind the *PIP2;5* promoter *in vitro*.

Figure 22: DNA affinity purification sequencing experiment with NAC24.

(A) Distribution of NAC24 binding sites across the maize V4 genome. DAP-seq experiment performed on primary root and 10th-leaf gDNA, performed with NAC24 and GFP as negative control (three biological replicates per tissue). Mapping and peak calling performed with CLC genomics (QIAGEN). In total, 1118 significant peaks were detected in roots and 2040 in leaves, with 38% and 26% of these peaks, respectively, located within genic regions (promoter region, gene, and downstream region).

(B) Venn diagram showing DAP-seq results for root and leaf DNA libraries. The diagram highlights 246 genes unique to the leaf dataset (orange), 176 genes unique to the root dataset (blue), and 176 genes common to both datasets.

(C) MEME WebLogos of NAC24 binding motifs identified by DAP-seq. Motif 1 displays a palindromic CGTG sequences separated by a 5-bp linker, forming a 21-bp motif that was found in 196 peak sequences. Motif 2 is a single 12-bp sequence containing the CACG consensus without palindromic repetition, identified in 128 peak sequences.

(D) Gene-ontology analysis performed on genes located near a NAC24 binding site in root gDNA and containing motif 1 or 2. The y-axis represents the fold enrichment of these genes compared to all the *Z. mays* genes, while the x-axis represents the biological processes for which the genes are overrepresented. The analysis was conducted using the PANTHER overrepresentation test with Fisher's exact test to assess statistical significance.

Production and purification of recombinant NAC24

The study of the interaction between NAC24 and target promoters by EMSA first required the production of the recombinant NAC24 protein in *Escherichia coli*, followed by purification steps. Two affinity chromatography methods were selected, using two different tags: a cleavable GST tag and a 6xHis-tag. The cleavable GST tag and the small 6xHis-tag were chosen to minimize interference with the purified protein and to preserve its binding ability during the EMSA.

Purification of NAC24-GST

A survey of the literature indicates that the glutathione S-transferase (GST) tag is an appropriate choice for the purification of NAC TFs (Jensen *et al.*, 2010; Olsen *et al.*, 2005; Tran *et al.*, 2004). The tag exploits the strong affinity of GST for reduced glutathione, its natural substrate, providing an efficient method for single-step purifications (Schäfer *et al.*, 2015). In addition, the GST protein accumulates in the cytoplasm and due to its large size, the solubility is expected to spread to the fusion partners (Smith & Johnson, 1988). To first produce and purify NAC24, the full-length NAC24 CDS was PCR-amplified and the sequence of the Tobacco Edge Virus protease (TEV) cleaving site was fused at its 5'. The inclusion of the TEV cleaving site would allow specific removal of the tag at 4°C and in various buffers (Lundbäck *et al.*, 2008; Tropea *et al.*, 2009; Sun *et al.*, 2012). The amplified fragment was digested and cloned into the pGEX4T-1 vector (Amersham Biosciences) to produce the GST-TEV-NAC24 fusion protein in *E. coli* C41. Derived from BL21, the C41 strain prevents cell death during recombinant protein expression and offers higher transformation and plasmid expression rates (Miroux & Walker, 1996; Dumon-Seignovet *et al.*, 2004).

Since it is impossible to predict whether recombinant proteins will remain soluble or accumulate in occlusion bodies, a trial experiment was set up to check solubility of GST-TEV-NAC24 and assess the optimal growth temperature (Figure 23). GST-TEV-NAC24 (~68,5 kDa) was found mainly in the soluble fraction, which means that the recombinant protein was sufficiently soluble in *E. coli* in all growth conditions. However, degradation bands were observed at all induction temperatures, a common issue due to *E. coli* endo- and exoproteases (Murby *et al.*, 1996; Ryan *et al.*, 2013). To avoid or limit

proteolysis, the expression and lysis conditions were optimized by adjusting the induction parameters and adding protease inhibitors to the lysis buffer.

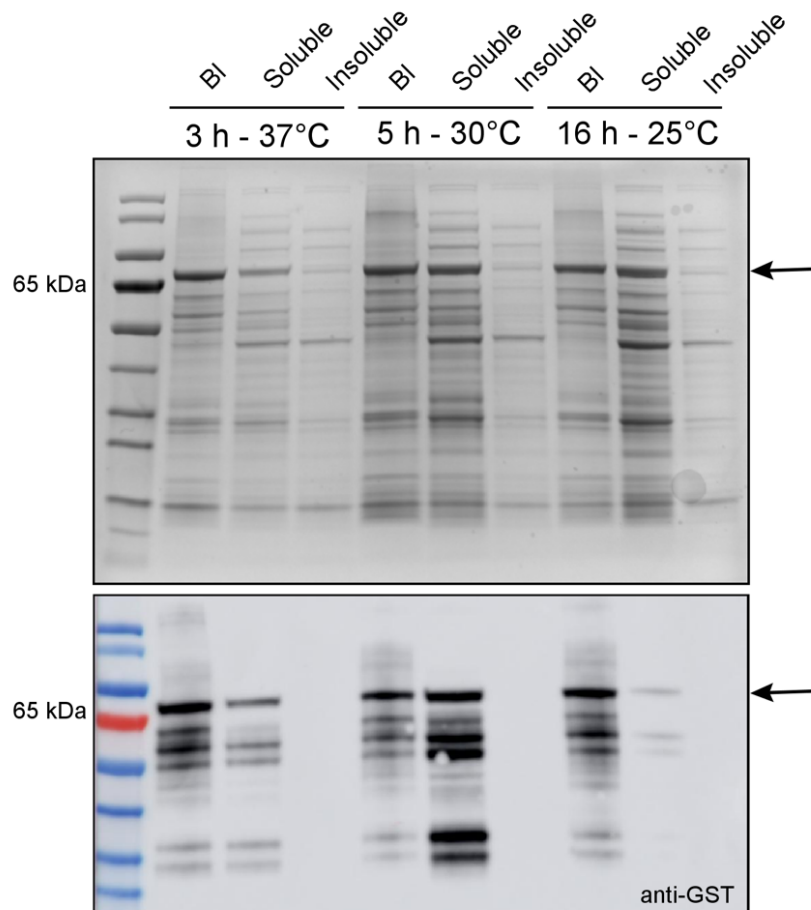


Figure 23: Screening experiment to assess protein expression levels and solubility of the GST-TEV-NAC24 fusion protein grown at different temperatures. Mini cultures of 50 ml *E. coli* were grown until OD_{600} of ~ 0.5 . IPTG was added and the cell cultures were induced at 25, 30, and 37°C for 3, 5, and 16 h respectively. Cell pellets were harvested before (BI) and after the different induction periods and homogenized in protein lysis buffer. The supernatant (soluble proteins) was recovered after sonication and ultracentrifugation. The pellet (insoluble proteins) was resuspended in the initial volume of protein lysis buffer. Each sample (5 μ l) was analyzed by SDS-PAGE, stained with Coomassie blue (top panel), and by anti-GST (Invitrogen) Western blot (bottom panel). A protein ladder was included in the gels (PageRuler, Prestained protein ladder, Thermo Scientific). The arrow points to the band corresponding to GST-TEV-NAC24 (~ 68.5 kDa).

The pGEX vectors have their expression controlled by the *tac* promoter, which is induced by the lactose analog isopropyl- β -d-1-thiogalactopyranoside (IPTG). However, we noticed a high protein expression in absence of the inducer, suggesting that the *tac* promoter of pGEX4T-1 was leaky or that the

repressor, *lacI*, is not well expressed. The Lac system has already been reported in the literature as leaky, with high-level production of recombinant proteins in *E. coli* occurring even without the inducer (Nielsen *et al.*, 2007; Penumetcha *et al.*, 2010; Zhang *et al.*, 2015). Finally, we found that culturing cells at 30°C before induction and applying IPTG at a lower OD (~0.3 - 0.4) reduced the leakiness of the Lac system. The screening experiment also showed that there were no major differences in solubility or degradation between the different growing temperatures.

Afterwards, cells were consistently, induced at 30 °C for 5 hours. The cells were harvested, washed, and resuspended in the lysis buffer. Cells were then lysed by sonication, which gave a higher protein yield than grinding (data not shown). Centrifugation was used to pellet unlysed cells and inclusion bodies, and the soluble fraction was saved for purification.

A purification protocol was established for NAC24 and is described in Materials & methods section. NAC24-GST fusion protein was purified using Glutathione-Sepharose chromatography followed by size exclusion chromatography (SEC) (Superdex 200 Increase 10/300 GL, GE Healthcare Bio-Sciences) (Figure 24A). The elution profile of NAC24 was analyzed by SDS-PAGE using fractions collected during SEC (Figure 24B). The NAC24 recombinant protein eluted near the void volume (~8 mL), instead of the anticipated elution volume of ~15 mL for a protein of its size. This earlier elution suggests potential oligomerization, aggregation, or non-specific interactions with the column matrix. Aggregation can occur as a random process involving protein misfolding and assembly into larger, often insoluble complexes, whereas oligomerization is an ordered association of protein subunits (Bondos & Bicknell, 2003). To evaluate whether non-specific interactions between NAC24 and the column matrix contributed to the early elution, the ionic strength of the mobile phase (initially 50 mM phosphate, 150 mM NaCl, pH 7.0) was increased to 500 mM NaCl. High salt concentrations reduce secondary ionic interactions, improving peak shape and protein size estimation (Fekete *et al.*, 2014). Despite this adjustment, the elution profile of NAC24 remained unchanged, indicating that non-specific ionic interactions were not a factor. Next, dithiothreitol (DTT) (5 mM) was added to determine if NAC24 oligomerizes via disulfide bond formation. DTT reduces disulfide bonds, potentially breaking oligomers into monomers, but no change in elution volume was observed, suggesting disulfide bonds were not responsible for the early elution. Finally, 0.5% DDM (n-dodecyl- β -D-maltoside), a detergent to reduce hydrophobic interactions, was added to the mobile phase to disrupt potential hydrophobic-driven aggregation. This modification also did not alter the elution profile (Figure 24C). This suggests that NAC24 aggregates are

probably stabilized by non-reducible, non-disruptible forces, making them resistant to dissociation.

Tackling the aggregation problem

Proteins can aggregate due to nonoptimal conditions during protein expression, purification, or storage that alter protein structure leading to loss of activity (Bondos & Bicknell, 2003). Hence, before performing any EMSA, the aggregation issue of NAC24 had to be addressed. Aggregation can be prevented by changing the protein environment (pH, salt concentration, appropriate additives, etc.). However, after modifying the composition of the extraction buffer and the protein storage conditions several times, no change was observed in the aggregation state of NAC24 (data not shown).

Proteins that are intrinsically disordered proteins or contain intrinsically disordered regions are prone to aggregation due to the absence of a stable tertiary structure. Some proteins also exhibit a higher aggregation propensity, often attributed to the presence of short aggregation-prone regions. These regions are defined as short sequence fragments (5-15 amino acids) characterized by high hydrophobicity and a low net charge, which contribute to aggregation under certain conditions (Conchillo-Solé *et al.*, 2007; Lebendiker *et al.*, 2015). In the case of NAC24, the C-terminal domain (CTD) is particularly enriched with these short hydrophobic sequences compared to the more structured N-terminal NAC domain (Figure S2A). The CTD of NAC24 was then evaluated for intrinsic disorder using the PONDR VL3 predictor (Xue *et al.*, 2010). The analysis confirmed that the CTD is significantly more disordered than the NAC domain, suggesting that it contributes to the aggregation of the NAC24 protein (Figure S2B).

A review of the literature reveals that for EMSAs, only the NAC domain, which harbors the DNA-binding function, is generally expressed and purified (Tran *et al.*, 2003; Olsen *et al.*, 2005; Chun *et al.*, 2023). Interestingly, Jensen *et al.* (2010) showed that full-length Arabidopsis ANAC019 binds a NACBS with similar affinity as its isolated NAC domain. Accordingly, the sequence encoding only the NAC domain (NAC24₁₄₋₁₇₅) was PCR-amplified and cloned into the pQE-60 vector (Addgene) to express the His-tagged NAC domain of NAC24. His-tags were chosen for their small size, which should minimize interference during EMSA binding. The fusion protein (~20 kDa) was expressed in *E. coli* C41 cells under the same conditions as the full-length GST-tagged NAC24. Purification was performed using Ni-NTA agarose, and the eluted product was passed on the SEC column to assess the molecular mass. The different elution peaks were examined by SDS-PAGE (Figure 25B). NAC24₁₄₋₁₇₅-6His was detected around an elution volume of 15 ml, which

corresponds to a molecular mass of ~44 kDa (estimated from calibration with a mix of molecular mass markers), indicating that NAC24₁₄₋₁₇₅ forms a dimer.

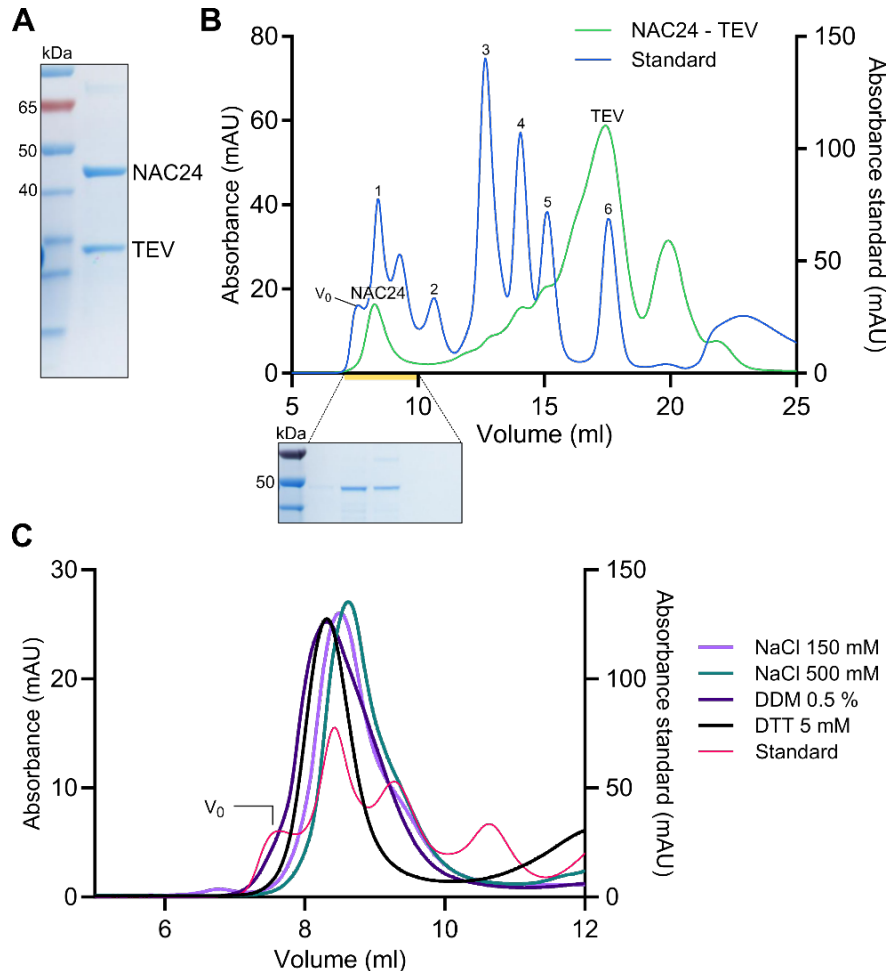


Figure 24: Purification and size exclusion chromatography of NAC24.

(A) Expression of GST-TEV-NAC24 was induced for 5 h at 30°C. After cell homogenization, the soluble fraction was incubated with Glutathione Sepharose resin. After washing, GST-TEV-NAC24 was cleaved on column using TEV protease. The resulting cleaved product was analyzed by SDS-page and stained with Coomassie blue. NAC24 has an expected molecular mass of 42.5 kDa, while the TEV protease is 27 kDa.

(B) NAC24 and TEV protease were separated using a Superdex 200 Increase 10/300 column on the ÄKTA Pure system. The elution profile of NAC24 and TEV (green) was compared to a standard protein mix (blue) with known MWs: 1. Thyroglobulin (670 kDa), 2. Ferritin (440 kDa), 3. Aldolase (158 kDa), 4. Conalbumin (75 kDa), 5. Ovalbumin (44 kDa), and 6. Ribonuclease (13 kDa). Elution fractions between 7.5 and 10 ml (yellow) were analyzed by SDS-PAGE. NAC24 was detected in fractions eluting between 8 and 9 ml. The x-axis represents elution volume and y-axis the absorbance.

(C) Evaluation of the effect of buffer additives on NAC24 elution profile using the Superdex 200 column. The SEC was repeated with modified mobile phases containing 500 mM NaCl (to minimize ionic interactions), DTT (to reduce oligomerization driven by disulfide bond), or 0.5% DDM (to disrupt hydrophobic interactions). The elution volume of NAC24 was unaffected by the modifications.

NAC24 dimer

NAC24 was found to elute as a dimer during SEC, consistent with previous findings that NAC TFs form functional homodimers capable to bind DNA (Chun *et al.*, 2023). The dimerization interface of NAC TFs is mediated by the conserved sequence LPPGFRFYPTDEEL, where two salt bridges form between the arginine residue in one monomer and a glutamic acid in the other (Olsen *et al.*, 2005; Jensen *et al.*, 2010). To evaluate the role of these salt bridges in NAC24, a NAC24(R26A) mutant, replacing Arg-26 with alanine, was generated. Both WT and mutant NAC domains were expressed, purified, and analyzed by SEC. The WT protein eluted at a volume (~15 ml) consistent with its dimeric form, whereas NAC24(R26A) eluted later (~17 ml), corresponding to a monomeric state (Figure 25A). These results emphasized the importance of Arg-26 and the associated salt bridges in maintaining the dimeric state of NAC24.

At this point, the NAC24 was adequately purified and non-aggregated, which made it possible to carry out EMSA experiments with the fractions coming from the SEC (Figure 25B).

Electrophoretic mobility shift assays

Gel electrophoresis mobility shift assays are used to detect protein complexes with nucleic acids. In summary, a binding reaction mixture of a protein and nucleic acids is subjected to electrophoresis under native conditions through a polyacrylamide or agarose gel. After electrophoresis, the distribution of nucleic acids in the gel is visualized. Generally, the formation of a protein-nucleic complex reduces the electrophoretic mobility of the nucleic acids in the gel matrix (Fried & Crothers, 1981; Lane *et al.*, 1992). Its ease of use, versatility and high sensitivity make the EMSA a powerful method that has already been used to study the binding ability of several NAC TFs (Ogo *et al.*, 2008; Liu *et al.*, 2010; Ge *et al.*, 2017; Xiang *et al.*, 2021). However, whether a complex existing in solution is detected as a retarded band on gel depends strongly on the design of the DNA probe, the binding reaction conditions, and gel running conditions (Hellman & Fried, 2007; Gaudreault *et al.*, 2009). Optimization of the experimental conditions must be done for each specific protein-DNA interaction.

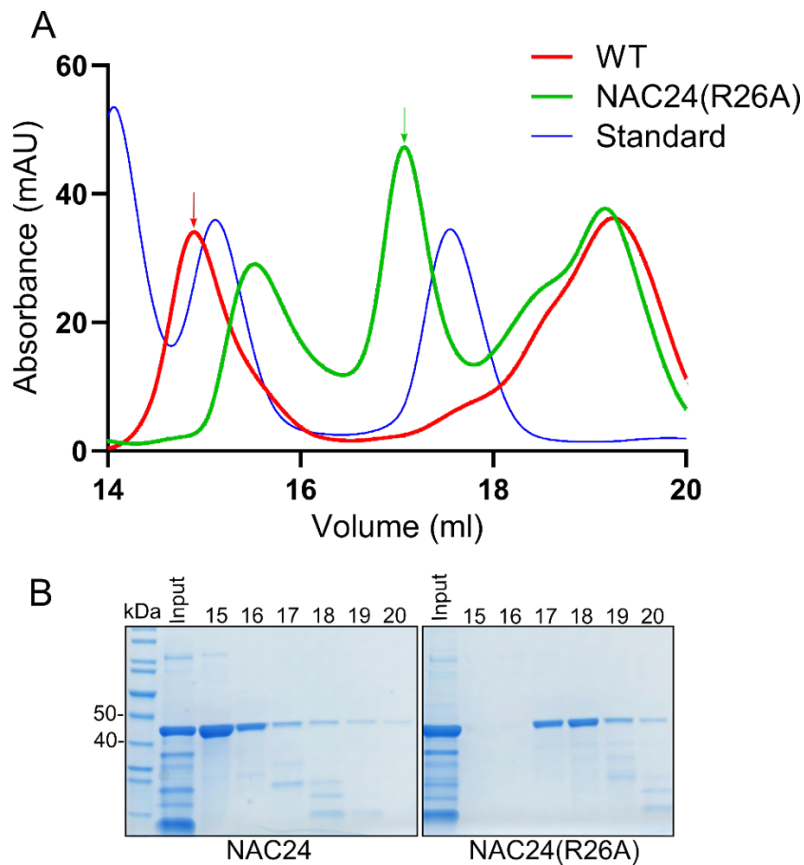


Figure 25: SEC analysis of NAC24₁₄₋₁₇₅ WT and R26A mutant.

(A) Elution profiles of WT NAC24 NAC domain (red) and R26A mutant NAC domain (green) compared to molecular weight standards (blue): 1. Ovalbumin (43 kDa) and 2. Ribonuclease (13 kDa). Proteins were separated using a Superdex 200 column after Ni-NTA agarose purification. The WT NAC domain eluted at ~15 ml (red arrow), consistent with its dimeric form (~45 kDa), while the R26A mutant eluted at ~17 ml (green arrow), consistent with a monomeric form (~22 kDa). The x-axis indicates elution volume, and the y-axis shows absorbance in milli-absorbance units (mAU).

(B) SDS-PAGE analysis of elution fractions (15–20 ml) confirms the elution volumes. WT NAC domain eluted at ~15 ml, whereas the R26A mutant eluted at ~17 ml, demonstrating that the R26A mutation disrupts NAC24 dimerization.

The NAC24-NACBS complex

In conventional EMSAs, DNA-protein complexes are generally detected using [³²P]-labeled deoxynucleotides (40–400 nt). While isotopic labeling is sensitive and cost-effective, it has drawbacks including short half-life of the samples, extended exposure requirements and safety issues. Other methods, like cyanine dye-labeled oligonucleotides, reduce the risks but can introduce structural artifacts that affect binding (Jing *et al.*, 2003; Hellman & Fried,

2007). For this study, we used short probes (20-200 nt) labeled with cyanine3 (CY3). The use of shorter probes reduces the migration time, which improves band resolution, and increases mobility shift upon protein binding.

To confirm the binding of NAC24 to motif 1 identified by DAP-seq, an EMSA was designed using a CY3-labeled synthetic oligonucleotide ("NACBS") and the NAC domain. The NACBS contains two palindromic CGTG sequences separated by a 5-nt linker. Mutant probes were also generated to test binding specificity: NACBS_{mut4} and NACBS_{mut6} (linker modifications from 5 to 4 and 6 nt, respectively) and NACBS_{mutTC} (CGTG to CGCG substitution) (Figure 26A). Protein-DNA binding is strongly affected by the buffer composition of the binding reaction, which usually comprises Tris-HCl/HEPES, salt, glycerol, and EDTA. Inclusion of BSA (or other carrier proteins) in the binding reaction seems to improve the stability of some complexes during electrophoresis (Hellman & Fried, 2007). Starting from frequently used buffer compositions, the binding conditions were optimized for the NAC24-NACBS interaction (see Materials and methods). Once the binding condition established, we tested the binding of NAC24 to NACBS, NACBS_{mut6}, NACBS_{mut4}, and NACBS_{mutTC}.

The DNA probes were incubated with increasing concentrations of NAC24 NAC domain and the binding reactions were analyzed by PAGE. Nucleic acids were visualized using the Amersham Typhoon imager (Cytiva) with the CY3 filter (560 to 580). Figure 26B presents representative EMSA results. In each panel, the first lane corresponded to unbound DNA, as NAC24 was omitted. We observed a concentration-dependent formation of the NAC24-NACBS complex, indicated by a DNA mobility shift and the gradual disappearance of unbound DNA. Binding was reduced for NACBS_{mut6}, further weakened for NACBS_{mut4} and nearly absent for NACBS_{mutTC}, emphasizing the importance of the linker length and the conserved thymine for NAC24 binding. This suggests that NAC24 binds as a dimer, which requires optimal spacing between the palindromic motifs for specific and stable interaction. The dimer appeared to have greater flexibility to accommodate a longer 6-bp linker compared to a shorter 4-bp linker, which disrupted binding more significantly.

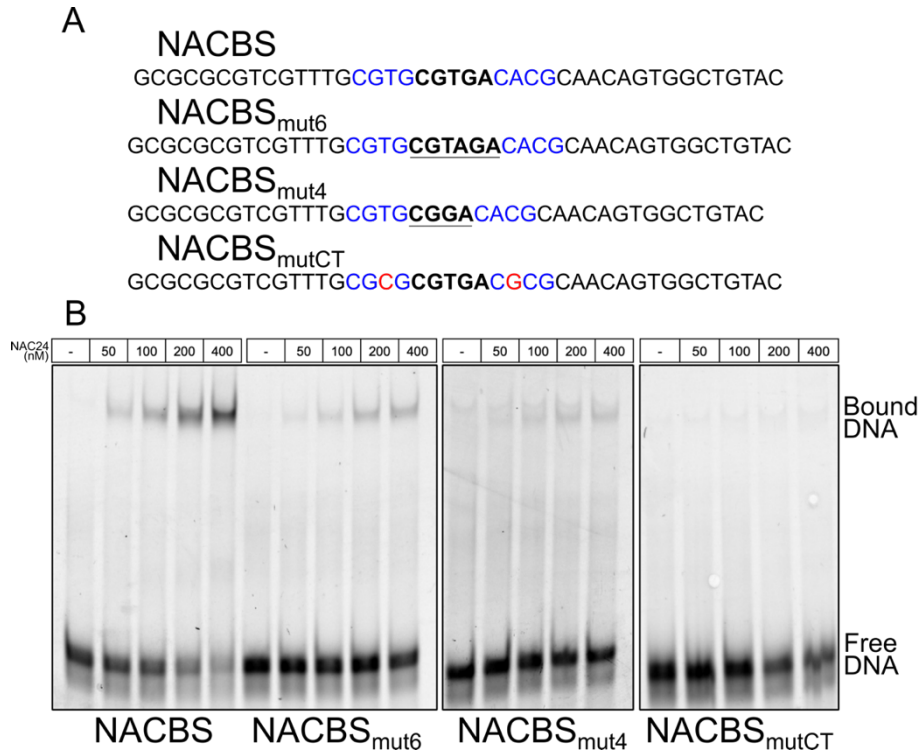


Figure 26: Gel mobility shift assay demonstrating the specific interaction of NAC24 with the consensus NACBS motif.

(A) Sequence details of NACBS and NACBSmut probes used in the EMSA. The core CGTG palindromes are shown in blue; linker is shown in bold, mutated linkers are underlined, and mutated nucleotides are marked in red.

(B) Representative EMSAs performed using the NAC domain of NAC24, expressed in *E. coli*, with NACBS and NACBSmut probes. CY3-labeled DNA probes (5 ng) were incubated with increasing, indicated, amounts of recombinant NAC24 NAC domain in DNA binding buffer for 20 min at 25°C. In each panel, the first lane represents unbound DNA (no NAC24 added). NAC24 produced shifted band for NACBS in a dose-dependent manner, with reduced binding to NACBSmut6 and NACBSmut4, and no detectable binding to NAC24mutTC, confirming the specificity of NAC24 for NACBS. The positions of the unbound DNA and the NAC24-NACBS complex are indicated to the right of the gel. All EMSAs were performed in duplicate with two independent protein preps.

DNA-binding residues of NAC24

To identify key DNA-binding residues in NAC24, we aligned its sequence with *Arabidopsis* ANAC019 and ORE1 (ANAC092), whose DNA-binding residues are known. The conserved positively charged residues (Lys79, Arg85, Arg88 in ANAC019; Lys85, Arg91, Arg94 in ORE1) are essential for binding, as their substitution with alanine abolished the interaction (Olsen *et al.*, 2005; Chun *et al.*, 2020). In NAC24, the corresponding residues are Lys86, Arg92, and Arg95

(Figure 27A). Using site-directed mutagenesis, we generated two mutant versions of the NAC24 NAC domain: a triple mutant (K86A/R92A/R95A) and a double mutant (R92A/R95A) and assessed their DNA-binding ability via EMSA.

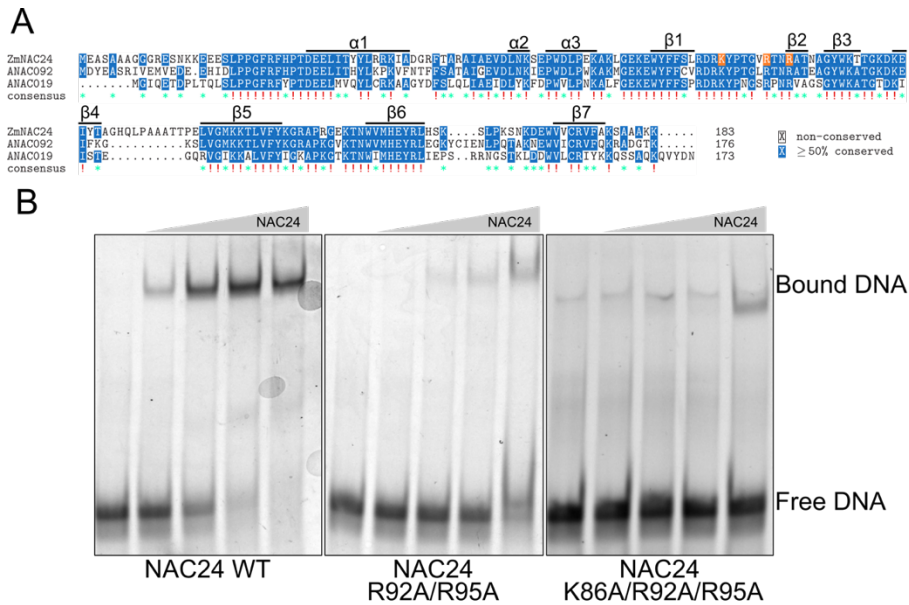


Figure 27: DNA-binding ability of NAC24 mutant NAC domains.

(A) Sequence alignment of NAC24, ANAC019 and ORE1 (ANAC092) NAC domains. Residues Lys86, Arg92, and Arg95 in NAC24, targeted by site-directed mutagenesis, are marked with asterisks. The secondary structure of the NAC24 NAC domain is indicated above the alignment. Alignment was performed using ClustalW (msa function in R). UniProt accession numbers: ANAC019 (Q9C932), ANAC092 (Q9FKA0), NAC24 (A0A1D6ESJ5).

(B) Representative EMSAs comparing the DNA-binding ability of WT NAC24 NAC domain and mutant domains (K86A/R92A/R95A and R92A/R95A) to the NACBS probe (Figure 19A). CY3-labeled NACBS was incubated with increasing amounts of recombinant NAC24 WT or mutant domains. The first lane in each gel represents unbound DNA (no protein added). Both mutants displayed reduced DNA binding compared to the WT domain. The positions of unbound DNA and DNA-protein complexes are indicated to the right of the gel. EMSAs were performed in duplicate.

The K86A/R92A/R95A modification showed a reduction in DNA binding compared to the WT (Figure 27B). Similarly, the R92A/R95A double mutant also showed reduced binding (Figure 27B). However, DNA binding was not completely abolished, suggesting Arg92 and/or Arg95 are critical for NAC24-DNA interaction, with potential contributions from additional residues or non-specific interactions.

AlphaFold Model of NAC24-DNA interactions

To validate the NAC24-DNA binding model, we predicted the structure of the NAC24 NAC domain bound to the NACBS using AlphaFold3 (Abramson *et al.*, 2024). The predicted structure and its Predicted Aligned Error (PAE), which estimates prediction quality, were visualized with the PAE viewer (Elfmann & Stülke, 2023) (Figure S3). PAE reflects AlphaFold3 confidence in the relative positions of two residues, measured in Å. A low PAE score between residues from different domains indicates high confidence, while a high score suggests uncertainty.

AlphaFold predicted structure for NAC24 shows high confidence for the central β -sheets. However, there is very low confidence in the N-terminal region and some loops between the β -sheets, probably due to intrinsically disordered segments or regions of low sequence complexity. This is consistent with previous findings that certain loop regions are intrinsically disordered (Chun *et al.*, 2022). Additionally, the predicted confidence for the relative positions of the DNA residues with the protein is low, indicating that the DNA-protein interactions in the model cannot be considered reliable. After assessing the quality of the structure, the output model was loaded into PyMol for further analysis and visualization (Figure 28A).

The predicted structure confirmed that the NAC24 NAC domain binds the NACBS probe as a dimer. Residues targeted by site-directed mutagenesis (Lys86, Arg92, and Arg95) are shown as sticks, and their interactions with the DNA strand were closely examined (Figure 28B).

In the structure predicted by AlphaFold3, Arg92 is positioned to interact with a specific nucleobase (thymine) from the NACBS. Interestingly, this thymine is found outside the arbitrarily defined conserved 4-nt palindrome (CGTG) and is instead located in an extended motif (TTnCGTG). This is consistent with the MEME analysis, which shows that the thymine is conserved. As a result, the consensus sequence recognized by NAC24 is redefined from a 4-nt palindrome (CGTG) to a 7-nt sequence (TTnCGTG) with a 5 nt linker. Arg92 likely mediates sequence-specific recognition by forming a hydrogen bond with the carbonyl group of the thymine.

In addition, Lys86 and Arg92 form a hydrogen-bond with the DNA phosphate backbone and may play a critical role in stabilizing the DNA-protein complex and maintaining overall binding affinity. However, no hydrogen-bond was observed between Arg95 and the DNA in the predicted structure, although the residue is close to the DNA. This differs with the findings of Chun *et al.* (2022), who observed that Arg95 forms a hydrogen bond with the DNA backbone in the X-ray crystallographic structure. Given the low confidence of DNA

nucleotide placement relative to the protein in the AlphaFold model, this interaction may have been lost.

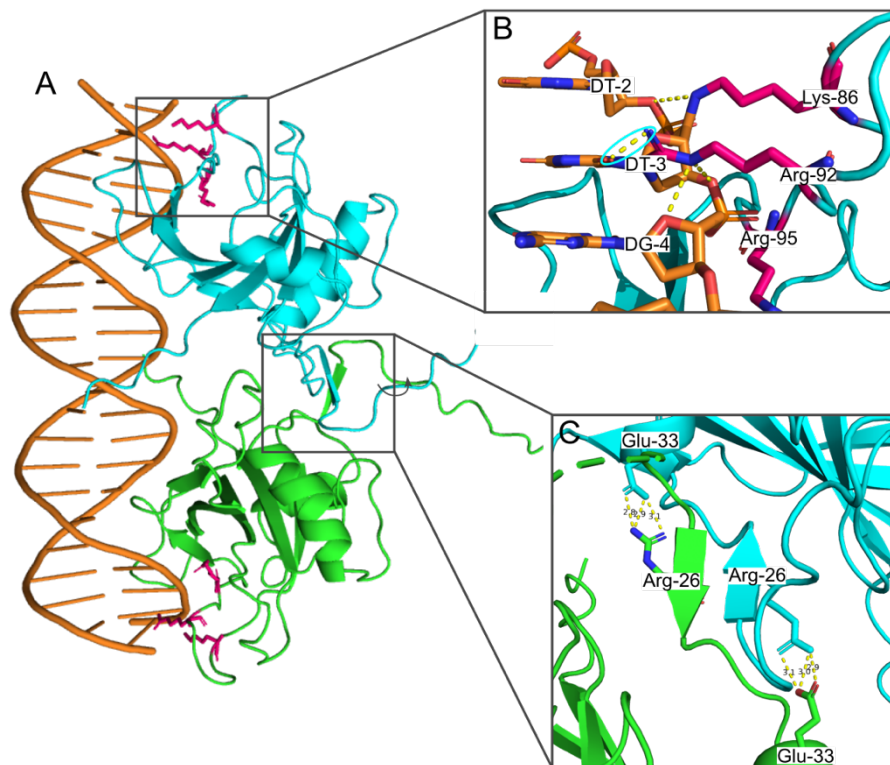


Figure 28: AlphaFold3 prediction of NAC24-NACBS complex.

(A) Cartoon representation of the NAC24 NAC domain dimer interacting with NACBS (orange). Monomer A is shown in green and monomer B in cyan. The residues Lys86, Arg92, and Arg95, targeted by site-directed mutagenesis are depicted as pink sticks (B) Interaction between NAC24 residues and DNA. Arg92 is positioned to form an H-bond (circled in cyan) with the carbonyl group of a specific nucleobase (DT-3) from the NACBS, which lies within the TTnCGTG consensus motif. Both Arg92 and Lys86 are positioned within <4 Å to form hydrogen bonds with the DNA phosphate backbone, likely playing a role in stabilizing the DNA-protein complex. No hydrogen-bond was observed between Arg95 and the DNA in the predicted structure, although the residue is close to the DNA.

(C) Dimerization interface of the NAC24 NAC domain dimer. Residues Arg26 and Glu33 are shown as sticks, with nitrogen atoms in blue and oxygen atoms in red. Arg26, located within the short antiparallel β -sheet of one monomer, forms three hydrogen bonds (yellow dots, <4 Å) with Glu33 of the other monomer. Together with the ionic interaction, these bonds form salt bridges stabilizing the dimerization interface. (Figures generated with PyMOL Version 2.2.5, Schrödinger).

Promoter binding by NAC24

With the EMSA successfully optimized and the specificity of the binding validated using the NACBS identified by DAP-seq, we proceeded to evaluate NAC24 binding to targets containing a NAC24 binding motif identified by DAP-seq. To validate these targets, we selected promoter regions of candidate genes that could potentially explain the mutant phenotypes observed in *nac24* KD plants. Additionally, we included a terminator and coding sequences containing peaks indicating NAC24 binding. The selected genes are highlighted in yellow in Table S1..

The promoter regions (~2–3 kb) were PCR-amplified and cloned, while CY3-labeled primers were used to amplify the specific DAP-seq peak sequences (~200–300 bp) for EMSA.

The selected gene targets, with their designated abbreviations and information about their encoded proteins, are presented below, along with the reasons for their selection. The list is ranked in ascending order p-value, starting with the most significant peak.

- Zm00001d029285 - ***pyruvate dehydrogenase (lipoamide) kinase1 (PDK1)***. This gene codes for a mitochondrial matrix protein kinase that phosphorylates the pyruvate dehydrogenase complex (PDC), which catalyzes the oxidative decarboxylation of pyruvate to produce acetyl-CoA and NADH for the Krebs cycle and oxidative phosphorylation. The kinase inactivates PDC via phosphorylation, while a phosphatase reactivates it, allowing physiological effectors to regulate PDC activity *in vivo* (Thelen *et al.*, 1998). This gene exhibited the most significant peak in its promoter region within the dataset.
- Zm00001d034189 - ***casparian-strip-membrane-domain-like 6 (CASP6)*** (peak located in terminator). CASPs (Casparian Strip Membrane Domain Proteins) are transmembrane proteins, essential for Casparian strip formation in the root endodermis (Roppolo *et al.*, 2011). CASPs and CASP-like proteins (CASPLs) play critical roles in root development, stress responses, and mineral uptake (Xue *et al.*, 2024). They organize lignin-based Casparian strips, creating diffusion barriers and restricting pathogen entry (Kolkman *et al.*, 2022). This gene had the second most significant peak overall in his terminator.
- Zm00001d043011 - ***LIGHT-DEPENDENT SHORT HYPOCOTYLS 5 (LDSH5)***. This gene encodes the ortholog of BSG1 (BEAK SHAPED GRAIN 1), which is associated with grain shape, size, and weight in

rice (Yan *et al.*, 2013). In maize, it is linked to a QTL associated with grain size and yield, which may explain the small grain size observed in *nac24* mutants (Zhou *et al.*, 2020).

- Zm00001d030998 - **Protein ROOT HAIR DEFECTIVE 3** homolog (**RHD3**). Encodes a homolog of Arabidopsis ROOT HAIR DEFECTIVE 3. Mutations in *AtRHD3* affect cell size, not number, and cause growth defects, including shortened root hairs and non-viable pollen (Wang *et al.*, 1997; Zhang *et al.*, 2013). *AtRHD3* may be involved in hormone signaling, particularly auxin and ethylene, but is not required for cell type specification. In maize, *RHD3* is a downstream target of PHR2 (Phosphate Starvation Response Regulator) and plays a role in root hair development, which is crucial for nutrient and water uptake. It is also upregulated under phosphate stress (Hu *et al.*, 2024).
- Zm00001d017977 - **hairy sheath frayed1** (*histidine kinase 1*) (**HK1**). This gene codes for a histidine kinase that perceives cytokinins (CKs) at the endoplasmic reticulum and plasma membrane. Specific missense mutations in the *HK1* gene lead to the *hsf1* phenotype, characterized by leaf outgrowths along the blade margin. This gain-of-function mutation alters CK binding, resulting in hypersensitivity to CKs and reprogramming developmental patterns (Muszynski *et al.*, 2020). Disruption of the CK signaling cascade could explain the *nac24* mutant phenotype.
- Zm00001d031798 - **BRI1 kinase inhibitor 1** (**BKI1**) (peak located in coding sequence). This gene codes for a plasma-membrane associated phosphoprotein that inhibits the kinase activity of BRASSINOSTEROID-INSENSITIVE1 (BRI1), a key receptor in brassinosteroid (BR) signaling. BRs regulate plant growth, development, and environmental responses. In the absence of BRs, BKI1 binds to the BRI1 kinase domain, maintaining BRI1 in an inactive state by preventing its interaction with its signaling partners, the receptor kinase BAK1 (Jing *et al.*, 2024).
- Zm00001d039776 - **Cell Number Regulator8** (**CNR8**). This gene codes for a plasma membrane protein with a conserved Cys-rich PLAC8 motif. Overexpression of certain family members (e.g., CNR1 and CNR2) reduces plant and organ size, while silencing their expression increases size. These size changes are attributed to variations in cell number, not cell size (Guo *et al.*, 2010). This gene may underlie the reduced size of *nac24* mutant plants.
- Zm00001d045203 – **Aux/IAA-transcription factor 14** (**AUX14**). This gene codes for a short-lived nuclear protein that is primarily

responsive to auxin induction and plays critical roles in early auxin signaling. Auxin is essential for various cellular and developmental processes, including embryogenesis, lateral root initiation, leaf expansion, inflorescence and fruit development (Wang *et al.*, 2010). ZmAux/IAA14 was shown to negatively regulate root dry weight under low nitrogen conditions (Wang *et al.*, 2024).

- Zm00001d016828– **yellow-green3 (YG3)**. This gene codes for a predicted pentatricopeptide repeat (PPR) protein, and its disruption in maize leads to a pale green or yellow phenotype, similar to that observed in *nac24* KD plants. (Sorgini & Crystal, 2018). PPR proteins are involved in various RNA-related processes, including transcription, RNA processing, splicing, stability, editing, and translation (Schmitz-Linneweber & Small, 2008).
- Zm00001d028344 - **metal tolerance protein4 (METP4)**. This gene codes for metal tolerance proteins (MTPs) belonging to the cation diffusion facilitator (CDF) family, which transport divalent transition metal cations (Zhao *et al.*, 2024). MTPs play a key role in heavy metal tolerance by mediating sequestration, chelation, and compartmentalization of these metals (Liu *et al.*, 2024). Associated with the seed maturation GO term, enriched in the NAC24 target genes.
- Zm00001d039352 - **abnormal chromosome organization in zygotene1 (ACOZ1)**. This gene codes for an F-box protein essential for proper meiotic chromosome morphology and successful progression through meiotic processes such as synapsis and recombination (Jing *et al.*, 2022). Although *aco1* mutant plants showed normal growth during the vegetative stage, their tassels are completely male-sterile. In addition, the plants are female sterile. Male fertility issues in *nac24* plants may result from disruption of ACOZ1.
- Zm00001d043083 – **PIN-like 6 (PILS6)**. This gene codes for an auxin efflux carrier localized to the ER and plays a crucial role in regulating root traits in maize by controlling the spatial distribution of auxin in primary roots. Mutants of *PILS6* exhibit significant root developmental defects, including reduced total root length, network area, fewer branch points, and lateral root tips, establishing PILS6 as a positive regulator of lateral root morphogenesis (Cowling *et al.*, 2024). Disruption of the auxin signaling pathway could explain the observed *nac24* phenotypes.
- Zm00001d025490 - **auxin regulated gene involved in organ size 1 (ARGOS1)**. This gene codes for a transmembrane protein that

functions as a negative regulator of ethylene signal transduction. *ARGOS1* OE reduces plant sensitivity to ethylene, which enhances drought resistance and increases grain yield and organ growth under both normal and drought conditions (Guo *et al.*, 2014; Shi *et al.*, 2015). The utility of *ARGOS1* for improving agronomic traits has been patented, highlighting its role in promoting growth, biomass production, and organ size in maize (Bruce *et al.*, 2017). *ARGOS1* KD plants exhibit reduced organ and plant size, which may partially explain the *nac24* phenotype.

- Zm00001d017609 – **REVEILLE 6 – MYB100 TF (MYB100)**. The peak is located in an intron and serves as a control due to the absence of an associated binding motif and a high p-value (0.04).

EMSAs were performed using the peak sequences of all selected DNA targets under the same conditions as for the short NACBS probes. Figure 29 shows representative EMSA results for the interaction of NAC24 with the different DNA targets.

The EMSAs confirmed NAC24 binding to all tested promoters, as well as to the *CASP6* terminator and *BK11* coding sequence. For the targets containing the motif 1 (palindromic "CGTG"), a second, slower-migrating band was detected, suggesting NAC24 homodimerization upon binding (Figure 29A). In contrast, the targets containing motif 2 ("CACGnAA") displayed a single shifted band, consistent with monomeric binding (Figure 29B). In contrast, no binding was observed for *MYB100*, which served as a negative control, since it was identified in the DAP-seq data but lacks known NAC24 motifs (Figure 29C).

Figure 29: Gel mobility shift assay demonstrating the specific interaction of NAC24 with gene targets identified through DAP-seq.

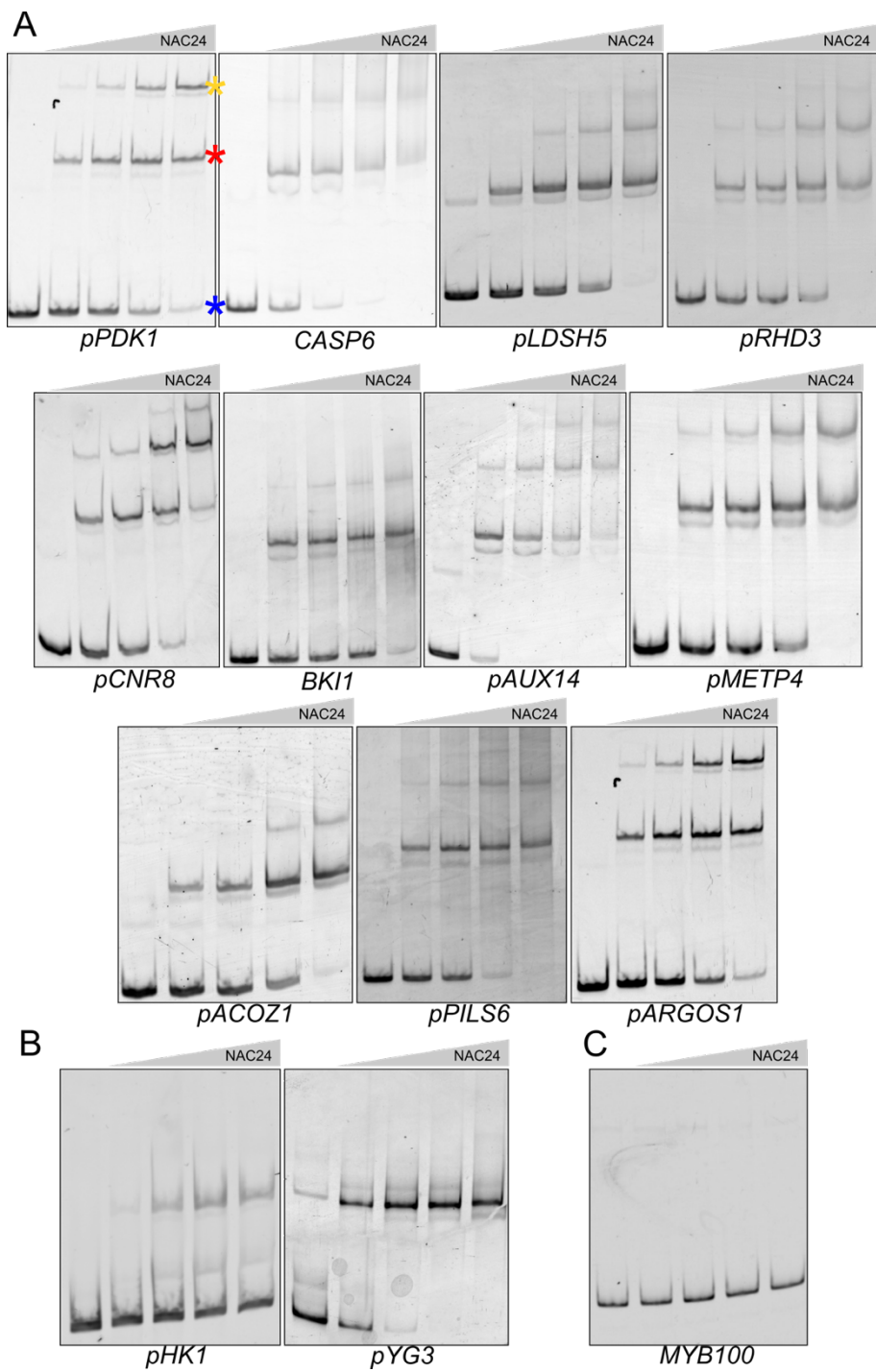
Representative EMSAs were performed using the NAC domain of NAC24, with CY3-labeled DNA probes corresponding to the peak sequences of selected target genes. CY3-labeled DNA probes (350 fmol) were incubated with increasing amounts of recombinant NAC24 NAC domain (0-400 nM) in DNA binding buffer. In each panel, the first lane represents unbound DNA (no NAC24 added).

(A) For targets containing the palindromic motif "TTnCGTG," two shifted bands were observed in a dose-dependent manner. The first shift corresponds to monomer binding, while the second shift likely represents homodimer binding. The positions of unbound DNA, monomer-DNA complex, and dimer-DNA complex are indicated on the first gel with blue, red, and yellow asterisks, respectively.

(B) For targets containing motif 2 ("CACGnAA") in a single repeat, only a single shifted band (monomer binding) was observed.

(C) For the *MYB100* target, identified in DAP-seq but lacking any known NAC24 motif, no shift was observed.

All EMSAs were performed in duplicate using two independent protein preparations, under identical conditions and protein concentrations.



Additionally, a minor band with slower migration was occasionally observed (e.g., with *RHD3* and *CNR8* targets). This may represent non-native protein-protein interactions, as reported in previous studies of DNA-binding proteins (Jensen *et al.*, 2010).

The *in vivo* transcriptional impact of NAC24 on these targets remains unclear and will be assessed using the DLR assays.

NAC24 binding to 35S and PIP2;5 promoters

Previous studies have shown that NAC TFs bind to a 30-bp region of the *CaMV 35S* promoter (-84 to -55), which contains an imperfect palindrome with core sequences "CGTA" and "CGTC" separated by six nucleotides (Xie *et al.*, 2000; Duval *et al.*, 2002; Hegedus *et al.*, 2003). It has also been shown that *Brassica napus* NAC proteins binds and *transactivate* the *35S* promoter (Hegedus *et al.*, 2003). The NACBS of *p35S* is shown in Figure 30A and compared to the consensus NAC24BS.

To test whether NAC24 binds to *p35S*, which was used in DLR and *transactivation* assays to drive the expression of the internal controls RLuc and mCherry, we assessed its binding affinity via EMSA. The *p35S* sequence (-160 to 0), which includes the imperfect palindrome, was amplified using CY3-labeled primers, and EMSAs were performed using the recombinant NAC domain of NAC24. No detectable binding to the promoter was observed compared with the *CASP6* terminator, which is a confirmed target of NAC24 (Figure 30B). Although the *p35S* NACBS resembles the NAC24 consensus motif, key differences—including a 6-nt spacer (instead of 5) and three nucleotide substitutions—abolish NAC24 binding, demonstrating its sequence specificity for NAC24BS (Figure 30A). These apparently minor modifications abolished the ability of NAC24 to bind, showing its specificity for NAC24BS. This result also confirms that not all NAC TFs bind to *p35S*, consistent with previous findings showing that SOG1, an Arabidopsis NAC TF, does not bind this sequence (Jensen *et al.*, 2010).

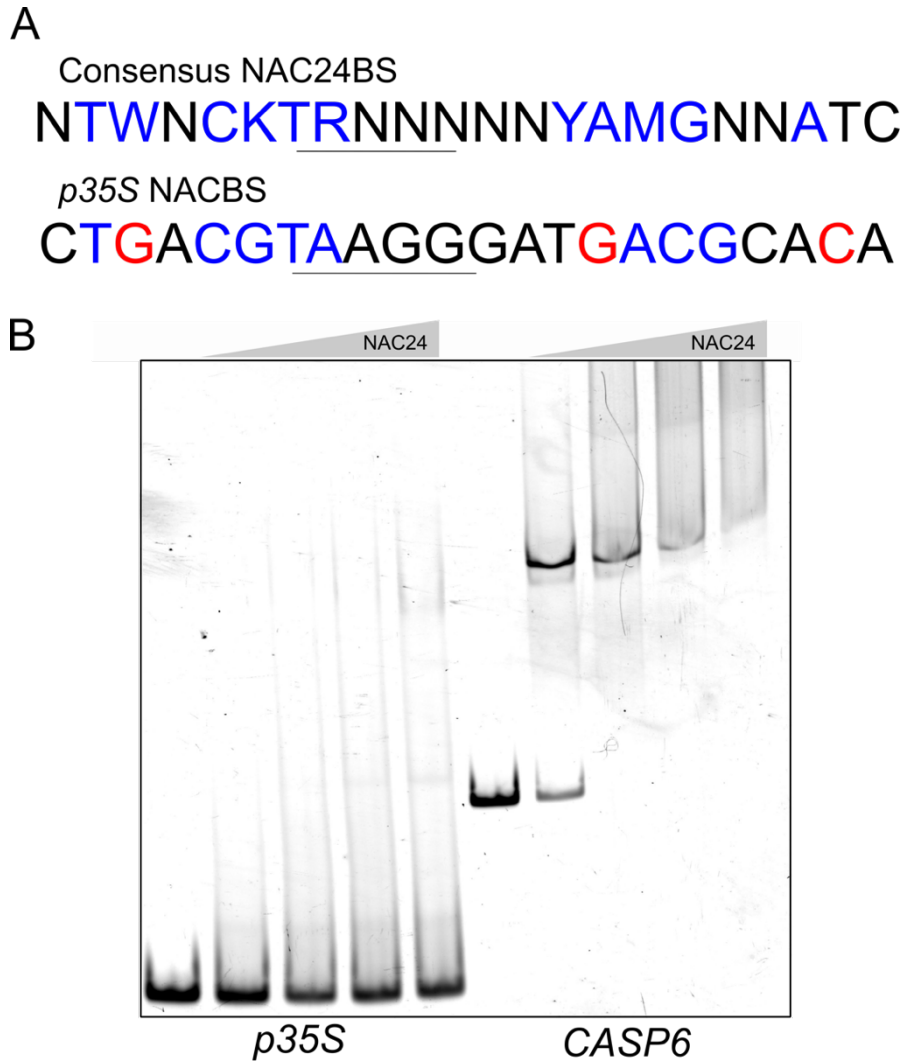


Figure 30: Gel mobility shift assay testing the interaction of NAC24 with p35S.
 (A) Sequence comparison of the consensus NAC24BS (in IUPAC nucleotide code) and the NACBS within p35S. The conserved palindrome sequences are highlighted in blue, the linker region is underlined, and nucleotides differing from the NAC24BS in the p35S sequence are marked in red. W = [A/T]; K = [G/ T]; R = [G/A]; Y = [C/T] and M = [C/A].
 (B) EMSA results showing the binding of the NAC domain of NAC24, expressed in *E. coli*, to CY3-labeled probes for p35S (160 bp) and CASP6 (207 bp). DNA probes (5 ng) were incubated with increasing amounts of recombinant NAC24 NAC domain protein in DNA binding buffer for 20 min at 25°C. In each panel, the first lane represents unbound DNA (no NAC24 added). NAC24 produced shifted bands for CASP6 in a dose-dependent manner, but no detectable binding to p35S was observed, confirming the specificity of NAC24 for the consensus NAC24BS.

The *PIP2;5* promoter contains two sequences (highlighted in Figure 8C) resembling NAC24 motif 2 (ACACGnnA), identified as potential NACBS using PlantRegMap prediction tool (Tian *et al.*, 2020). To assess NAC24 binding, the 1.8 kb promoter was divided into six overlapping fragments (230–400 bp), PCR-amplified with CY3-labeled primers, and tested via EMSA. The first ACACGnnA motif is located in fragment 3, while the second is in fragment 4. NAC24 potentially binds both fragments, but all six fragments were tested in the EMSAs.

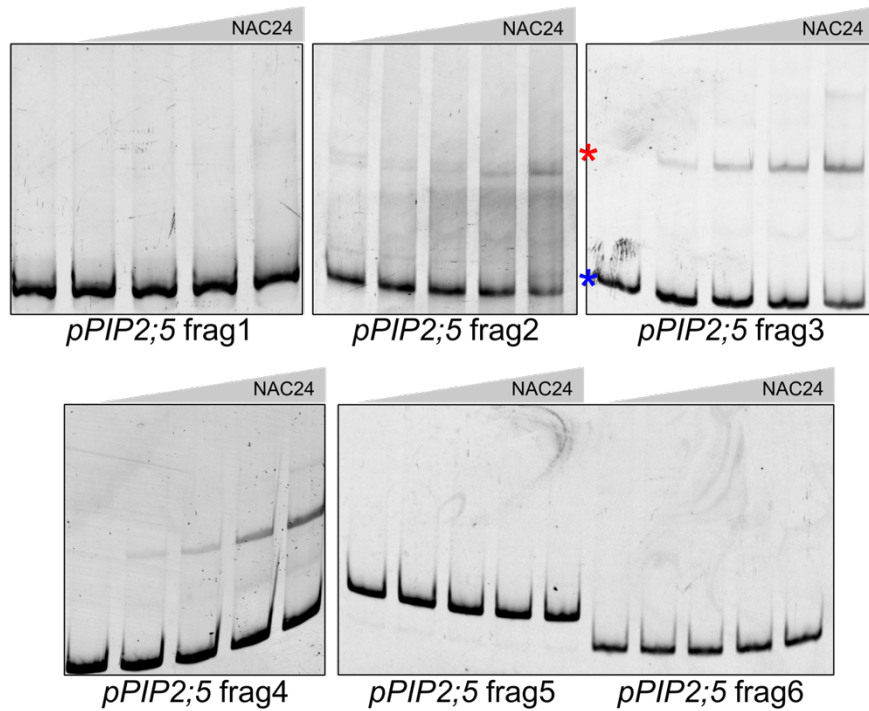


Figure 31: Gel mobility shift assay testing the interaction of NAC24 with pPIP2;5. EMSA results showing the binding of the NAC domain of NAC24 to CY3-labeled overlapping PCR fragments of the *PIP2;5* promoter. DNA probes were incubated with increasing amounts of recombinant protein (0–400 nM) in DNA binding buffer. The first lane in each panel represents unbound DNA (blue asterisk). Shifted bands were observed for fragments 3 and 4 (red asterisk), likely due to interactions with the ACACGnnA motif, whereas no detectable binding was observed for fragments 1, 2, 5, and 6.

The EMSA results demonstrated that NAC24 did not bind to fragments 1, 2, 5, or 6 of the *PIP2;5* promoter, but a mobility shift was observed for fragments 3 and 4 (Figure 31), which contain the ACACGnnA motif. However, the binding was weaker than for previously tested targets, as unbound DNA remained visible even at the highest concentrations of NAC24. This data suggests that

NAC24 binds to fragments 3 and 4 with reduced affinity, likely due to differences in the nucleotides flanking the conserved CACG region. In DAP-seq targets, this position is consistently a cytosine, whereas in *PIP2;5*, it varies. However, further mutational analysis is needed to confirm its role. Overall, NAC24 binds the *PIP2;5* promoter *in vitro*, but with lower affinity than DAP-seq-validated targets.

The effect of NAC24 on DAP-seq targets

To assess the impact of NAC24 on the activity of the promoter to which it was found to bind, we performed DLR assays. Promoter regions (~2.5 kb upstream of the ATG) were cloned upstream of *FLuc*, with *RLuc* under the control of the *CaMV 35S* promoter as an internal normalizer. NAC24 was co-expressed under the control of *CaMV 35S* promoter or without a promoter (negative control).

In maize mesophyll protoplasts, most of the promoters were inactive, showing *FLuc* levels comparable to the negative control (i.e., no promoter driving *FLuc* expression) (data not shown).

The constructs were then tested in *Nicotiana tabacum* leaf protoplasts and five promoters (*LDSH5*, *ARGOS1*, *PILS6*, *AUX14*, *ACOZ1*) showed activity in *Nicotiana tabacum* leaf protoplasts, while *PDK1*, *METP4*, and *RHD3* showed no activity. The remaining promoters (*HK1*, *YG3*, *CNR8*) were not tested. This suggests that promoter activity may vary depending on species- and cell-specific regulatory environments.

The results of the DLR assay in *N. tabacum* protoplasts are shown in Figure 32. *ARGOS1* and *LDSH5* promoters showed significantly higher normalized *FLuc* activity with NAC24 expression ($p < 0.001$), indicating activation. *AUX14* promoter activity decreased significantly ($p < 0.001$), suggesting repression. No significant effect was observed for *ACOZ1* and *PILS6* promoters. These results confirm that NAC24 not only binds to some target promoters but also functions as a transcriptional regulator, activating or repressing specific genes.

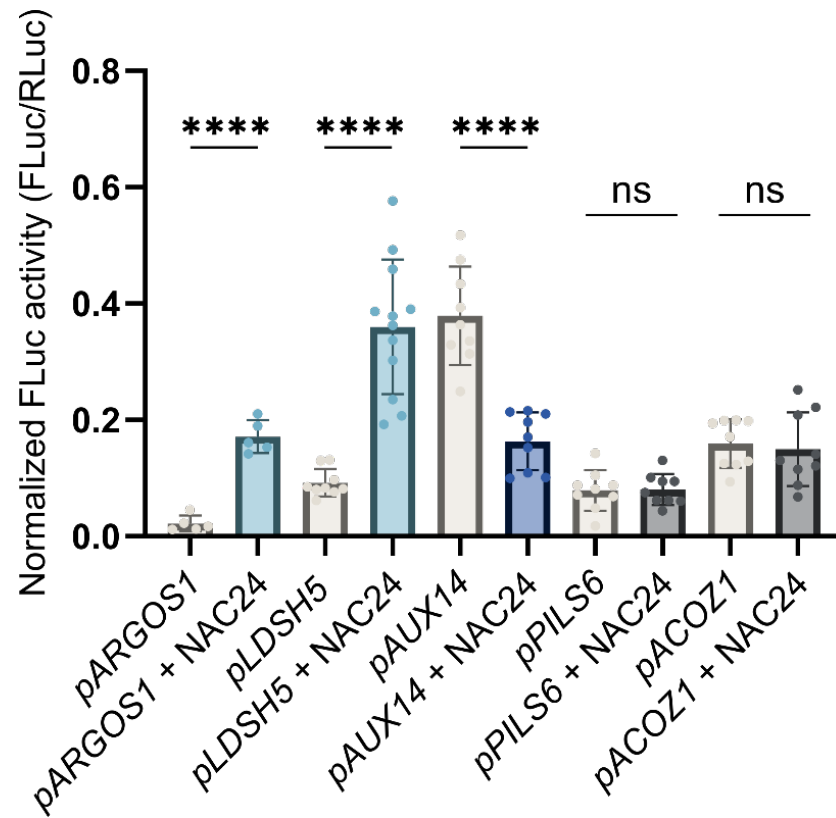


Figure 32: Dual luciferase reporter assay evaluating the effect of NAC24 on various promoters in *N. tabacum* leaf protoplasts. The constructs were transfected in *N. tabacum* protoplasts, and FLuc and RLuc activities were measured with the FB12 Single Tube Luminometer (Berthold). Normalized FLuc activity (FLuc/RLuc) revealed that NAC24 expression (p35s::NAC24) significantly increased the activity of the ARGOS1 and LDSH5 promoters compared to the control (p0::NAC24) (p-value<0.001, ****). In contrast, the AUX14 promoter exhibited significantly lower activity in the presence of NAC24 (p<0.001). No significant effect of NAC24 was observed on the ACOZ1 and PILS6 promoters. The data represents the mean ($\pm$ SE) of three independent experiments, each with three technical replicates. A linear mixed model was used to test the NAC24 presence effect, incorporating a random intercept taking the experience replication dependency into account (lmer function in R).

PART II: Take-home messages

- The *nac24* KD affects plant and leaf morphology, producing a variety of phenotypes. The mutant plants also exhibited reduced fertility and germination rate, indicating its role in plant development and reproduction.
- A DAP-seq experiment was successfully conducted using the primary root and leaf gDNA, identifying direct target genes of NAC24. *PIP2;5* or other *AQP* genes were not detected in the DAP-seq data.
- Motif analysis revealed two consensus binding motifs for NAC24: a 7-nt palindrome (TTnCGTG) with a 5-bp linker and a 12-bp sequence containing a single CACG motif with no palindromic repetition.
- The NAC24 NAC domain was adequately purified, and the protein forms a homodimer stabilized by Arg-26 and associated salt bridges, which are essential for dimerization.
- EMSA experiments confirmed binding specificities of NAC24. NAC24 binds the TTnCGTG motif as a dimer, with spacing between palindromes being critical for proper binding. The thymine conserved in the CGTG motif is essential for recognition and binding. Arg-92 and/or Arg-95 are crucial for the interaction between the NAC24 domain and DNA.
- NAC24 binds as a dimer to the promoter, terminator, and coding regions containing the TTnCGTG motif, and binds as a monomer to sequences containing CACGC[A/C]A.
- NAC24 has low binding affinity for to the *PIP2;5* promoter fragments and no detectable binding to the *35S* promoter.
- NAC24 differentially regulates target promoters in *N. tabacum* protoplasts: it activates *ARGOS1* and *LDSH5* promoters, represses *AUX14*, and has no effect on *ACOZ1* and *PILS6*.

DISCUSSION AND PERSPECTIVES

Structural and functional features of NAC24

As the first study to specifically investigate NAC24, we have characterized this TF by integrating literature mining, bioinformatics analyses, and experimental data. While this approach has provided useful insights into NAC24, additional research is required to more precisely characterize its structural features and clarify their connection to its physiological functions.

Multiple sequence alignment with other NAC family members revealed that NAC24 possesses the conserved NAC domain in its N-terminal region, divided into five subdomains (A–E). Phylogenetic analyses focused on maize consistently place NAC24 within the NAM subfamily, one of the twelve NAC subfamilies (Fan *et al.*, 2014; Shiriga *et al.*, 2014; Wang *et al.*, 2020). Members of this subfamily are primarily associated with developmental regulation (Ooka *et al.*, 2003), which corresponds to the role of NAC24, as mutants exhibit developmental defects.

We also confirmed its nuclear localization and, using the NLS mapper prediction tool (NLSExplorer), identified VGMKKTLV within the D subdomain as the putative nuclear localization signal. This sequence resembles a monopartite NLS (a single cluster of basic residues). The importance of the lysine residues in this sequence has already been demonstrated, as the mutant phenotype of the *cuc1-1* allele suggests that Lys115 (corresponding to Lys131 in NAC24) is essential for its function (Takada *et al.*, 2001). Since this residue is not essential for DNA binding, it is likely involved in nuclear localization. However, a mutational analysis will be required to confirm its role in nuclear localization of NAC24.

NAC24 binds a typical NACBS as a dimer

NAC24 contains the conserved NAC dimerization interface LPPGFRFHPTDEEL, where arginine and glutamic acid residues form salt bridges critical for dimerization (Olsen *et al.*, 2005). Through site-directed mutagenesis, we identified Arg26 as essential for the dimerization of the NAC24 NAC domain. Substitution of Arg26 with alanine disrupted the two salt bridges at the dimerization interface, effectively converting the NAC domain into a monomeric form, as evidenced by a peak shift in size exclusion chromatography. The importance of this residue is reinforced by its conservation across NAC domains, with all but one NAC protein in maize

containing an Arg residue capable of salt bridge formation at the position corresponding to Arg26.

Members of the NAC family preferentially bind to palindromic DNA sequences composed of two consensus motifs separated by a linker of 5 to 7 bp. The dimeric flexibility of the NAC domain was thought to facilitate recognition of palindromic sequences with variable linker lengths (Welner *et al.*, 2012). However, *in vitro* screening assays actually suggest that each NAC TF prefers a specific linker length within its palindromic target sequence (O'Malley *et al.*, 2016).

Members of the NAC family preferentially bind to palindromic DNA sequences composed of two consensus motifs separated by a linker of 5 to 7 bp. In this study, we identified the consensus binding sites for ZmNAC24 from the DAP-seq results. EMSA assays confirmed that the NAC domain of ZmNAC24 specifically binds to a palindromic 7-bp consensus motif (TTnCGTG) linked by a 5-bp linker, in agreement with previously identified NAC binding motifs. For example, AtORE1 binds a similar motif, TTnCACG, with a 5-bp linker (Matallana-Ramirez *et al.*, 2013) and AtANAC019 binds to the palindromic sequence TTnCGTG, but with a 6-bp linker (Olsen *et al.*, 2005). The identified motif is also consistent with known NAC binding sites in Arabidopsis, as revealed by DAP-seq and Protein Binding. The mutation of the linker showed that NAC domain of NAC24 has the strongest affinity for the palindromic sequence separated by a 5-bp linker. Notably, shortening the linker by one bp led to a more pronounced reduction in NAC24-DNA binding than lengthening it by one bp, suggesting that the NAC24 dimer has a constrained closing angle but greater opening, a characteristic that has already been observed for ORE1 (Chun *et al.*, 2023). Rather than allowing the recognition of different palindromic sequences with varying spacer lengths, this dimer flexibility in the NAC domain was considered crucial for maintaining binding to structurally flexible DNA substrates (Chun *et al.*, 2023). In addition, thymine, the most conserved nucleotide in the CGTG motif, is essential for recognition and binding of ZmNAC24, as substituting it with cytosine completely abolished DNA-protein complex formation.

Further supporting the specificity of NAC24 binding, it showed no detectable interaction with the *p35S* promoter, which is recognized by multiple NAC proteins (Xie *et al.*, 2000; Duval *et al.*, 2002; Olsen *et al.*, 2005). Although the NACBS within *p35S* shares similarities with the NAC24BS, key differences, such as a 6-bp instead of a 5-bp linker and three nucleotide variations, were sufficient to prevent NAC24 binding. This specificity was particularly advantageous, because both DLR and transient fluorescence assays use *p35S* for the expression of internal controls. If NAC24 had bound to *p35S*, an

alternative constitutive promoter would have been required to drive the normalizer genes (*RLuc* and *mCherry*).

EMSAs on various promoter fragments containing NAC24BS confirmed that the NAC domain of NAC24 binds as a dimer to the palindromic sequences, as shown by two distinct bands corresponding to monomeric and dimeric binding states. EMSAs also demonstrated that NAC24 can bind to single NACBS motifs as a monomer, indicating that a single NACBS can be sufficient for promoter recognition. Similar results have been reported for ANAC072/019/055 and ANAC096 binding to the *ERD1* and RD29A promoter, respectively (Tran *et al.*, 2004; Xu *et al.*, 2013).

Similarly, in maize, NAC128 and NAC130 bind the core binding site ACGCAA contained within their target promoter regions (Zang *et al.*, 2019).

The ability of NAC24 to bind both single and palindromic NACBS motifs introduces an additional layer of gene regulation. Promoters containing palindromic dimer sites may be differentially regulated through distinct combinations of NAC homo- and heterodimers (Lindemose *et al.*, 2014), potentially expanding the functional diversity of NAC-mediated transcriptional control.

Notably, EMSA analysis with the dimerization mutant NAC24R26A yielded ambiguous results (Figure S4). The mutant formed a DNA-protein complex consistent with monomer binding at low protein concentrations. However, at higher concentrations, a second, higher-migrating and more diffuse band appeared, which did not correspond to dimer binding. This could indicate reduced protein stability in solution due to the mutation, but further investigations are needed.

Mutational analysis of the NAC24 NAC domain revealed that both the triple mutant (K86A/R92A/R95A) and the double mutant (R92A/R95A) exhibited impaired DNA binding. In the structure predicted by AlphaFold, Arg92, located in the loop between $\beta 1$ and $\beta 2$, is positioned to interact with a specific nucleobase (thymine) within the NACBS (TTnCGTG). Arg92 likely mediates sequence-specific recognition by forming a hydrogen bond with the carbonyl group of thymine. This is consistent with the crystal structure of ORE1 (ANAC092)-DNA, where the corresponding Arg91 plays a critical role in thymine recognition at the same position in the consensus sequence (Chun *et al.*, 2023). Sequence alignment of Arabidopsis NAC TF members indicates that Arg91 in ORE1 (corresponding to Arg92 in NAC24) is conserved in 77% of NAC TFs, highlighting its functional importance (Olsen *et al.*, 2005). Arg95 may also contribute to DNA binding, particularly in non-specific interactions,

which precede specific target site recognition and rely on electrostatic forces (Kalomindos *et al.*, 2004).

The ORE1–DNA structure further revealed that DNA recognition involves two highly conserved residues, Lys102 and Thr104, within the WRKY motif. These residues interact with key bases in the NAC binding site: Lys102 recognizes the conserved cytosine, while Thr104 interacts with the thymine of the CGTG core binding site (Chun *et al.*, 2023). In NAC24 these residues correspond to Lys103 and Thr105, and mutational analysis could be interesting to confirm their role in DNA binding.

Understanding the DNA-binding preferences of TFs and identifying their target genes are crucial steps towards breeding and genome editing projects. In particular, minor modifications to NACBS could be exploited to fine-tune gene regulatory networks (GRNs) and improve desirable agronomic traits.

The identification of NAC24BS in maize provides a basis for exploring natural genetic variations, such as SNPs within NACBSs of promoters of genes linked to key morphological traits. These variations could be exploited to modulate TF-DNA binding affinity and transcriptional output, thus offering new avenues for crop improvement.

Furthermore, precision genome editing tools like CRISPR-Cas9 (Bortesi & Fischer, 2015) could be applied to engineer NACBSs, thereby enabling targeted rewiring of GRNs. By leveraging the knowledge gained from this study, it may be possible to strategically modify NAC24BS with high precision to optimize gene expression patterns and enhance specific maize traits.

NAC24 upregulates *PIP2;5* expression

Many similarities can be drawn between NAC TFs and AQPs. First NACs and AQPs are both involved in lateral root formation (Guo *et al.*, 2005; Péret *et al.*, 2012; Li *et al.*, 2012). In addition, NACs regulate petal development, as do several PIPs and TIPs, which are subject to ethylene-driven regulation (Pei *et al.*, 2013; Shibuya *et al.*, 2014). Interestingly, *RhPIP1;1* and *RhPIP2;1*, which are involved in rose petal expansion, are directly bound and regulated by the ethylene-induced NAC TF RhNAC100, whose overexpression inhibits petal cell expansion (Pei *et al.*, 2013). Finally, NACs and AQPs both play indisputable roles in stress responses and adaptation. In this context, ThNAC12 from *Tamarix hispida* is a direct regulator of *ThPIP2;5* to improve salt tolerance by modulating reactive oxygen species (Wang *et al.*, 2021). Similarly, ThNAC7 improves salt and osmotic stress tolerance in Arabidopsis, with upregulation of *PIP1;5*, *PIP2;1*, and *TIP1;1* in *ThNAC7*-OE lines subjected to salt stress, suggesting NAC-mediated activation of AQP

expression (He *et al.*, 2019). Finally, several *PIP* and *TIP* genes were deregulated by overexpressing drought-inducible NACs in rice. Some of them were identified as direct targets of these NAC TFs (Chung *et al.*, 2018). Thus, while NACs and AQPs are involved in overlapping biological processes, and a few direct regulatory interactions have been identified, concrete evidence of a widespread direct link between NACs and *PIP* genes remains limited.

This study investigated the regulatory relationship between NAC24, identified in a distant eQTL associated with *PIP2;5* expression in the mature leaf zone (Maistriaux *et al.*, 2024). We demonstrated the ability of ZmNAC24 to *transactivate* the *ZmPIP2;5* promoter using transient expression and fluorescence quantification in maize epidermal cells, as well as DLR assays in maize mesophyll protoplasts. The DLR assay, successfully established in maize mesophyll protoplasts, used a single plasmid containing cassettes coding for the Firefly and Renilla luciferase reporters along with an effector cassette. NAC24 activation of the *PIP2;5* promoter further revealed that its transcriptional regulatory domain is located in the C-terminal region. NAC24 OE in BMS suspension cells also increased the level of *PIP2;5* mRNA. Overall, these *in vivo* validation of NAC24 as a transcriptional activator of *PIP2;5* was confirmed in transposon-induced *nac24* KD plants, which exhibited reduced *PIP2;5* expression in primary roots.

In EMSA experiments, the NAC domain of NAC24 bound to a *PIP2;5* CACG sequence *in vitro*, albeit with a much lower affinity than other targets. A point mutation of this CACG sequence could help confirm whether it is indeed the binding site for NAC24.

However, DAP-seq experiments did not identify the *PIP2;5* gDNA as a direct target of NAC24, suggesting that either the methylation state of the extracted gDNA prevented its direct binding (O'Malley *et al.*, 2016) or NAC24 did not bind directly to the *PIP2;5* promoter *in vivo* or bound to an unidentified distant enhancer of *PIP2;5* (Spitz and Furlong, 2012), and/or other TF partners could act in concert with NAC24 to control *PIP2;5* expression. Identification of the *PIP2;5* TF network involved in its expression could help test these hypotheses.

We therefore turned to gene co-expression network (GCNs) analysis to identify potential candidates involved in the NAC24-*PIP2;5* regulatory cascade. GCNs are valuable tools for discovering genes involved in specific pathways or processes, facilitating the characterization of TF-target interactions (Krouk *et al.*, 2013; Schaefer *et al.*, 2018). These networks are constructed using inference methods that analyze transcript abundance data,

revealing connections between genes. We analyzed the TF-target gene associations identified by Zhou *et al.* (2020), who constructed 45 GCNs using publicly available expression data. These networks represent potential regulatory relationships between TFs and other genes across different sample populations, but they are based on expression correlations rather than experimentally validated interactions. Therefore, they need to be validated and do not establish the hierarchical structure of genes within each regulatory pathway. We identified six TFs associated with the expression of *PIP2;5* and *NAC24*: homeobox64 (HB64), MYB62, WRKY14, bZIP7, bZIP70, and bZIP79. Although none of them were identified as direct *NAC24* targets by DAP-seq, their common association with *PIP2;5* and *NAC24* could serve as a starting point for uncovering the missing link in the indirect *transactivation* of *PIP2;5* mediated by *NAC24*. Furthermore, MYB and bZIP have already been shown to bind directly to *AQP* promoters. A MYB TF directly regulates *PtrPIP1;1* in poplar (Jiang *et al.*, 2020), while MabZIP53 directly interacts with the *MaPIP1;1* promoter in banana (Xu *et al.*, 2021).

Although *PIP2;5* has been extensively characterized, to our knowledge, *NAC24* is only the second transcriptional regulator identified for this gene. An AHL transcription factor binds to the RGRP motif ("ATTATTTTATGAA") in the *PIP2;5* promoter, where a natural A/T variation influences expression and Pb tolerance in maize seedlings by altering promoter activity. DLR assays revealed that the AHL protein negatively regulated the "A" variant (Haplotype 1, corresponding to the B73 promoter used in this study), but positively regulated the "T" variant (Haplotype 2), explaining expression differences among maize lines (He *et al.*, 2023).

Now that we have reaffirmed the findings of the original eQTL study identifying *NAC24* as a potential regulator of *PIP2;5*, it would be useful to locate the specific DNA sequence polymorphism near *NAC24* that ultimately influences *PIP2;5* expression. To achieve this, we should analyze maize lines that diverge at the "NAC24-eQTL" to identify potential causal variants and determine whether they affect *NAC24* expression or protein function.

We performed a preliminary comparison of *NAC24* coding sequences across several sequenced maize accessions (<https://www.maizegdb.org>), focusing on non-neutral polymorphisms that could alter the function *NAC24*. Several missense mutations were identified, along with deletions in some accessions. For example, PH207 and W22 have 222 and 9 nucleotides missing in the *NAC24* coding sequence, respectively, compared to the B73 reference genome (Figure S5). The functional impact of these non-neutral polymorphisms on *PIP2;5* expression could be assessed through

transactivation assays using different NAC24 variants. It is interesting to mention that the expression of *PIP2;5* in leaf is lower in PH207 than in B73 (Maistriaux *et al.*, 2024).

Additionally, comparing NAC24 promoter regions across maize accessions could help identify polymorphisms that influence its regulation. NAC24 expression in lines differing at the NAC24-eQTL could be examined via RT-qPCR and correlated with promoter variations. Alternatively, different promoter variants could be tested in *transactivation* assays to assess their activity.

Finally, it would be interesting to evaluate the regulatory effect of NAC24 on the *PIP2;5* promoter from other maize lines, particularly those differing in a local *cis*-acting eQTL. Previous research has shown that point mutations in the *PIP2;5* promoter can alter the regulatory effect of an AHL transcription factor, contributing to expression differences among maize lines (He *et al.*, 2023). Investigating similar regulatory mechanisms involving NAC24 could provide further insights into how genetic variation shapes *PIP2;5* expression.

Physiological significance of the NAC24 regulation of *PIP2;5*

The physiological role of the NAC24-*PIP2;5* relationship was not fully explored in this study, but some hypotheses can be put forward. NAC24 could regulate *PIP2;5* expression in roots, as both show specific expression in roots and low expression elsewhere, according to the maizeGDB eFP Atlas Browser. In *nac24* mutants, the expression of *PIP2;5* was reduced in primary roots, establishing an *in vivo* link.

The NAC24-*PIP2;5* relationship could be involved in lateral root development, as overexpression of a NAC isoforms has been associated to increased lateral root formation (Xie *et al.*, 2000; He *et al.*, 2005; Hao *et al.*, 2011; Zhang *et al.*, 2018). Spatial regulation of *PIP* and *TIP* gene expression was shown to be crucial in lateral root emergence (Peret *et al.*, 2012; Reinhardt *et al.*, 2016). Indeed, the turgescence of the cells through which the lateral root is moving has to be regulated.

In addition, NAC24 could also regulate other genes associated with root development (see “Unveiling NAC24 regulatory role in maize”). Future root phenotyping of *nac24* mutants could provide additional information on the function of NAC24 in root development and the possible involvement of *PIP2;5* or other target genes. This analysis could be carried out using the root phenotyping platform at the host university, set up by Xavier Draye, to study root growth and architectural dynamics.

The activation of *PIP2;5* by *NAC24* could also contribute to root water uptake in response to abiotic stress. *NAC24* expression was found to be stimulated by drought and repressed upon rewatering, although this phenomenon was not as evident as other *NACs* (Wang *et al.*, 2020). In addition, stress browser data indicate an early increase in *NAC24* expression in primary root following water stress (24 h) (Makarevitch *et al.*, 2015; Opitz *et al.*, 2014). Short term PEG-induced osmotic stress also led to upregulation of *PIP2;5* RNA level in roots, accompanied with increased protein levels (Hachez *et al.*, 2012). Since both *NAC24* and *PIP2;5* are more highly expressed under water stress, *NAC24* may activate *PIP2;5* under these conditions. The elevated *PIP2;5* expression improves root water uptake during mild water deficits, as demonstrated by the increased performance of *PIP2;5* OE lines compared to control plants (Ding *et al.*, 2022).

NAC24 may also regulate *PIP2;5* expression in leaves, as the original eQTL study linking the two genes was conducted on leaf samples. In leaf, *PIP2;5* is mostly expressed in guard cells (Ding *et al.*, 2022) and its overexpression under the control of the 35S promoter enhances leaf expansion under mild water deficit (Ding *et al.*, 2020). In fact, *PIP2;5* accelerates stomata closure after ABA treatment compared to the KD plants, making the plant more tolerant to water deficit (Ding *et al.*, 2020 and 2022). Overall, these observations suggest that *NAC24*-*PIP2;5* *transactivation* could play a role in stress adaptation by controlling the hydraulic conductivity of roots and leaves and promoting leaf expansion under specific abiotic stress conditions.

Finally, while *pip2;5* KD lines in the maize B104 background showed no significant growth differences in well water conditions (Ding *et al.*, 2020), those in the ND101 background were significantly shorter and had smaller leaves compared to WT plants (He *et al.*, 2023). These phenotypes resemble those observed in the *nac24* KD plants, suggesting that the regulation of *PIP2;5* by *NAC24* could partly explain the phenotype. However, given that *NAC24* KD did not completely eliminate *PIP2;5* expression and leads in a more severe phenotype, additional target genes are likely involved.

Challenges and insights for *NAC24* mutants

We successfully generated BMS cell lines expressing NAC24-FLAG using biolistic transformation. Unlike whole plants, which consist of diverse cell types with distinct gene expression profiles, BMS cells provide a homogeneous, low-complexity system that is ideal for analyzing TF regulation in a controlled environment. However, the main drawback of this approach is the lengthy regeneration process, as callus formation and cell regeneration require approximately three months. To accelerate the screening of transgenic events, we included an mCherry cassette, which proved highly effective. However, in addition to silencing of the endogenous *NAC24* gene following transgene introduction, the NAC24-FLAG protein, initially well-expressed, became undetectable on Western blot after approximately six months. This suggests the activation of post-transcriptional gene silencing (PTGS), likely triggered by the strong *p35S* promoter, leading to sequence-specific degradation of NAC24 mRNA (Eamens *et al.*, 2008). Consequently, the window of usability for these BMS lines in experimental work was very narrow, that would have required frequent re-transformation.

Despite these difficulties, we successfully obtained four transgenic lines OE NAC24, with different levels of transgene expression. This variation is likely due to random insertion of the T-DNA in the BMS genome, where chromosomal environment influences expression levels. We observed a correlation between NAC24 OE and changes in *PIP2;5* expression via RT-qPCR. However, the PIP2;5 protein was not detectable via Western blot, which aligns with previous findings (Moshelion *et al.*, 2009) where *PIP2;5* mRNA was barely detectable in BMS cells.

This single-cell system could allow us to investigate whether increased *PIP2;5* expression enhances the osmotic water permeability coefficient (Pf) of the plasma membrane using biophysical techniques like the cell pressure probe or protoplast swelling assays (Moshelion *et al.*, 2004). Furthermore, ChIP-seq or RNA-seq on the NAC24-FLAG expressing cells could provide valuable insights into NAC24-mediated transcriptional regulation. However, the lack of a fully assembled BMS genome poses a major challenge for data analysis in such experiments. Nevertheless, these approaches should be considered as valuable complementary strategies to whole-plant studies.

Transposon insertion lines, such as those from the BonnMu system, are valuable tools for studying gene function. However, PCR genotyping can be labor-intensive, and linking observed phenotypes to a specific insertion can

be challenging. Despite these difficulties, we successfully established a PCR genotyping assay. However, we were unable to amplify a PCR fragment using the recommended TIR6 primer in combination with the gene-specific reverse primer. This is not uncommon, as *Mu* elements often contain non-identical TIRs, resulting in variable primer efficiencies (McCarty *et al.*, 2013). Fortunately, our study confirmed that the transposon insertion was located in the expected region indicated in MaizeGDB: within the first intron of *NAC24*. Initially, it was unclear whether this insertion would result in a full gene KO or a KD. However, our expression analysis revealed a significant reduction in *NAC24* transcript levels, indicating a gene KD rather than a complete loss of function. This partial reduction in expression was advantageous, as a full KO could have been lethal or prevented plant propagation.

***NAC24* mutants reveal its role in plant development**

While propagating the *nac24* mutant maize plants, we observed pleiotropic phenotypes. To establish a link between these phenotypes and the insertion, we analyzed segregating F₄ families, where ears produced seeds representing all three genotypes: WT, heterozygous, and homozygous. In the F₄ generation, phenotypes consistently correlated with genotype, with homozygous plants displaying more pronounced phenotypes than heterozygous plants when compared to their nonmutant siblings.

The *nac24* KD significantly affected plant and leaf morphology. Compared to WT plants, *nac24* mutants exhibited pleiotropic developmental abnormalities, including reduced plant height and narrowed, shortened leaves. The dwarf phenotype resulted from inhibited internode elongation rather than a reduction in the number of internodes. To better understand the underlying cause of this phenotype, we could examine anatomical features such as cell number and cell growth, vascular bundle patterning, and secondary cell wall structure.

Hormonal signaling likely plays a key role in the observed dwarf and narrow-leaf phenotypes by regulating cell growth. Several phytohormones, including brassinosteroids (BRs), cytokinin (CK), auxins and ethylene, are known to influence plant architecture traits such as plant height, leaf morphology, and grain size (Wang *et al.*, 2018). Many previously identified genes associated with maize plant height and leaf size are involved in phytohormone biosynthesis or signal transduction. For example, genes involved in polar auxin transport, such as *Brachytic2* and *ZmPIN1a*, play a crucial role in mediating and regulating internode elongation in maize (Multani *et al.*, 2003; Li *et al.*, 2018). Additionally, reduced expression of genes like *NARROW*

LEAF 7 and *FISH BONE*, essential for auxin biosynthesis, result in a narrow-leaf phenotype. Mutants with impaired BR biosynthesis or signaling, including the *BRASSINOSTEROID INSENSITIVE1* KD line, also display dwarf phenotypes (Zhang *et al.*, 2014).

The observed *nac24* mutant phenotype suggests that NAC24 may regulate plant height and leaf shape by either modulating phytohormone synthesis, signal transduction, or hormone crosstalk to ensure proper development. To further support this hypothesis, future studies could quantify endogenous phytohormone levels (BRs, ethylene, auxin) in *nac24* mutants compared to WT plants.

In *nac24* KD plants, we also observed the presence of yellow spots on the leaves, suggesting a possible reduction in chlorophyll content, which is essential for photosynthesis. Decreased chlorophyll levels can impair light absorption, energy transfer, and starch synthesis, ultimately leading to stunted growth, dwarfism, and sterility (Li *et al.*, 2020).

One hypothesis for this phenotype is the disruption of the *yellow-green3* (*yg3*) gene in mutants, as NAC24 was found to bind to its promoter *in vitro*. *yg3* encodes a predicted pentatricopeptide repeat (PPR) protein, which plays a crucial role in RNA-related processes such as transcription, RNA processing, splicing, stability, editing, and translation. A mutation of this gene has been associated with pale green or yellow leaf phenotypes in maize (Sorgini & Crystal, 2018). The transcriptional regulation of *yg3* mediated by NAC24 could therefore influence leaf coloration by affecting chlorophyll biosynthesis, degradation, or chloroplast development. Further research on chlorophyll content, electron transport rate, and net photosynthetic rate in the mutant plants would provide a better understanding of the role of NAC24, and its potential regulatory relationship with *yg3*.

Reproductive development was also affected in the mutant. The tassel had fewer branches, while the ears occasionally failed to develop. Pollen viability was also affected by *NAC24* KD, as shown by an FDA test on fresh pollen. Pollen viability relies heavily on the accumulation of sufficient reserve metabolites, which are essential for energy supply during germination. This suggests that reduced enzymatic activity in *nac24* mutant pollen could be directly related to impaired starch and/or sugar biosynthesis, as well as decreased energy metabolism (De Storme & Geelen, 2014). Consequently, when this pollen was used for crossing, a reduction in the number of seeds per ear was observed (data not shown). This is not the first report of a maize NAC TF influencing male fertility. NAC84 and NAC132 affect pollen viability

and germination (Yang *et al.*, 2018; Yuan *et al.*, 2023), highlighting the broader role of NAC TFs in regulating male fertility in maize.

When we initially requested Bonn*Mu* mutant seeds, only one transposon insertion line in *NAC24* was available. However, with recent expansions of the Bonn*Mu* library (Win *et al.*, 2024), two additional lines are now available: one with a transposon insertion in the core promoter and another with an insertion in the proximal promoter. Comparing the gene expression and phenotypes of these new lines with our existing data would confirm the function of *NAC24*. While transposon-based mutants provided valuable information, CRISPR-Cas9-mediated *NAC24* KO would be a necessary complementary approach. Another complementary strategy would be to introduce a functional *NAC24* transgene into the *nac24* mutant line and assess whether the WT phenotype is restored. However, the B73 background of these plants presents a challenge, as this variety is known difficult to transform. Given the largely deleterious phenotype of the *nac24* mutant, OE of *NAC24* in Arabidopsis or maize could potentially yield beneficial effects, as many NAC TFs are known to enhance stress tolerance or promote development. A gain-of-function phenotype would provide additional information on the role of *NAC24* in maize development and/or stress responses.

Unveiling *NAC24* regulatory role in maize

DAP-seq is a powerful method to identify TFBS *in vitro*. However, it carries a risk of contamination of the analysis with false positives and false negatives. The integration of different layers of evidence, such as co-expression data, differential expression in mutant plants, motif identification, and *transactivation* assays, can help accurately identify functional target genes that are significant for the TF of interest. This characterization of TF binding preferences and the identification of target genes provide valuable information for predicting the genetic circuits that control gene expression in response to environmental, developmental, and physiological signals.

We used DAP-seq to map *NAC24* binding events in two distinct tissues: the primary root and the tenth leaf at the 14-leaf stage. These tissues were selected because *NAC24* is most expressed in the primary root, and the original eQTL analysis was performed on leaf tissue at the same developmental stage. We identified 1,119 peaks on the root gDNA dataset and 2,040 peaks on the leaf gDNA dataset. Due to the *in vitro* nature of DAP-

seq, these peaks represent all potential binding events, independent of tissue type or condition (O'Malley *et al.*, 2016).

Putative target genes were assigned to peaks located within 3 kb upstream of the TSS and 1 kb downstream of the TTS, resulting in 420 and 531 target genes in the root and leaf dataset, respectively.

By comparing our datasets with a recent study that mapped TF binding events for 166 maize TFs (excluding NAC24) from 30 distinct families (Galli *et al.*, 2024), we found that our peak counts were lower. Their study reported between 1,153 and 122,695 peaks per TF, with an average of ~33,000 peaks. They assigned target genes to peaks within 10 kb of the TSS, yielding an average of 8,400 target genes per dataset. Several factors may explain this difference. First, we applied stringent filtering, discarding reads mapped to multiple locations in the genome. In addition, differences in peak-calling algorithms may have contributed to the variation in peak counts. The decision to use a 10 kb window for target gene assignment in their study was based on the large size of the maize genome and the frequent insertion of transposons near genic regions, which can displace regulatory elements away from TSSs (Liu *et al.*, 2025). Applying similar parameters to our dataset could reveal additional NAC24 target genes whose regulation is influenced by distal *cis*-regulatory elements.

Furthermore, heterodimerization with other TFs may alter the binding specificity of NAC24, thereby expanding its binding sites and potential target genes, but these are not detected in a DAP-seq analysis.

We performed a GO enrichment analysis for biological processes using PANTHER, but no enriched biological processes were identified initially. However, when we focused on genes located near the peaks containing one of the two motifs, we observed significant enrichment in biological processes. These included responses to hormones, positive regulation of organ growth, seed maturation, and regulation of stress responses, among others. A closer examination of the genes identified in our DAP-seq results revealed genes involved in auxin, BR, ethylene, and CK synthesis or signaling. For example, auxin response genes included: *Aux/IAA transcription factor 14*, *Small Auxin Up RNA11*, *Small Auxin Up RNA12*) and *Auxin response factor*. In addition, we identified ethylene-responsive TFs, such as *AP2-EREBP 7* and *Tassels Replace Upper Ears1*, as well as *ARGOS1*, a regulator of ethylene signaling.

After confirming the binding of NAC24 to various gene promoters by EMSA, we performed DLR assays to evaluate its regulatory influence on these promoters. We arbitrarily defined the promoter region as 2.5–3 kb upstream of the ATG start codon and cloned it upstream of *FLuc*. Surprisingly, for the

DLR assay, we had to switch from maize leaf protoplasts to *N. tabacum* leaf protoplasts as nearly all tested promoters showed FLuc activity at background levels in maize mesophyll protoplasts (i.e. absence of promoter-driven *FLuc* expression). However, five promoters (*LDSH5*, *ARGOS1*, *PILS6*, *AUX14*, and *ACOZ1*) showed activity in *N. tabacum* leaf protoplasts, whereas *PDK1*, *METP4*, and *RHD3* still displayed no activity. This suggests that promoter activity is influenced by species- and cell-specific regulatory environments, indicating the presence of some promoter-specific silencing or repression mechanisms in maize that are absent in *N. tabacum*.

Nevertheless, we confirmed that NAC24 activates the *ARGOS1* and *LDSH5* promoters while repressing *AUX14*. No significant regulatory effect was observed for *ACOZ1* and *PILS6*. We discuss below, the potential physiological implications of these regulatory interactions and how they integrate into whole-plant regulation to potentially explain the mutant phenotype.

NAC24 activates *LDSH5* by binding to its promoter

Light-Dependent Short Hypocotyl 5 (*LDSH5*) is a direct target of NAC24, whose DLR assays in *N. tabacum* confirm its activation via promoter binding. *LDSH5* encodes an ortholog of *BEAK SHAPED GRAIN 1* (*BSG1*), a gene involved in determining the shape, size, and weight of rice grains (Yan *et al.*, 2013). In a broader context, *LDSH5* belongs to the ALOG/LSH (ARABIDOPSIS LIGHT-DEPENDENT SHORT HYPOCOTYLS 1 and *Oryza sativa* G1/LIGHT-DEPENDENT SHORT HYPOCOTYLS) family of plant-specific DNA-binding proteins acting as TFs or repressive chromatin recruiters. The ALOG/LSH proteins play highly conserved roles in plant development, including meristem identity, cotyledon formation, seedling photomorphogenesis, and leaf and shoot development (Takeda *et al.*, 2011). Furthermore, they are implicated in inflorescence architecture and floral development, influencing male and female reproductive organ formation (Yan *et al.*, 2013; Yoshida *et al.*, 2009). Maize *LDSH5* is not the first *LSH* gene found to be activated by a NAC TF. In *Arabidopsis*, *LSH3* and *LSH4*, expressed at the boundary between the shoot apical meristem and lateral organs, are transcriptionally activated by CUP-SHAPED COTYLEDON 1 (*CUC1*), a key regulator of organ boundary formation (Takeda *et al.*, 2011). The functional diversity among *LSH* family members suggests that the transcriptional activation of *LDSH5* by NAC24-mediated in maize is part of a broader regulatory network that influences plant growth and development. This regulatory relationship probably influences various developmental processes, including leaf and cotyledon formation, organ boundary determination, and inflorescence architecture. In addition, *LDSH5* is linked to

a QTL associated with grain size and yield (Zhou *et al.*, 2020). Disruption of this pathway in the *nac24* KD mutants could explain the observed phenotypes, such as altered leaf formation, male inflorescence abnormalities, and reduction in seed size.

NAC24 as regulator of ethylene sensitivity

Ethylene regulates various aspects of plant growth and development, including seed germination, leaf expansion, organ senescence, and fruit ripening, while mediating responses to abiotic stresses (Dubois *et al.*, 2018). Plants are extremely sensitive to ethylene through mechanisms that amplify responses to low concentrations and adapt or desensitize systems under constant or high exposure. Members of the ARGOS family, induced by ethylene, play a primary role in a negative feedback loop that desensitizes plants to ethylene, thus helping to regulate ethylene sensitivity (Azhar *et al.*, 2020). Overexpression of ARGOS family members from Arabidopsis and maize reduces ethylene sensitivity, as evidenced by faster seedling desensitization, higher growth rates, and altered expression of ethylene-dependent genes (Rai *et al.*, 2015). ARGOS OE improves drought tolerance, promotes biomass accumulation, and increases organ growth and grain yield by reducing plant sensitivity to ethylene (Shi *et al.*, 2015).

In this study, we identified *ARGOS1* (*Auxin-Regulated Gene Involved in Organ Size 1*) as a direct target of NAC24, which activates the *ARGOS1* promoter. ARGOS1 interacts with ethylene receptors in the signaling pathway to modulate ethylene perception and early signal transduction and acts as a negative regulator of ethylene responses (Shi *et al.*, 2015). The NAC24-ARGOS1 *transactivation* may be an integral part of this negative feedback loop, contributing to ethylene desensitization (Figure 33). *Cis*-regulatory elements identified within the 2-kb promoter region of *NAC24* suggest regulation via Ethylene-Responsive Factors (ERFs). The expression of ERFs is induced by EIN3/EIL1 TFs as part of the ethylene signaling cascade (Azhar *et al.*, 2020), which could activate NAC24, leading to ARGOS1 activation. In turn, ZmARGOS1 inhibits ethylene signal output by interacting with ethylene receptors, forming a regulatory circuit that fine-tunes ethylene perception and response.

In addition, OE of *ARGOS1* in both Arabidopsis and maize enhances drought tolerance, improves biomass accumulation, and increases organ growth and grain yield by reducing plant sensitivity to ethylene. Conversely, loss-of-function mutations or down-regulation of *ARGOS1* result in reduced plant and organ growth (Shi *et al.*, 2015a). The impact of ARGOS mutants on organ

growth can be linked to ethylene inhibitory effects on cell expansion and proliferation (Dubois et al., 2018). Thus, the small leaf and stunted plant phenotype observed in the *nac24* mutant may, in part, be due to the deregulation of *ARGOS1*, leading to an ethylene-sensitive phenotype that inhibits cell expansion and proliferation.

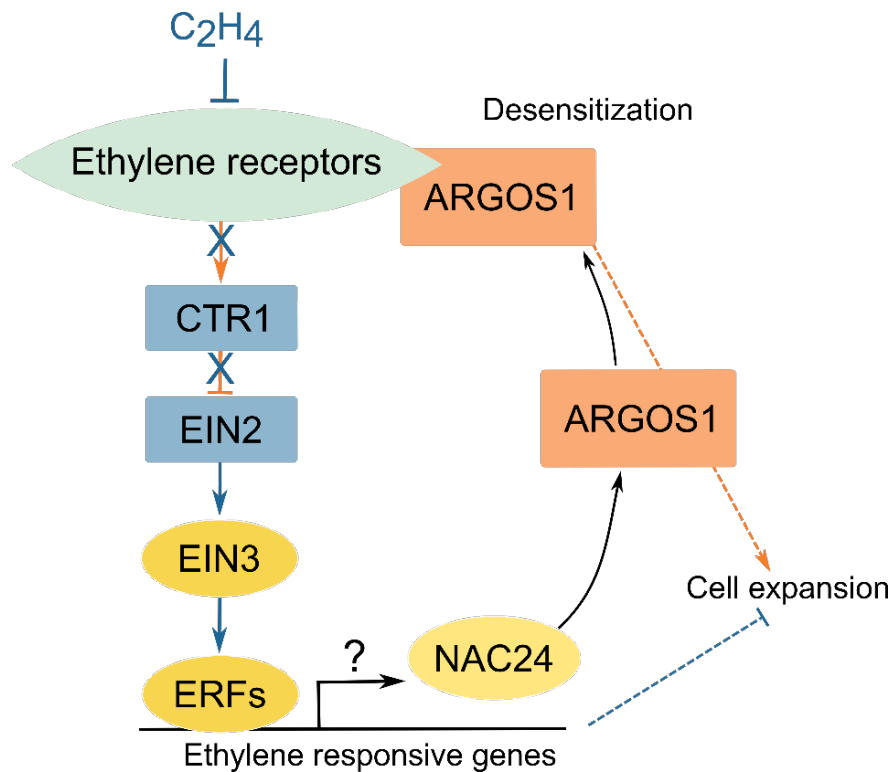


Figure 33: NAC24-mediated transcriptional regulation of ARGOS1 in maize could contribute to a negative feedback loop that modulates ethylene sensitivity.

In the presence of ethylene, ethylene receptors in the ER membrane become inactivated, initiating a signaling cascade that activates EIN3 and ERF TFs (blue pathway). Cis-regulatory elements within the NAC24 promoter suggest regulation by ERFs, which may induce NAC24 expression, leading to the activation of ARGOS1. In turn, ARGOS1 interacts with ethylene receptors to suppress ethylene signaling, forming a regulatory circuit that fine-tunes ethylene perception and response (orange pathway). The inhibition of ethylene signaling by ARGOS1 positively influences cell expansion and proliferation.

Ovals represent nuclear TFs, while rectangles depict ER membrane proteins. CTR1 = CONSTITUTIVE TRIPLE RESPONSE1; EIN2/3 = ETHYLENE INSENSITIVE 2/3; ERF = Ethylene-Responsive Factor; ARGOS1 = Auxin-Regulated Gene Involved in Organ Size 1.

Given its role in modulating ethylene sensitivity, activation of *ARGOS1* mediated by NAC24 could have significant agronomic potential.

NAC24 and auxin: link to root morphogenesis?

Auxin plays a crucial role in various aspects of plant growth and development, including cell division, vascular tissue formation, and root development. As key regulators, auxins modulate gene expression through specific TFs and proteins that integrate environmental signals into developmental responses. Auxin synthesis occurs in actively dividing tissues and is distributed via specialized transmembrane transporters that control its efflux and influx (Gomes *et al.*, 2020).

Through DAP-seq analysis, we identified several auxin-responsive genes as direct targets of NAC24, including *Aux/IAA14*, *Small Auxin Up RNA 11-12* (*SAUR 11 and 12*), *Auxin Response Factor (ARF)*, and *PIN-Like 6 (PILS6)*. NAC24 binds to the promoters of *Aux/IAA14* and *PILS6*, repressing *Aux/IAA14* promoter activity, while showing no effect on *PILS6*. Aux/IAAs, repressors of ARF TFs, regulate primary responses to auxin. In the presence of auxin, Aux/IAAs is degraded, activating ARF TFs and the transcription of auxin-induced genes (Luo *et al.*, 2018). In maize, it was shown that *Aux/IAA14* negatively regulates root dry weight (Wang *et al.*, 2024).

PILS proteins, localized in the ER, control intracellular auxin transport, influencing root architecture. In maize, *PILS6* mutants exhibit reduced root system architecture, fewer lateral roots, reduced shoot height and altered auxin response (Cowling *et al.*, 2024). NAC24 could contribute to the auxin signaling pathway through direct repression of *Aux/IAA14*, which encodes a transcriptional repressor of auxin signaling (Figure 34). Although we did not observe any effect of NAC24 on *PILS6* promoter activity when co-expressed in *N. tabacum*, a potential regulation in maize cannot be excluded. Future phenotyping of the roots of *nac24* mutant plants in different growing conditions could provide further insights into NAC24 function in root development and on the possible involvement of *PIP2;5*, and the auxin-responsive genes *Aux/IAA14* and *PILS6*.

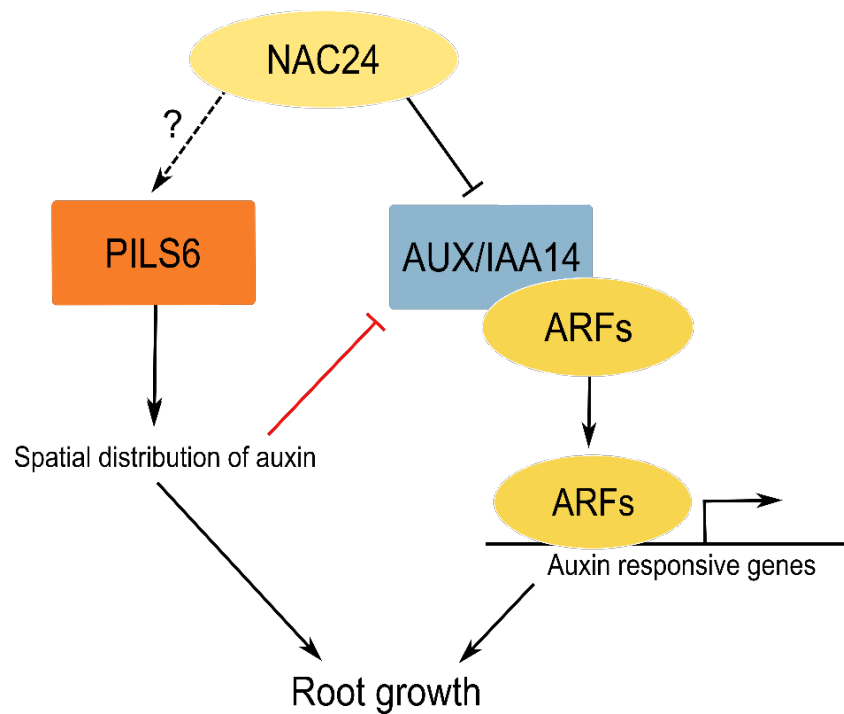


Figure 34: Proposed model of NAC24 transcriptional regulation of AUX/IAA14 and PILS6 involved in maize root development.

NAC24 could function in the auxin signaling pathway to promote root morphogenesis. It represses the transcription of the auxin signaling repressor AUX/IAA14 by directly interacting with its promoter, thereby regulating auxin signaling. This repression may free ARFs, enabling the activation of auxin-responsive genes. Additionally, NAC24 could influence intracellular auxin transport in root cells by regulating PILS6, though this remains unconfirmed. Auxin accumulation in the root may activate auxin signaling, which, in turn, could trigger the proteolysis of AUX/IAA14 (indicated by the red arrow).

Recent research highlights extensive crosstalk between auxin and other plant hormones such as BRs, CKs, ABA, and ethylene in the regulation of diverse growth and developmental processes. While auxin and BRs, for example, function independently in many pathways, increasing evidence suggests that they also function cooperatively in certain contexts (Bashri *et al.*, 2022). BRs and auxin regulate root growth in Arabidopsis through an antagonistic relationship, and maintaining the right balance between their opposing signals is essential for proper stem cell function and root development (Ma *et al.*, 2024). Furthermore, BR signaling controls PILS-dependent nuclear auxin levels by repressing PILS protein accumulation at the ER, both at the transcriptional and post-translational levels. This increases nuclear auxin abundance and signaling, linking BR pathways to auxin-driven organ growth (Sun *et al.*, 2020).

Recently, NAC24 was found to be differentially expressed (specifically upregulated) in response to epibrassinolide, an active BR, as shown by RNA-seq and RT-qPCR (Zhao *et al.*, 2022). An analysis using PlantRegMap (Tian *et al.*, 2020) identified *cis*-regulatory elements of BES1 (BRI1-EMS-Suppressor1) in the 2-kb promoter region of NAC24. Since BES1 TFs regulate BR-responsive genes, NAC24 could be activated by the BR signaling pathway via BES1 (Shi *et al.*, 2022).

BRs are perceived at the cell surface by the membrane-localized receptor BRASSINOSTEROID-INSENSITIVE1 (BRI1). Upon BR perception, BRI1 phosphorylates BRI1 KINASE INHIBITOR1, causing its release from the receptor. This triggers a signaling cascade that activates BZR1 and BES1, which then translocate into the nucleus to induce the expression of BR-responsive genes, including potentially NAC24 (Jing *et al.*, 2024). Interestingly, our EMSA data revealed that NAC24 can bind to an intron of *BK11*. This interaction could influence the expression of *BK11* gene or its alternative splicing, as some TFs are known to regulate such processes by binding to introns (Ullah *et al.*, 2024). Although the exact mechanisms remain unclear, and the effect of NAC24 on *BK11* is not yet known, it is plausible that NAC24 participates in a negative feedback loop regulating BR signaling.

Based on the data obtained in this study, NAC24 is likely involved in multiple hormonal pathways, potentially functioning in ethylene-responsive processes as well as auxin and BR signaling. NAC24 could even serve as a link between different hormonal signaling pathways to ensure proper development and possibly responses to abiotic stress. Although these ideas are currently speculative, they open promising avenues for future research. For example, integrating additional data layers with the existing DAP-seq results could provide further insights. An ideal next step would be conducting RNA-seq analysis on *nac24* mutants versus WT plants to generate complementary high-throughput expression data that can be directly compared with the target genes identified by DAP-seq.

MATERIAL & METHODS

Plant material

Zea mays

Zea mays inbred lines B104, B73, and the B73 × Co125 Bonn*Mu* population (Marcon *et al.*, 2020) were used. Seeds were surface sterilized with 50% sodium hypochlorite, rinsed with sterile water, and germinated vertically between moist filter paper at 30°C in the dark for two days. Seedlings were transferred to Jiffy pots with a 70:30 (v/v) potting soil (DCM) and vermiculite (Agra-vermiculite) mix and grown in an 8 h/16 h dark/light cycle at 25°C. For long-term growth, plants were transplanted as needed and maintained in growth chambers under a 16 h/8 h light/dark cycle at 25°C/18°C (day/night), ~70% humidity, and ~250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity. Granular fertilizer (Blaukorn Classic) was applied, when necessary, in the final pots.

For crossing and seed production, plants were maintained in growth chambers until flowering. Tassels were covered with paper bags once anthers began emerging and shedding pollen. Pollinations were performed around 10 a.m. using fresh pollen to pollinate growing silks. Plants were watered regularly for 25 days after pollination (DAP) and allowed to dry naturally until 35 DAP. Seeds were harvested and left to dry in paper bags for at least two additional weeks before further use.

For DNA and RNA extraction, primary roots (~1 mm pieces) or leaf sections (~2 cm) were flash-frozen in liquid nitrogen and ground into fine powder using glass beads (425–600 μm) and a Precellys 24 homogenizer (5000 rpm, 3 × 40 s). RNA was extracted using the Spectrum Plant Total RNA Kit (Sigma-Aldrich), incorporating on-column DNA digestion with DNase I (Sigma-Aldrich). Genomic DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega).

Nicotiana tabacum

Nicotiana tabacum (Petit Havana SR1) seeds were germinated in potting soil at 27°C. After eight days, seedlings were transferred to Jiffy pots for one week before being transplanted into plastic pots with potting soil. Plants were grown at 25°C under a 16 h/8 h light/dark cycle and watered daily.

Transactivation assay in maize epidermal cells

Genetic construct for the transactivation assay

Constructs used for the *transactivation* assay were derived from the pEGB *p35S::Luciferase::tNOS* plasmid (GB0110, Addgene plasmid #68216; Sarrion-Perdigones et al., 2013). This vector was previously modified by Maistriaux et al. (2024) to generate GB0110m-B73mVenus-K7mCherry, containing the *PIP2;5* promoter driving mVenus expression. The *NAC24* (Zm00001d006053, GRMZM2G008374) CDS was cloned from *Z. mays* B73 cDNA and fused to *tNOS* via triple PCR. The minimal *p35S* promoter and *NAC24::tNOS* fragments were inserted into the MCS, generating GB0110m-*pPIP2;5::mVenus::tNOS-p35S::mCherry::tNOS-p35S::NAC24::tNOS*. A control construct lacking *p35S* in the MCS was also created: GB0110m-*pPIP2;5::mVenus::tNOS-p35S::mCherry::tNOS-p0::NAC24::tNOS*. Plasmids were validated via restriction enzyme analysis and sequencing. Primer sequences used are listed in Table S2 and plasmids are listed in Table S3.

Biolistic transient transfection of leaf epidermal cells

Transient transfection of maize leaf epidermal cells was performed using the Biolistic® PDS-1000/He Particle Delivery System (BioRad), following the manufacturer's instructions and adapting the protocol described by Maistriaux et al. (2014).

Per bombardment, 1 mg of gold microbeads (0.6 µm, BioRad) was coated with 1.5 µg of plasmid DNA. Twelve-day-old B104 maize plants were selected, and the median portion of the fourth fully developed leaf (adaxial face) was secured on a Petri dish lid and positioned on the target shelf. The leaf was placed at the minimum possible distance from the microcarrier launch assembly, ~3 cm. The macrocarrier flight distance and the gap distance were set to 10 mm. Vacuum pressure was applied at 29" Hg before firing using a 1,100-psi rupture disk.

After bombardment, plants were maintained under 16 h light/8 h dark conditions for 40 h before imaging. Imaging was conducted using a Zeiss LSM710 confocal microscope equipped with a 40× aqueous immersion objective. The mCherry fluorescence was excited at 561 nm (helium-neon laser), while mVenus was excited at 514 nm (argon laser). Emission signals were collected using detectors set to 583–650 nm for mCherry and 519–564 nm for mVenus. Imaging settings were kept constant across all experiments. Fluorescence signals for mVenus and mCherry were quantified using Fiji software (Schindelin et al., 2012). Fluorescence was measured within

manually defined masks to ensure accurate region selection. Saturated fluorescence signals and signals from potentially adjacent transformed cells were excluded.

Fluorescence ratios were analyzed using a linear mixed model (*lmer* function, R) with random intercepts to account for experimental dependency. Experiments were performed independently four times, with bombardment, imaging, and analysis conducted blind.

Dual Luciferase Reporter Assay

Genetic constructs for Dual Luciferase Reporter assay

Plasmids for the DLR assay were constructed using the Golden Gate Modular Cloning Toolbox for Plants (Engler et al., 2014). Golden Gate cloning is a DNA assembly method using Type II restriction enzymes to assemble multiple DNA fragments in a single reaction, using 4 bp fusion site overhangs to ensure correct order. Starting modules needed to be domesticated by removing the Internal recognition sites for the Type II enzymes present in their sequence (Bird et al., 2022).

NAC24 CDS, the *NAC24* NAC domain (first 531 bp), and the *Renilla* CDS (from GB0109, Addgene #68215; Sarrion-Perdigones et al., 2013) were domesticated via triple PCR. The domesticated fragments were cloned into the level 0 acceptor (pICH41308). Promoters of interest were PCR-amplified from *Z. mays* B73 gDNA, domesticated, and inserted into pICH41295. Level 1 constructs were assembled using level 0 modules and proCaMV35SShort5U (pICH51277), CDSLuciferase (pICSL80001), and 3'UTR AtuNOS (pICH41421), into level 1 acceptors (pICH47802, pICH47811, pICH47751). Level 1 constructs, p35S::RLuc::tNOS, Pro::FLuc::tNOS, and p35S/p0::NAC24::tNOS, were assembled in the level 2 acceptor vector (pAGM4673). The final constructs consisted of p35S::RLuc::tNOS in position 1 Rv, Pro::FLuc::tNOS in position 2 Rv, and p35S/p0::NAC24::tNOS in position 3 Fw.

Golden Gate reactions (20 fmol of each plasmid, 1.5 μ L T4 Ligase, T4 ligase buffer, 1 μ L BsaI/BbsI, 25 μ L total volume) were cycled 50 times (37°C for 5 min, 16°C for 5 min) and inactivated at 80°C for 10 min. Verification of the constructs was performed by restriction for level 0 and level 1 plasmids, while level 2 plasmids were confirmed through sequencing.

Isolation and transfection of protoplasts

Maize mesophyll protoplasts were isolated from etiolated *Zea mays* B104 leaves and transfected following Zelazny et al., 2007. Protoplasts (120,000

cells) were resuspended in transfection medium (0.6 M mannitol, 15 mM CaCl₂, 4 mM MES, pH 5.7) and incubated with 10 µg plasmid.

The protoplasts were kept in the dark at 20°C for 16 to 20h in 1 ml culture medium (0.6 M mannitol, 4 mM KCl, and 4 mM MES, pH 5.7) in the presence of 90 µg/ml of cefotaxime.

N. tabacum protoplasts were isolated from 3-week-old leaves as described by (Lukaszewicz et al., 1998). Protoplasts were recovered by adding flotation medium (2 mL/leaf) (0.6 M sucrose, 15 mM CaCl₂, and 7.5 mM MES at pH 6.0) and centrifugation (7 min at 100 g). The floating layer was collected, diluted four times with washing medium (0.44 M mannitol, 80 mM KCl, 4 mM CaCl₂, and 0.1% BSA at pH 7.0) and centrifuged (7 min at 100 g). The pellet was washed with washing medium, and cell density estimated using a Fuchs-Rosenthal chamber. Protoplasts were transfected like maize protoplasts, with 10,000 protoplasts resuspended in 100 µL transfection medium and incubated with 10 µg plasmid.

Dual-luciferase reporter assay

DLR assays were performed using the Dual-Luciferase® Reporter Assay kit (Promega). Protoplasts were recovered 20 h after transfection by centrifugation (3 min at 100 g), resuspended in 50 µl of PLB, and incubated for 15 min. Luminescence quantification was performed using the GloMax® 20/20 Luminometer (Promega) for maize protoplasts and the FB12 Single Tube Luminometer (Berthold) for *N. tabacum* protoplasts. FLuc activity was measured by adding 50 µl of LAR II to 20 µl of lysed protoplasts, followed by RLuc quantification using 50 µL Stop & Glo® Reagent.

Subcellular localization

The *NAC24* CDS was fused to the *tNOS* terminator and inserted into pCAMBIA vectors (USER pCAMBIA2300 35S N-/C-terminal mYFP; Nour-Eldin et al. 2006; Bienert et al. 2011) to generate N-terminal and C-terminal YFP fusion constructs for subcellular localization. The plasmids were transfected into maize leaf mesophyll protoplasts. Before visualizing (16 – 20 h post-transfection), protoplasts were stained with Hoechst 33342 nuclear marker (20 µg/mL, Sigma-Aldrich) in the dark for 20 min, then washed with culture medium (centrifugation at 100 g for 3 min). Confocal images were acquired using a Zeiss LSM710 confocal microscope with a 40x aqueous immersion objective. YFP fluorescence was excited at 514 nm (argon laser) and detected at 519–579 nm. Hoechst staining was excited at 405 nm (diode laser) and detected at 410–503 nm.

Black Maize Sweet suspension cells

BMS suspension cells were grown in 50 mL BMS medium (4.4 g/L Murashige and Skoog basal salts with minimal organics (Sigma), 30 g/L sucrose, 2 mg/L 2,4-dichlorophenoxyacetic acid (Sigma), and 0.2 g/L of L-Asparagine (Sigma), pH 5.8) at 25°C in the dark with constant shaking (120 rpm). Cultures were diluted every 14 days to 5% plant cell volume (PCV).

For RNA extraction from BMS suspension cells, 2 mL of cells (15% PCV) were collected by centrifugation through a Miracloth filter, snap-frozen in liquid nitrogen, and homogenized (~100 mg) in 1 mL TRIzol™ reagent (Invitrogen). Total RNA was extracted following the manufacturer's protocol. The resulting RNA pellet was resuspended in 75 µl of RNase-free H₂O, and the RNA concentration was determined using a spectrophotometer.

For protein extraction, frozen cells were homogenized with glass beads (425–600 µm) in extraction buffer (250 mM sorbitol, 60 mM Tris-HCl, 2 mM Na₂EDTA, pH 8.0) supplemented with PMSF (1 mM) and protease inhibitors (2 µg/mL leupeptin, aprotinin, pepstatin, chymostatin). The cells were disrupted using a Precellys 24 homogenizer (Bertin) (5000 rpm, 3 × 40 s cycles). Samples were centrifuged at 2500 g for 5 min at 4° C, and the supernatant was further centrifuged at 7500 g for 10 min to isolate the nuclear fraction. The pellet was resuspended in extraction buffer, and 40 µg of protein from each fraction was analyzed by western blot using FLAG (Sigma-Aldrich) and mCherry (Rockland Immunochemicals) antibodies.

Stable transformation of BMS cells

Stable transformation constructs for BMS cells were generated using the Golden Gate Modular Cloning Toolbox for Plants (Engler *et al.*, 2014). The construct included a *NAC24-FLAG* and *mCherry* cassette driven by *p35S*, and a *kanamycin* resistance gene.

Stable transformation of BMS cells was performed using the PDS-1000/He Biolistic Particle Delivery System (Bio-Rad). Seven-day-old BMS cells were vacuum-filtered, spread onto Whatman filters on BMS agar, and bombarded with gold microbeads (600 µg; 0.6 µm; Bio-Rad), coated with 2 µg of DNA construct, at a 4 cm distance. After three days in the dark, filters were transferred to selective BMS medium with 150 µg/mL kanamycin, refreshed every two weeks. After ~2 months, growing calli were observed and transferred to new plates without filters. mCherry fluorescence was detected using an Amersham Imager 600 with EpiRGB filters (red, 630 nm). Fluorescent calli were subsequently cultured in liquid medium, and transgene presence was confirmed by western blot.

Bonn*Mu nac24* mutants

Genotyping of BonnMu mutants via PCR

Mu insertions in the *NAC24* intron were validated for each plant by PCR-based genotyping using *NAC24*-specific and *MuTIR6* primers (Table S2) to amplify the genomic region flanking the insertion. Leaf tissue from the third leaf was used for gDNA extraction. PCR reaction: 0.3 μ L GoTaq polymerase, 4 μ L 50 $\times$ diluted gDNA, 4 μ L 5 $\times$ GoTaq buffer, 0.5 μ L dNTPs (10 mM), and 0.5 μ L of each primer (10 μ M). The thermocycling program: 94 °C for 2 min, followed by 35 cycles of 94 °C for 25 s, 60 °C for 30 s, and 72 °C for 1 min. PCR products were purified and sequenced to confirm *Mu* insertion sites.

Metabolic viability of pollen

Mature pollen grains were collected in the morning and immediately tested for metabolic viability using fluorescein diacetate (FDA) staining. Fresh pollen was incubated in 8 μ g/mL FDA solution (diluted from a 50 mg/mL DMSO stock in PBS) for 15 min, then washed three times with PBS. Stained pollen was visualized using a Zeiss Axio Observer Z1 epifluorescence microscope with 450 nm excitation and 520 nm emission filters. Fluorescence intensity was quantified using ICY software (Institut Pasteur), analyzing 30 pollen grains per plant and six plants per genotype.

***NAC24* and *PIP2;5* expression by RT-qPCR**

cDNA synthesis

cDNA synthesis for RT-qPCR was performed with 1 μ g of total RNA. A Poly-T tail was added by incubating with 1 μ L Poly-T-18 (100 μ M) at 70°C for 5 min, followed by immediate cooling on ice. Reverse transcription was carried out using M-MLV reverse transcriptase (Promega) and RNasin (Promega), following the manufacturer's instructions. The final cDNA volume was 200 μ L.

RT-qPCR

RT-qPCR primers are listed in Table S2. Gene expression analysis of *NAC24* and *PIP2;5* was performed on cDNA derived from BMS cells overexpressing *NAC24* and from primary root tissue of transposon-induced *nac24* mutants. Reactions (20 μ L) contained 5 μ L cDNA, 0.5 μ L of each primer (10 μ M), and 10 μ L SYBR Green Master Mix 2 $\times$ (Eurogentec). Cycling conditions: 95°C for

10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s, with a melting curve analysis from 60°C to 95°C. Cycling was conducted using the StepOnePlus™ Real-Time PCR System (Applied Biosystems).

Melting curve analysis was performed from 60 °C to 95 °C to verify primer specificity, ensuring that a single discrete PCR product was generated. The resulting PCR products were purified and sequenced to confirm amplification of the correct target.

Gene expression of *NAC24* and *PIP2;5*, along with reference genes (*EF1 α*, *GAPDH*, and *actin*), was measured in triplicate biological samples with two technical replicates, repeated across two independent plates. In BMS cells, *actin* and *EF1 α* were used as controls, while *GAPDH* and *actin* were used for *nac24* mutant root tissue. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008).

DAP-seq and data analysis

Dap-seq experiment

The DNA library preparation and DAP-seq experiment were performed as described by O'Malley et al. (2016). Briefly, gDNA was extracted from primary roots and the tenth leaves of 14-leaf stage plants (B73 accession) and 20 μ g was fragmented using a sonication bath for 30 min. Sheared DNA was end-repaired using the Fast DNA End-Repair Kit (Thermo Scientific), A-tailed with Klenow Fragment (3'→5' exo-) (NEB), and Y-adapters were added with T4 DNA ligase (NEB). After final cleanup, the concentration of the DNA library was quantified using the Qubit dsDNA Assay (Invitrogen).

The pENTR::NAC24 construct was LR recombined into pIX-HALO::ccdB (Bartlett et al., 2017), and the NAC24-Halo fusion protein was expressed using the TNT SP6 Wheat Germ Protein Expression System (Promega). The NAC24-HALO protein was incubated with Magne® HaloTag® Beads (Promega) for 1 h at 25°C with gentle rotation. After washing, 1 μ g of gDNA library was added, and samples were incubated for 1 h at 25°C. Unbound DNA was removed with six washes, and bound DNA was eluted in Elution Buffer (QIAGEN) at 98°C for 10 min. Eluted fragments were PCR-amplified (20 cycles) to incorporate index sequences (NEBNext® Multiplex Oligos for Illumina®, NEB). PCR products were analyzed on an agarose gel, purified using the MinElute PCR Purification Kit (QIAGEN), and quantified with the Qubit dsDNA Assay (Invitrogen). Mock DAP-Seq libraries were prepared under identical conditions using GFP-Halo proteins as a negative control. Libraries were sequenced on NovaSeqX (paired-end, 150 bp reads).

Data analysis

DAP-seq data analysis was performed using the CLC Genomics Workbench (QIAGEN). Reads were trimmed to remove ambiguous nucleotides, adaptors, and polymer tails before being uniquely mapped to the maize B73_V4 reference genome using default mapping parameters. The mapped reads were then analyzed using the Transcription Factor ChIP-seq tool for peak calling, with GFP samples serving as the negative control.

Peak sequences from NAC24-bound regions were extracted using the extract sequences tool in CLC Genomics Workbench. These sequences were analyzed using MEME (Multiple EM for Motif Elicitation) (<https://meme-suite.org>) to identify enriched motifs. Identified motifs were compared to known TFBS in the JASPAR database (<https://jaspar.elixir.no>) using the Tomtom motif comparison tool in MEME. To locate matching sequences in the *Zea mays* B73RefGen v4 upstream gene database, the MAST (Motif Alignment & Search Tool) web tool was used.

GO enrichment analysis for biological processes was performed using the PANTHER (Protein ANalysis THrough Evolutionary Relationships) statistical overrepresentation test (<https://www.pantherdb.org/>). The analysis was performed on genes containing a peak with enriched motifs. Fisher's exact test was used, and the false discovery rate (FDR) was calculated to correct for multiple testing.

Production and purification of recombinant NAC24

Genetic constructs for production of recombinant NAC24

To produce recombinant NAC24 protein, cDNA from NAC24 (B73 inbred line) was amplified to obtain either the full coding sequence or the sequence encoding only the NAC domain (NAC24₁₄₋₁₇₅). Mutant versions (NAC24 R26A, NAC24 K85A/R92A/R96A and NAC24 R92A/R96A) were generated by site-directed mutagenesis.

The PCR products were digested and cloned into pGEX 4T-1 (with EcoRI and XhoI) (Amersham Biosciences) or pQE-60 (with BamHI and MluI) (QIAGEN) expression vectors. Expression plasmids were transformed into *Escherichia coli* strain C41 (DE3) for recombinant protein production.

Production of recombinant NAC24 in E. coli

Transformed *E. coli* cultures were grown to OD₆₀₀ 0.3–0.4 at 37°C, and protein expression was induced with 0.5 mM isopropyl β-D-thiogalactoside (IPTG) for 5 h at 30°C. Cells were harvested by centrifugation (5 min, 5000 g, 4° C) and

resuspended in lysis buffer (10 mM Tris-HCl (pH 8.0), 50 mM NaCl, 20 mM imidazole, 5 mM DTT, 1 mM PMSF) supplemented with 0.8 mg lysozyme and incubated on ice for 15 min. The samples were sonicated (4x 20 s; amplitude 40, Ultrasonic processor, Vibra-Cell) and the lysates were cleared by centrifugation at 40.000 *g* for 30 min (Beckman-coulter Optima LE-80K ultracentrifuge, Ti-50 rotor).

Purification of NAC24-GST

The supernatant was mixed with 2 ml bed volume of Glutathione-sepharose for fast flow (Cytiva), and incubated for 2 h at 4°C. The mixture was loaded onto a Poly-Prep 20 ml column (Bio-Rad), and the flow-through was discarded. The Glutathione-Sepharose was washed with 10 bed volumes of lysis buffer. Bound proteins were cleaved on the column by incubating the resin with 6-His-TEV protease, diluted in PBS with 10% glycerol (w/v). The TEV protease, produced and purified in-house as described by Tropea *et al.* (2009), was added at an empirically determined amount specific for the fusion protein. For each milliliter of resin containing bound protein, 10 mg TEV was added. The resulting flow-through was collected, frozen in liquid nitrogen, and stored at -80°C. Protein purity was evaluated using SDS-PAGE followed by Coomassie blue staining. When necessary, the sample was concentrated using a Millipore Amicon Ultra 15 filter unit (30 kDa cut-off), according to the manufacturer's instructions, to a volume of 500 µl for SEC. SEC was performed on a SuperDex 200 column (GE Healthcare) connected to an ÄKTA Pure system (GE Healthcare), with concentrated samples eluted in SEC buffer (25 mM NaH₂PO₄, 200 mM NaCl, 10% glycerol (w/v), pH 7.5). Protein elution was monitored at 280 nm, and 500 µl fractions were collected and analyzed by SDS-PAGE.

Purification of NAC24 NAC domain

The NAC24₁₄₋₁₇₅-His fusion proteins and mutated variants were purified by incubating the supernatant with 600 µl of Ni-NTA agarose (QIAGEN) for 2 h at 4°C. The samples were then loaded onto a Poly-Prep 10 ml column (Bio-Rad), and the columns were washed with 10 column volumes of TK40 buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 40 mM imidazole, 1 mM DTT, 1 mM PMSF). The proteins were eluted in 300 µl fractions using TK300 buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 300 mM imidazole, 10% glycerol (w/v)). The elution fractions were pooled and concentrated using a Millipore Amicon Ultra 15 (10 kDa cut-off). SEC was carried out using a SuperDex 200 column (GE Healthcare) connected to an ÄKTA Pure system (GE Healthcare). Samples were eluted in SEC buffer, and collected fractions were analyzed by

SDS-PAGE. Fractions containing the NAC24 NAC domain were pooled, aliquoted, frozen in liquid nitrogen, and stored at -80°C. Protein concentration was determined using the Bradford method.

Electrophoretic mobility shift assay

DNA probes

For gel mobility shift assays, a Cyanine-3-tagged oligonucleotides were prepared by annealing complementary single-stranded oligonucleotides, with one strand tagged at the 5' end with CY-3. Annealing was performed in TE buffer by heating at 100°C for 5 min, followed by cooling to room temperature. The annealed oligonucleotides were purified using the MinElute PCR Purification Kit (QIAGEN).

Peak sequences from promoter regions and the fragmented *PIP2;5* promoter were PCR-amplified using a CY-3-tagged Fw primer, separated on agarose gels, and purified with the Nucleospin Gel and PCR Clean-up Kit (Macherey-Nagel). Primers used for EMSA probe generation are listed in Table S2. All CY-3-tagged DNA probes were stored in the dark.

Gel mobility assays

For the gel mobility shift assay, 350 fmol of DNA probes were incubated with increasing concentrations of NAC2414-177-His (up to 400 nM) in DNA binding buffer (25 mM Tris-HCl, 150 mM NaCl, 8% glycerol, 1 mg/mL BSA, 50 µg/mL sonicated salmon sperm DNA, pH 7.5) to a final volume of 40 µL. After 20 min incubation at room temperature, samples were loaded onto an 8% polyacrylamide/TAE gel and electrophoresed at 45 mA in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) at 4° C for 2–4 hours. A lane with bromophenol blue was included to monitor migration. Nucleic acids were visualized using the CY3 filter of the Typhoon Biomolecular Imager (Cytiva).

Bioinformatics

The theoretical molecular weight and isoelectric point (pI) of NAC24 were predicted using the ExPASy ProtParam tool (<https://web.expasy.org/protparam/>). Nuclear localization sites (NLS) within NAC24 were identified using the NLSExplorer prediction tool (<http://www.csbio.sjtu.edu.cn/bioinf/NLSExplorer/>). Multiple sequence alignment between NAC24 and other NAC members was performed using the msa function in R (<https://bioconductor.org/>).

Cis-regulatory elements in the 2-kb promoter regions of *NAC24* and *PIP2;5* were identified using PlantRegMap (<https://plantregmap.gao-lab.org/>). The structure of the NAC24 NAC domain bound to the NAC24 NACBS was predicted using AlphaFold3 (<https://alphafoldserver.com/>). The predicted structure and its Predicted Aligned Error, were visualized with the PAE viewer (<https://subtiwiki.uni-goettingen.de/v4/paeViewerDemo>). The output model was loaded into PyMol for further analysis and visualization (version 2.2.5, Schrödinger).

Accession numbers

The Uniprot accession numbers of the selected NAC proteins: the Arabidopsis ATAF1 (Q39013); ATAF2 (Q9C598); AtNAM (Q9ZNU2); ANAC 019 (Q9C932); CUC1 (Q9FRV4); CUC2 (O04017); ANAC 092 (Q9FKA0); the maize ZmNAC60 (A0A1D6 GER6); ZmSNAC1 (B5A4B4); ZmSNAC13 (A0A804QVK5); ZmNAC24 (A0A1D6 ESJ5).

Gene accessions of selected maize genes: *NAC24* (Zm00001d006053); *PIP2;5* (Zm00001d003006); *PDK1* (Zm00001d029285); *CASP6* (Zm00001d034189); *RHD3* (Zm00001d030998); *LDSH5* (Zm00001d043011); *HK1* (Zm00001d017977); *BKI1* (Zm00001d031798); *CNR8* (Zm00001d039776); *AUX14* (Zm00001d045203); *YG3* (Zm00001d016828); *METP4* (Zm00001d028344); *ACOZ1* (Zm00001d039352); *PILS6* (Zm00001d043083); *ARGOS1* (Zm00001d025490).

SUPPLEMENTAL DATA

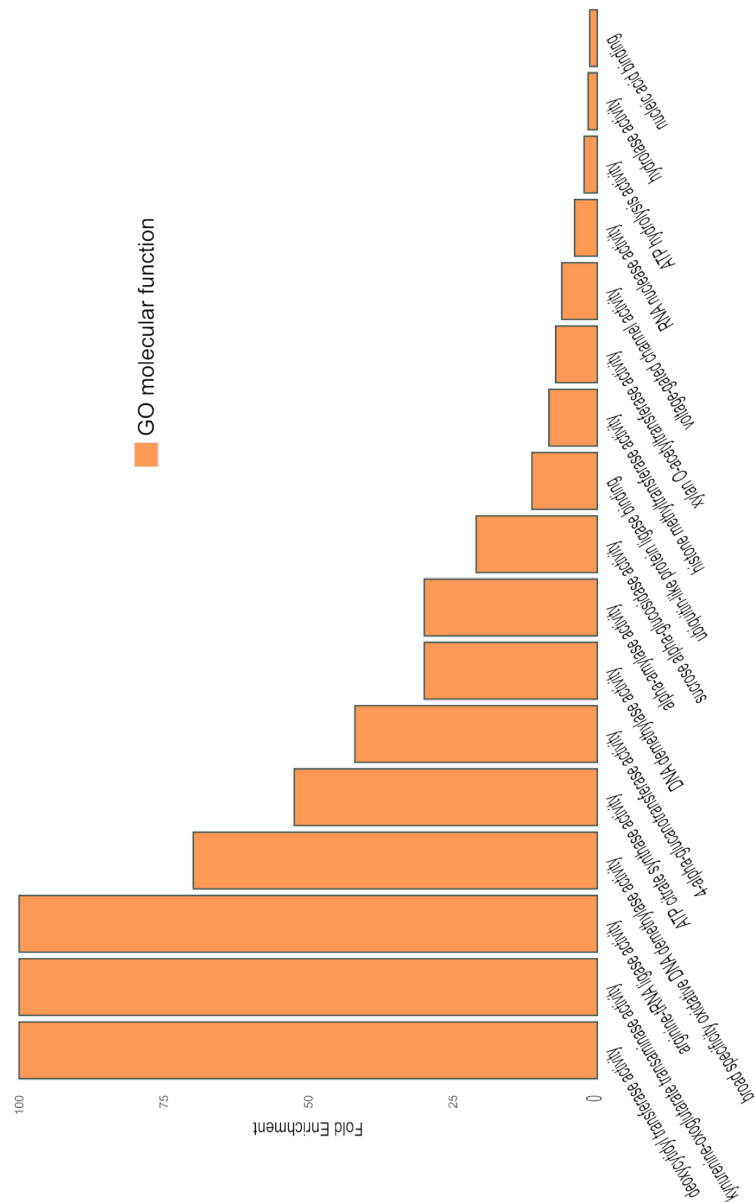


Figure S1: Gene-ontology analysis performed on genes located near a NAC24 binding site in root gDNA. The y-axis represents the fold enrichment of these genes compared to all the *Z. mays* genes, while the x-axis represents the GO molecular function for which the genes are overrepresented. The analysis was conducted using the PANTHER overrepresentation test with Fisher's exact test to assess statistical significance.

A

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MEASAAAGGGRESNKEEESLPPGFRFHPTDEELITYYLRRKIA
DGRFTARAIAEVDLNKSEPWDLPEKAKLGEKEWYFFSLRDRKYP
TGVRTNRRATNAGYWKTGKDKIYTAGHQLPAATTPELVGMKKT
LVFYKGRAPRGEKTNWVMHEYRLHSKSLPKSNKDEWVVCRVF
AKSAAKKYPSNNAHAHARSSHHHPYALDMFPPLLP TLLQHDPF
VARRHHHHHPYMAPADLAELARFARGTPGLHPIQPHPGTSSS
APYMNPAVAAPPTLSGGGRLNLNLGATPAMPSSPPALHAMSM
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GCVIVPGADGGFGADSAAGARYQGLDVEQLVERYWPAGGYQV
```

B

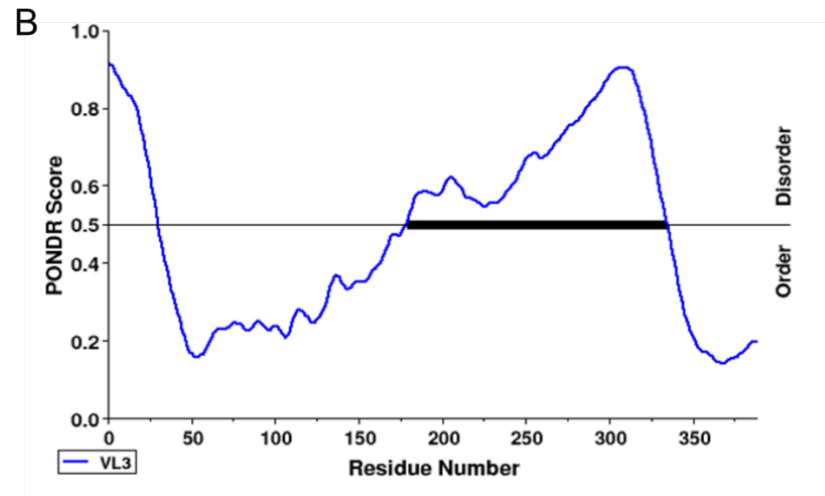


Figure S2: NAC24 C-terminal domain is prone to aggregation.

(A) NAC24 amino acid sequence with the NAC domain highlighted in yellow. Aggregation-prone regions, identified as 5–15 amino acid fragments with high hydrophobicity and low net charge, are marked in red and predominantly located within the CTD.

(B) Prediction of intrinsically disordered regions in NAC24 using the VL3 PONDR score (<http://www.pondr.com/>). A threshold (disorder assigned to values ≥ 0.5 , indicated by the black bar) reveals that the CTD (residues 170–330) exceeds the threshold, indicating this region is disordered and prone to aggregation. The x-axis represents amino acid positions, and the y-axis represents PONDR scores predicting natural disordered regions.

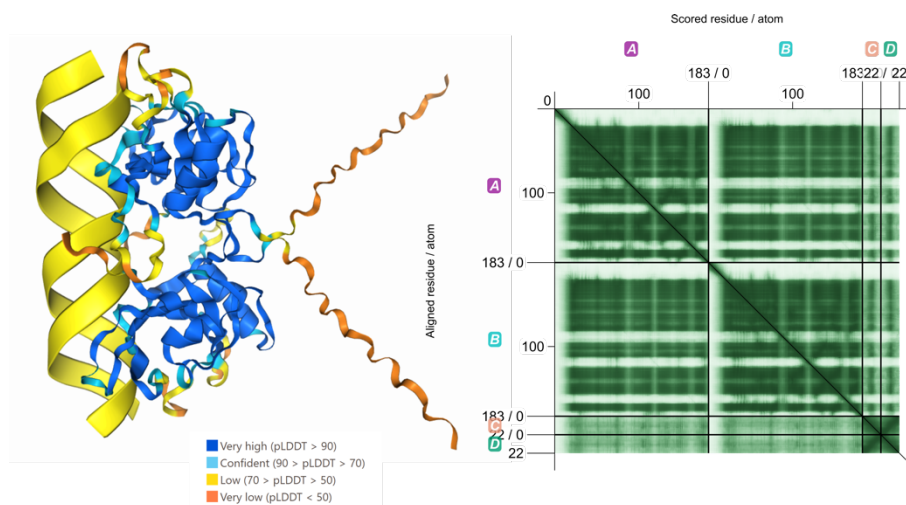


Figure S3: AlphaFold-predicted structure of the NAC24-NACBS complex with corresponding Predicted Aligned Error (PAE) plot. The left panel shows the NAC24-NACBS complex, with residues color-coded based on confidence scores: dark blue indicates very high confidence, while orange represents very low confidence. The right panel shows the PAE plot, representing the expected positional error in Angströms (Å) for a pair of residues, with the shade of green in each square reflecting confidence. Dark green tiles represent high confidence (low error), while light green tiles indicate low confidence (high error). The dark green diagonal line reflects residues aligned against themselves, which always have high confidence. Protein chains A and B correspond to the NAC24 monomers, while DNA strands are labeled as chains C and D.

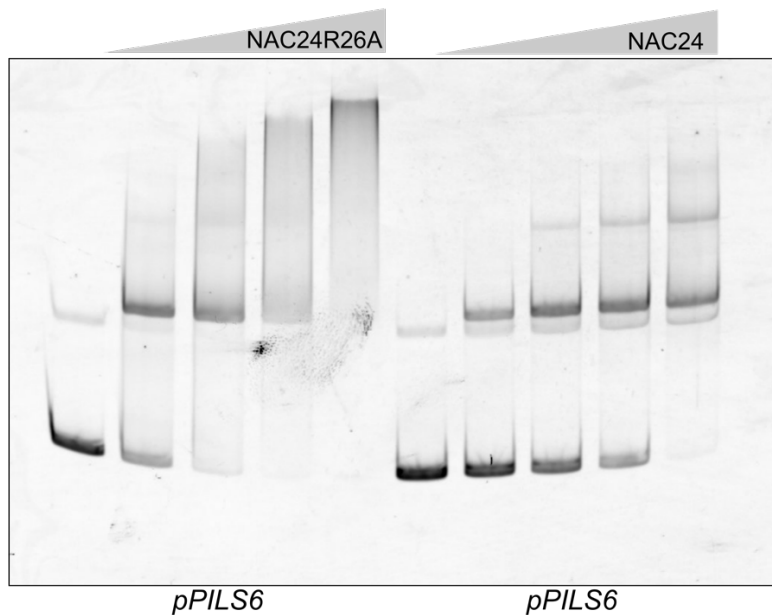


Figure S4: DNA-binding ability of NAC24 dimerization mutant.

Representative EMSAs comparing the DNA-binding ability of WT NAC24 NAC domain and mutant domain (R26A) to the PILS6 promoter. CY3-labeled probe was incubated with increasing amounts of recombinant NAC24 WT or mutant domains. The R26A mutant formed a DNA-protein complex consistent with monomer binding at low protein concentrations. At higher concentrations, a higher migrating, more diffuse band appeared, which did not correspond to dimer binding, suggesting that the mutation may reduce protein stability in solution.

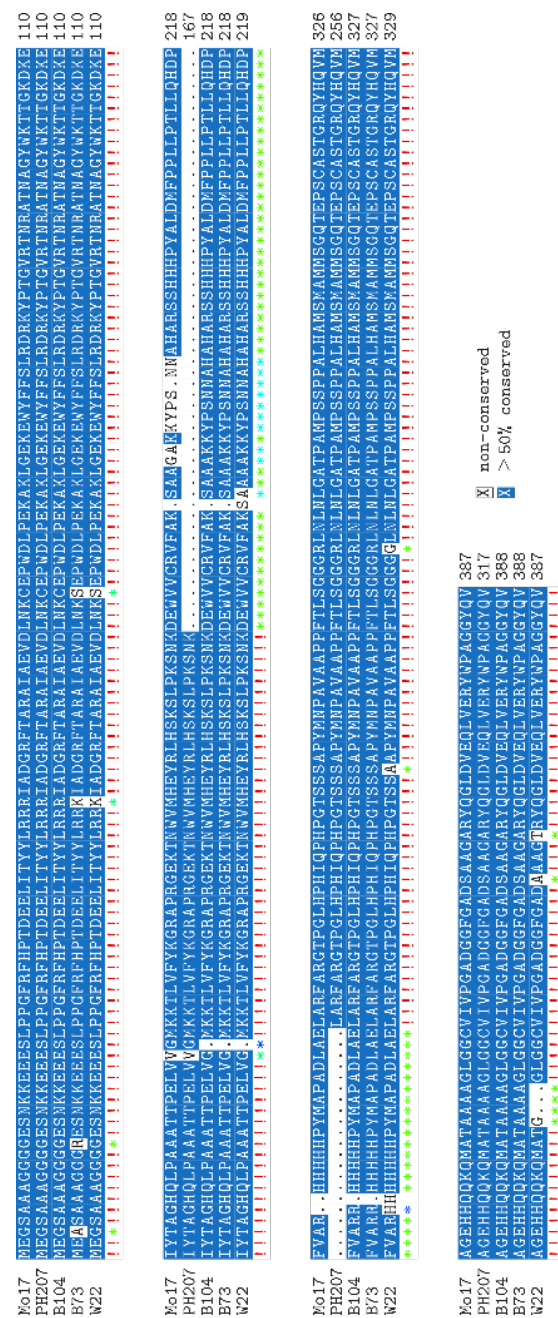


Figure S5: Multiple sequence alignment of NAC24 protein sequences from various maize accessions. NAC24 protein sequences from five maize accessions (Mo17, PH207, B104, B73, and W22) were aligned using ClustalW (msa function in R). The alignment revealed several missense mutations and deletions, with PH207 and W22 lacking 74 and 9 amino acid residues, respectively, compared to the B73 reference.

Table S1: List of genes identified through DAP-seq analysis performed on root gDNA with NAC24. This includes maize gene accessions showing a significant peak (p -value < 0.05) within their genic regions (promoter, terminator, intron, exon, UTR3', or UTR5') and the corresponding proteins they encode (as found on NCBI, www.ncbi.nlm.nih.gov). Only genes containing consensus NAC binding sites (motif 1 and motif 2, identified using MEME) are included. Genes encoding unknown proteins were excluded to streamline the list. The genes are ordered from the most significant peak to the least significant. Genes selected for EMSA and DLR assays are highlighted in yellow.

Gene	Protein(NCBI)	Chr	pvalue	Location	Motif
Zm00001d029285	pyruvate dehydrogenase (lipoamide) kinase1	Chr1	2,15297E-44	Promoter	motif1
Zm00001d034189	CASP-like protein	Chr1	1,38651E-39	Terminator	motif1
Zm00001d043011	LIGHT-DEPENDENT SHORT HYPOCOTYLS 5	Chr3	1,37851E-24	Promoter	motif1
Zm00001d002707	Glycosyltransferase	Chr2	2,02513E-24	Promoter	motif1
Zm00001d048775	Putative polyol transporter 1	Chr4	9,13505E-19	Exon	motif1
Zm00001d033463	small auxin up RNA11	Chr1	7,53786E-14	Promoter	motif1
Zm00001d033464	small auxin up RNA12	Chr1	7,53786E-14	Promoter	motif1
Zm00001d028074	GNAT-transcription factor 2	Chr1	3,56338E-13	Terminator	motif1
Zm00001d018820	FAD-dependent oxidoreductase family protein	Chr7	6,25549E-13	UTR5	motif1
Zm00001d022155	Leucine-rich repeat transmembrane kinase	Chr7	7,60938E-12	Promoter	motif2
Zm00001d012515	Proline-rich family protein	Chr8	1,3766E-10	Promoter	motif2
Zm00001d011555	NAC-transcription factor 1	Chr8	4,62627E-10	UTR5	motif2
Zm00001d009140	Peroxidase	Chr8	4,64616E-10	Promoter	motif2
Zm00001d034628	40S ribosomal protein S23	Chr1	9,65812E-09	Promoter	motif1
Zm00001d021791	Fiber expressed protein	Chr7	9,8019E-09	Promoter	motif2
Zm00001d011821	Calcium-dependent phospholipid-binding protein	Chr8	9,92447E-09	Promoter	motif2
Zm00001d013992	Pyridoxal phosphate (PLP)-dependent transferase	Chr5	1,30525E-08	Promoter	motif1
Zm00001d011846	Calcium-dependent lipid-binding family protein	Chr8	2,08087E-08	Promoter	motif2
Zm00001d036963	Transcription factor bHLH144	Chr6	4,03795E-08	Promoter	motif2
Zm00001d034428	Pentatricopeptide repeat 78	Chr1	4,14268E-08	Exon	motif1
Zm00001d008283	Serpin-Z1	Chr8	6,2435E-08	Promoter	motif2
Zm00001d029832	Protein trichome birefringence-like 12	Chr1	6,90388E-08	Promoter	motif1
Zm00001d014525	Glycosyltransferases	Chr5	7,72663E-08	UTR5	motif1
Zm00001d044704	Alpha/beta-Hydrolases superfamily protein	Chr3	8,2632E-08	UTR3	motif1
Zm00001d044705	phosphatidylethanolamine-binding protein1	Chr3	8,2632E-08	UTR3	motif1
Zm00001d017177	Serine/arginine-rich splicing factor RSZ22	Chr5	8,78017E-08	Promoter	motif1
Zm00001d030998	Protein ROOT HAIR DEFECTIVE 3 homolog	Chr1	1,02689E-07	Promoter	motif1
Zm00001d016118	ATP synthase subunit 1	Chr5	1,7538E-07	Exon	motif1
Zm00001d034621	Protein trichome birefringence-like 32	Chr1	1,3493E-06	Promoter	motif1
Zm00001d007039	SWI/SNF complex component SNF12-like protein	Chr2	1,99076E-06	Promoter	motif1
Zm00001d022038	protein S-acyltransferase32	Chr7	3,85659E-06	Promoter	motif2
Zm00001d036307	DNA-directed RNA polymerase subunit	Chr6	4,31976E-06	UTR5	motif2
Zm00001d012624	SNF2 domain-containing protein	Chr8	4,33146E-06	Intron	motif2
Zm00001d013709	WRKY-transcription factor 109	Chr5	5,08022E-06	UTR5	motif1
Zm00001d025185	Eukaryotic translation initiation factor 3 subunit H	Chr10	6,43258E-06	UTR5	motif2
Zm00001d014890	Transcription elongation factor (TFIIS) protein	Chr5	9,85128E-06	Promoter	motif1
Zm00001d015124	Pentatricopeptide repeat-containing protein	Chr5	1,12213E-05	Promoter	motif1

Zm00001d026632	Stem-specific protein TSJT1	Chr10	1,31866E-05	Promoter	motif2
Zm00001d048621	ABC transporter G family member 11	Chr4	1,34546E-05	UTR5	motif1
Zm00001d010452	Nudix hydrolase 11	Chr8	1,49348E-05	UTR5	motif2
Zm00001d025919	Aldolase-type TIM barrel family protein	Chr10	1,59421E-05	UTR5	motif2
Zm00001d012809	Cysteine-type peptidase	Chr5	2,12664E-05	Intron	motif1
Zm00001d013415	Receptor-like kinase TMK2	Chr5	2,34147E-05	Promoter	motif1
Zm00001d044468	Maternal effect embryo arrest 18	Chr3	2,69257E-05	Promoter	motif1
Zm00001d019920	Protein SMG7	Chr7	3,73172E-05	Terminator	motif1
Zm00001d022538	Hexosyltransferase	Chr7	3,8673E-05	Intron	motif2
Zm00001d052125	WRC domain-containing protein	Chr4	3,89574E-05	Promoter	motif1
Zm00001d013532	Defense-related protein	Chr5	5,63859E-05	UTR5	motif1
Zm00001d005573	Mediator of RNA polymerase II transcription 25	Chr2	5,83657E-05	Terminator	motif1
Zm00001d049380	Putative aminotransferase class III protein	Chr4	6,68138E-05	Terminator	motif1
Zm00001d010062	Rop guanine nucleotide exchange factor 7	Chr8	6,77727E-05	UTR3	motif2
Zm00001d017977	hairy sheath frayed1	Chr5	7,18232E-05	Promoter	motif2
Zm00001d006233	Lung seven transmembrane receptor protein	Chr2	7,56847E-05	Promoter	motif1
Zm00001d016230	ADP/ATP translocase	Chr5	0,00011997	Terminator	motif1
Zm00001d022206	4-alpha-glucanotransferase	Chr7	0,00014412	UTR5	motif2
Zm00001d010525	Kinesin-like protein	Chr8	0,00015329	UTR5	motif2
Zm00001d010526	O-fucosyltransferase family protein	Chr8	0,00015329	Promoter	motif2
Zm00001d003894	Double Clip-N motif-containing hydrolase protein	Chr2	0,00015477	Promoter	motif1
Zm00001d032609	Ser/Thr protein phosphatase family	Chr1	0,00015797	Exon	motif1
Zm00001d038644	Protein RNA-directed DNA methylation 3	Chr6	0,00016595	UTR5	motif2
Zm00001d014695	Terpene cyclase/mutase family member	Chr5	0,00017108	UTR5	motif1
Zm00001d022198	SAP domain-containing protein	Chr7	0,0001956	UTR5	motif2
Zm00001d032301	methyltransferase superfamily protein	Chr1	0,00021712	UTR3	motif1
Zm00001d017467	3-isopropylmalate dehydratase small subunit 2	Chr5	0,00024517	UTR5	motif1
Zm00001d007571	Hexosyltransferase	Chr2	0,00027442	Promoter	motif1
Zm00001d043986	Pyruvate kinase	Chr3	0,00028211	Promoter	motif1
Zm00001d014852	Ser/Thr-rich protein T10 in DGCR region	Chr5	0,00033870	Promoter	motif1
Zm00001d012042	small ubiquitin-related modifier1a	Chr8	0,00033995	Promoter	motif2
Zm00001d026366	RING finger domain/helicase domain protein	Chr10	0,00034500	UTR3	motif2
Zm00001d022338	transcription domain-associated protein1	Chr7	0,00035392	Promoter	motif2
Zm00001d013359	DNA binding	Chr5	0,00036040	Terminator	motif1
Zm00001d013360	Chaperone DnaJ-domain superfamily protein	Chr5	0,00036040	Terminator	motif1
Zm00001d010172	fructose bisphosphatase1	Chr8	0,00047950	Promoter	motif2
Zm00001d048332	Pentatricopeptide repeat-containing protein	Chr9	0,00048774	Promoter	motif2
Zm00001d046774	Zinc finger protein WIP2	Chr9	0,00060026	UTR5	motif2
Zm00001d043938	Peroxidase	Chr3	0,00064489	Promoter	motif1
Zm00001d008726	DUF4050 family protein	Chr8	0,00073710	Promoter	motif2
Zm00001d052167	AP2-EREBP-transcription factor 7	Chr4	0,00074534	Promoter	motif1
Zm00001d016815	Pentatricopeptide repeat-containing protein	Chr5	0,00081378	Terminator	motif1
Zm00001d050816	Alfin-like-transcription factor 7	Chr4	0,00093824	Promoter	motif1
Zm00001d009316	Tetratricopeptide repeat protein 5	Chr8	0,00097917	UTR5	motif2
Zm00001d016979	Prohibitin3	Chr5	0,00099160	Promoter	motif1
Zm00001d011828	splicing regulator45_2	Chr8	0,00100215	UTR5	motif2
Zm00001d023499	Retrovirus-related Pol polyprotein LINE-1	Chr10	0,00103063	UTR5	motif2
Zm00001d031454	MYB-related-transcription factor 103	Chr1	0,00104344	UTR5	motif1
Zm00001d028511	ARM repeat superfamily protein	Chr1	0,00112458	UTR5	motif1

Zm00001d043264	Pentatricopeptide repeat-containing protein	Chr3	0,00133611	Intron	motif1
Zm00001d045217	Subtilisin-like protease SBT6.1	Chr9	0,00138147	UTR5	motif2
Zm00001d051393	SPT2 chromatin protein	Chr4	0,00162340	Promoter	motif1
Zm00001d031798	BRI1 kinase inhibitor 1	Chr1	0,00165002	Intron	motif1
Zm00001d039776	Cell Number Regulator8	Chr3	0,00165472	Promoter	motif1
Zm00001d039777	phosphoglycerate mutase	Chr3	0,00165472	Terminator	motif1
Zm00001d039096	Pentatricopeptide repeat-containing protein	Chr6	0,00172676	Promoter	motif2
Zm00001d044641	Transducin protein / WD-40 repeat protein	Chr3	0,00185201	UTR5	motif1
Zm00001d013116	Cullin-4	Chr5	0,00188806	UTR5	motif1
Zm00001d022005	Transcriptional regulatory protein algP	Chr7	0,00192283	Terminator	motif2
Zm00001d051956	NAC-transcription factor 51	Chr4	0,00205060	UTR5	motif1
Zm00001d050372	Dentin sialophosphoprotein	Chr4	0,00207767	Promoter	motif1
Zm00001d011430	glycerol-3-phosphate acyltransferase13	Chr8	0,00211230	UTR5	motif2
Zm00001d018390	RING/U-box superfamily protein	Chr5	0,00222419	UTR3	motif1
Zm00001d022538	Hexosyltransferase	Chr7	0,00229810	Terminator	motif2
Zm00001d014692	Putative gamma-glutamylcyclotransferase	Chr5	0,002326	UTR5	motif1
Zm00001d028486	Chemocyanin	Chr1	0,00236273	Promoter	motif1
Zm00001d037035	Cyclic nucleotide-gated ion channel 1	Chr6	0,00236538	UTR5	motif2
Zm00001d051484	Putative RING zinc finger domain protein	Chr4	0,00257738	Terminator	motif1
Zm00001d028205	Mitochondrial substrate carrier family protein	Chr1	0,00268786	Promoter	motif1
Zm00001d027625	Multidrug resistance-associated protein 4	Chr1	0,00274308	UTR5	motif1
Zm00001d016477	aspartyl protease family protein	Chr5	0,0029552	Terminator	motif1
Zm00001d023669	NAC-transcription factor 67	Chr10	0,00299406	UTR3	motif2
Zm00001d034777	Nicotinate phosphoribosyltransferase	Chr1	0,00312031	Intron	motif1
Zm00001d041910	SHSP domain-containing protein	Chr3	0,00321278	UTR5	motif1
Zm00001d045203	Aux/IAA-transcription factor 14	Chr9	0,00338439	Promoter	motif1
Zm00001d045002	Nucleolar protein 10	Chr9	0,00369325	UTR5	motif2
Zm00001d002673	amino acid/auxin permease8	Chr2	0,00382538	Promoter	motif1
Zm00001d027480	Calcium-dependent protein kinase 6	Chr1	0,00403043	UTR3	motif1
Zm00001d046366	Transcriptional elongation regulator MIN1YO	Chr9	0,00439559	Terminator	motif2
Zm00001d033528	Protein NRT1/ PTR FAMILY 2.9	Chr1	0,00440878	UTR5	motif1
Zm00001d024679	bHLH-transcription factor 162	Chr10	0,00445343	UTR3	motif2
Zm00001d027546	bHLH-transcription factor 35	Chr1	0,00474047	Promoter	motif1
Zm00001d051337	Ubiquitin carboxyl-terminal hydrolase	Chr4	0,00474840	Intron	motif1
Zm00001d039314	slow anion channel-associated4	Chr3	0,00497264	Intron	motif1
Zm00001d011096	argonaute1	Chr8	0,00519851	Exon	motif2
Zm00001d011097	12-oxo-phytodienoic acid reductase4	Chr8	0,00519851	Promoter	motif2
Zm00001d011964	Putative beta-14-xylosyltransferase IRX10L	Chr8	0,00524882	Promoter	motif2
Zm00001d038988	Hydroxyproline-rich glycoprotein family protein	Chr6	0,00543044	Promoter	motif2
Zm00001d027266	DeSI-like protein	Chr1	0,00587635	Terminator	motif1
Zm00001d027267	Alpha/beta-Hydrolases superfamily protein	Chr1	0,00587635	Promoter	motif1
Zm00001d029598	Transcription initiation factor TFIID subunit 4b	Chr1	0,00604257	UTR5	motif1
Zm00001d024128	GINS complex protein	Chr10	0,00661211	UTR5	motif2
Zm00001d026392	LSM7-like	Chr10	0,00664249	UTR5	motif2
Zm00001d046663	RING/U-box superfamily protein	Chr9	0,00675812	Promoter	motif2
Zm00001d021071	Hydroxyproline-rich glycoprotein family protein	Chr7	0,00692900	Terminator	motif1
Zm00001d013361	Zinc ion binding	Chr5	0,00700566	UTR5	motif1
Zm00001d038471	RING-type E3 ubiquitin transferase	Chr6	0,00705157	Terminator	motif2

Zm00001d044717	outward rectifying potassium channel2	Chr9	0,00712276	UTR5	motif2
Zm00001d016828	yellow-green3	Chr5	0,00739906	Promoter	motif2
Zm00001d045837	Transducin/WD40 repeat-like superfamily protein	Chr9	0,00749229	Promoter	motif2
Zm00001d027731	YTH domain-containing family protein	Chr1	0,00783514	Promoter	motif1
Zm00001d044515	F-box protein SKIP8	Chr3	0,00802427	Terminator	motif1
Zm00001d044516	Development and Cell Death domain protein	Chr3	0,00802427	UTR5	motif1
Zm00001d031194	bZIP-transcription factor 31	Chr1	0,00825493	Promoter	motif1
Zm00001d041941	Leucine-rich repeat protein kinase family protein	Chr3	0,00904020	Terminator	motif1
Zm00001d049516	C3H transcription factor 38	Chr4	0,00915312	Promoter	motif1
Zm00001d018251	Knottin scorpion toxin-like domain-containing	Chr5	0,00922533	Promoter	motif1
Zm00001d028344	Putative metal tolerance protein C3	Chr1	0,00996732	Promoter	motif1
Zm00001d018124	mTERF domain protein21	Chr5	0,01057224	Promoter	motif1
Zm00001d002613	Glucose-methanol-choline oxidoreductase protein	Chr2	0,01066755	UTR5	motif1
Zm00001d032838	Growth-regulating factor	Chr1	0,01119074	Intron	motif1
Zm00001d003234	Zinc finger, C3HC4 type family protein	Chr2	0,01147075	Promoter	motif1
Zm00001d040639	RING-type E3 ubiquitin transferase	Chr3	0,01184076	Exon	motif1
Zm00001d018795	187-kDa microtubule-associated protein AIR9	Chr7	0,01221810	Promoter	motif1
Zm00001d002843	MYB-transcription factor 54	Chr2	0,01247661	UTR3	motif1
Zm00001d008794	WRKY-transcription factor 26	Chr8	0,01255406	Promoter	motif2
Zm00001d020351	Alpha-amylase	Chr7	0,01276461	UTR5	motif1
Zm00001d004933	Carbohydrate-binding X8 domain protein	Chr2	0,01327974	Promoter	motif1
Zm00001d039352	abnormal chromosome organization in zygotene1	Chr3	0,01345185	Promoter	motif1
Zm00001d025073	Protein argonaute 1	Chr10	0,01412995	UTR5	motif2
Zm00001d012788	Glycosyltransferase	Chr8	0,01427867	Promoter	motif2
Zm00001d050913	Intracellular transport protein USO1-related	Chr4	0,01445313	Promoter	motif1
Zm00001d040603	ATP citrate synthase	Chr3	0,01464676	Promoter	motif1
Zm00001d018135	ER6 protein	Chr5	0,01530749	Promoter	motif1
Zm00001d008782	FCS-like zinc finger32	Chr8	0,01585240	Promoter	motif2
Zm00001d040735	invertase alkaline neutral10	Chr3	0,01600805	UTR5	motif1
Zm00001d027481	DUF3598 domain-containing protein	Chr1	0,01610526	UTR5	motif1
Zm00001d039416	Alpha/beta-Hydrolases superfamily protein	Chr3	0,01614546	UTR5	motif1
Zm00001d013380	FIP1[V]-like protein	Chr5	0,01650825	Intron	motif1
Zm00001d007468	Agnet domain-containing protein	Chr2	0,01672961	UTR3	motif1
Zm00001d011550	S-locus receptor protein kinase family protein	Chr8	0,01695496	UTR5	motif2
Zm00001d005813	ZIM-transcription factor 13	Chr2	0,01698962	Intron	motif1
Zm00001d009740	Plasminogen activator inhibitor 1 RNA-binding	Chr8	0,01712971	Promoter	motif2
Zm00001d008744	Transaminase/transferase	Chr8	0,01722937	UTR5	motif2
Zm00001d014669	heavy metal ATPase2	Chr5	0,01808585	Promoter	motif1
Zm00001d018014	Kinesin-like protein	Chr5	0,01926279	UTR5	motif1
Zm00001d013440	H15 domain-containing protein	Chr5	0,01994452	Terminator	motif1
Zm00001d023728	DNAJ protein JJJ1-like protein	Chr10	0,02010623	Promoter	motif2
Zm00001d019684	Cyclin-L1-1	Chr7	0,02030341	UTR5	motif1
Zm00001d037172	Fucosyltransferase	Chr6	0,02033325	Terminator	motif2
Zm00001d037173	EF hand family protein	Chr6	0,02033325	Terminator	motif2
Zm00001d023345	Non-specific lipid-transfer protein 1	Chr10	0,02056568	Promoter	motif2
Zm00001d043121	Osmotin-like protein OSM34	Chr3	0,02081491	Promoter	motif1
Zm00001d052435	transporter of mugineic acid2	Chr4	0,02082507	UTR5	motif1
Zm00001d045475	Histone H3	Chr9	0,02135218	UTR5	motif2
Zm00001d047574	WRKY-transcription factor 65	Chr9	0,02151257	Promoter	motif2
Zm00001d010965	Yippee domain-containing protein	Chr8	0,02228764	UTR5	motif2
Zm00001d043783	CO CO-LIKE TIMING OF CAB1 protein domain16	Chr3	0,02249034	Promoter	motif1
Zm00001d011969	NAC-transcription factor 9	Chr8	0,02253094	UTR5	motif2

Zm00001d029408	ARF-related protein	Chr1	0,02255613	Promoter	motif1
Zm00001d035086	Polyadenylate-binding -interacting protein 10	Chr6	0,02289007	UTR5	motif2
Zm00001d033159	Chromatin associated protein	Chr1	0,02293155	UTR5	motif1
Zm00001d037032	Putative DUF231 domain containing family protein	Chr6	0,0230373	Promoter	motif2
Zm00001d021592	potassium high-affinity transporter14	Chr7	0,02305862	UTR5	motif2
Zm00001d023281	UDP-N-acetylglucosamine transferase ALG14	Chr10	0,02326660	Exon	motif2
Zm00001d029226	Rhomboid-like protein 19	Chr1	0,02344611	UTR3	motif1
Zm00001d016606	Protein MEI2-like 1	Chr5	0,02404296	Promoter	motif1
Zm00001d047673	DUF4378 domain protein	Chr9	0,02437153	Terminator	motif2
Zm00001d014239	Myosin-15	Chr5	0,02458931	Promoter	motif1
Zm00001d051666	invertase alkaline neutral1	Chr4	0,02472120	Intron	motif1
Zm00001d030760	Asparagine synthase family protein	Chr1	0,02477453	UTR5	motif1
Zm00001d019348	RING/FYVE/PHD-type zinc finger family protein	Chr7	0,02482497	Intron	motif1
Zm00001d030174	Lecithin-cholesterol acyltransferase-like 1	Chr1	0,02496374	Promoter	motif1
Zm00001d048948	Hevein-like preproprotein	Chr4	0,02503465	UTR3	motif1
Zm00001d020378	Type IV inositol polyphosphate 5-phosphatase 9	Chr7	0,02602789	UTR5	motif1
Zm00001d037538	Arginyl-tRNA synthetase	Chr6	0,02637265	Intron	motif2
Zm00001d014568	DNA repair protein REV1	Chr5	0,02647606	UTR5	motif1
Zm00001d022538	Hexosyltransferase	Chr7	0,02689277	Terminator	motif2
Zm00001d018416	bHLH-transcription factor 168	Chr5	0,02702826	UTR5	motif1
Zm00001d003473	Sugar transport protein 5	Chr2	0,02715064	UTR5	motif1
Zm00001d043083	PIN-formed protein14	Chr3	0,02729131	Promoter	motif1
Zm00001d025490	ARGOS1	Chr10	0,02738402	Promoter	motif1
Zm00001d027749	sulfate transporter4	Chr1	0,02827697	UTR5	motif1
Zm00001d042468	Structural maintenance of chromosomes protein	Chr3	0,028314889	UTR5	motif1
Zm00001d054001	Protein arginine N-methyltransferase	Chr4	0,029484393	UTR3	motif1
Zm00001d040033	Factor of DNA methylation 1	Chr3	0,029568079	UTR5	motif1
Zm00001d052198	Polyadenylate-binding protein 2	Chr4	0,030393697	UTR5	motif1
Zm00001d032938	DUF1677 family protein	Chr1	0,030555186	Terminator	motif1
Zm00001d002452	WRKY-transcription factor 70	Chr2	0,032367822	UTR5	motif1
Zm00001d042111	Tassels replace upper ears1	Chr3	0,032473149	Promoter	motif1
Zm00001d025131	protein disulfide isomerase11	Chr10	0,032974826	UTR5	motif2
Zm00001d046677	Nuclear pore complex protein GP210	Chr9	0,033655953	Promoter	motif2
Zm00001d045782	DNA oxidative demethylase ALKBH2	Chr9	0,035152955	UTR5	motif2
Zm00001d021015	DNA topoisomerase	Chr7	0,035396388	UTR5	motif1
Zm00001d047247	Putative RING zinc finger domain protein	Chr9	0,035785946	Terminator	motif2
Zm00001d053316	Casein kinase I isoform delta-like	Chr4	0,036365594	UTR5	motif1
Zm00001d037463	Fiber protein Fb34	Chr6	0,037176445	UTR5	motif2
Zm00001d023706	Thioredoxin M1	Chr10	0,037404912	UTR5	motif2
Zm00001d038963	DUF1639 family protein	Chr6	0,037431321	Promoter	motif2
Zm00001d008266	Peroxidase	Chr8	0,037446202	Promoter	motif2
Zm00001d017307	Two-component response regulator ARR16	Chr5	0,037449923	Promoter	motif1
Zm00001d021418	PRLI-interacting factor A	Chr7	0,037518883	Promoter	motif2
Zm00001d029272	peptidylprolyl isomerase	Chr1	0,037707749	UTR5	motif1
Zm00001d002729	Acyl-acyl carrier protein thioesterase ATL3	Chr2	0,03788457	Terminator	motif1
Zm00001d018204	DUF1644 family protein	Chr5	0,037921887	Promoter	motif1
Zm00001d017175	La protein 1	Chr5	0,038458687	UTR3	motif1
Zm00001d038815	CASC3/Barentsz eIF4AIII binding	Chr6	0,038971821	Terminator	motif2
Zm00001d034152	Mitogen-activated protein kinase 5	Chr1	0,03978828	Terminator	motif1
Zm00001d032078	Cell division cycle 2-related protein kinase 7	Chr1	0,040434476	UTR5	motif1
Zm00001d046374	Premnaspirodiene oxygenase	Chr9	0,040669018	Promoter	motif2
Zm00001d038192	glutathione transferase41	Chr6	0,040903637	Promoter	motif2
Zm00001d008693	Alpha/beta-Hydrolases superfamily protein	Chr8	0,040930678	Promoter	motif2

Zm00001d042933	Zinc finger protein ZAT11	Chr3	0,041746094	Promoter	motif1
Zm00001d037004	Ras-related protein RABA1d	Chr6	0,042564293	UTR5	motif2
Zm00001d013840	Ypt/Rab-GAP domain of gyp1p protein	Chr5	0,043300352	UTR5	motif1
Zm00001d043784	RING-type E3 ubiquitin transferase	Chr3	0,043336833	Promoter	motif1
Zm00001d013244	UDP-glycosyltransferase 83A1	Chr5	0,043613351	Terminator	motif1
Zm00001d033258	math-btb30	Chr1	0,046009676	Intron	motif1
Zm00001d048559	glutathione transferase35	Chr9	0,046807292	Promoter	motif2
Zm00001d044017	Putative plastid-lipid-associated protein 14	Chr3	0,04698859	UTR5	motif1
Zm00001d021565	Disease resistance protein RPM1	Chr7	0,047001868	UTR5	motif2
Zm00001d023333	set domain gene127	Chr10	0,04713839	UTR5	motif2
Zm00001d031062	Homeobox prospero protein	Chr1	0,047317184	Promoter	motif1
Zm00001d049860	NAC domain transcription factor 75	Chr4	0,047403245	UTR5	motif1
Zm00001d013653	Transcription regulator NOT2/NOT3/NOT5 protein	Chr5	0,047726453	UTR5	motif1
Zm00001d020837	C2C2-Dof-transcription factor 16	Chr7	0,047974539	Exon	motif1
Zm00001d038820	NSP (Nuclear shuttle protein)-interacting GTPase	Chr6	0,048656789	Promoter	motif2
Zm00001d049087	Exocyst complex component SEC3A	Chr4	0,048678069	Exon	motif1

Table S2: Primers used for molecular cloning, RTqPCR, and EMSA. Primers sequences are given in the 5'-3' orientation. F stands for forward, R stands for reverse primer and GG stands for Golden Gate.

Primer name	Sequence	Purpose
NAC24_F	ATGGAAGCATCAGCAGCAG	NAC24 cDNA cloning
NAC24_R	CTAGACTTGGTACCCGCCG	
p35S_F	GCGGCCGCTGAGACTTTTCAACAAAGGG	Amplification and insertion of p35S into GB010m
p35S_R	CTCGAGGTCCTCTCCAAATGAAATGAAC	
tNOS_F	GATCGTTCAAACATTTGGC	tNOS amplification
tNOS_R	GATCTAGTAACATAGATGACACC	
NAC24G_G_F	TTGAAGACAAAATGGAAGCATCAGCAGCAGC	Amplification and GG Cloning NAC24
NAC24G_G_R	TTGAAGACAAAAGCCTAGACTTGGTACCCGCCGGC	
RLucGG_F	TTGAAGACAAAATGACTTCGAAAGTTTATGATC	Amplification and GG Cloning <i>Rluc</i>
RLucGG_R	TTGAAGACAAAAGCTTATTGTTTATTTTGAAGAAC	
pPIP2;5G_G_F	TTGAAGACAAGGAGCGAGTGTAATAAGTGGCTAG	Amplification and GG Cloning <i>pPIP2;5</i>
pPIP2;5G_G_R	TTGAAGACAACATTTCTCGCGCTAGCCGCTAGTCTG	
pPDK1G_G_F	TTGAAGACAAGGAGGTGCCCCATCAGCACAC	Amplification and GG Cloning <i>pPDK1</i>
pPDK1G_G_R	TTGAAGACAACATTGGGATGATGAAGGGATG	
pLDSH5G_G_F	TTGAAGACAAGGAGGACTTCTGGTCATAGAAG	Amplification and GG Cloning <i>pLDSH5</i>
pLDSH5G_G_R	TTGAAGACAACATTGAGCTGGCGAGCGAGCAAGAGACTG	
pRHD3G_G_F	TTGAAGACAAGGAGCACCGCTACCAGTTACATTGC	Amplification and GG Cloning <i>pRHD3</i>
pRHD3G_G_R	TTGAAGACAACATTCCACTCCTGGTTTACACTTGTACC	
pTBL32G_G_F	TTGAAGACAAGGAGGGACGATCGTGATGTATAC	Amplification and GG Cloning <i>pTBL32</i>
pTBL32G_G_R	TTGAAGACAACATTGGCCGATGACTGCACGTAC	
pAux14G_G_F	TTGAAGACAAGGAGGAATCATTCTATCAAGTGGTTTG	Amplification and GG Cloning <i>pAUX14</i>
pAux14G_G_R	TTGAAGACAACATTCGGTCACTTCTCCTTACTTGGTCAG	
pMETP4G_G_F	TTGAAGACAAGGAGGCTAGTTTAGGCTATAGCAGG	Amplification and GG Cloning <i>pMETP4</i>
pMETP4G_G_R	TTGAAGACAACATTTACGCTCTGCTCGACTGCTC	

pACOZ1G G_F	TTGAAGACAAGGAGCTTCATCTTAGCAATCATTTTGC	Amplification and GG Cloning <i>pACOZ1</i>
pACOZ1G G_R	TTGAAGACAACATTGGCGAGGAGTGCCTGCATATATAAG	
pPILS6G G_F	TTGAAGACAAGGAGGTCTGTCGCTGCTTTGCCCTGTC	Amplification and GG Cloning <i>pPILS6</i>
pPILS6G G_R	TTGAAGACAACATTGGCTTGACCCAGACCTGACTACTG	
pARGOS 1GG_F	TTGAAGACAAGGAGGATGGAACGACACCACTTG	Amplification and GG Cloning <i>pARGOS1</i>
pARGOS 1GG_R	TTGAAGACAACATTCTCAAGAACCTGATGAGCGAA	
NAC24US ER_F	GGCTTAAUATGGAAGCATCAGCAGCAG	Amplification and insertion of <i>NAC24</i> into USER pCAMBIA230 0
NAC24US ER_R	GGTTTAAUCTAGACTTGGTACCCCGCCG	
BonnMu NAC24_F	AGGGCTACCTAGGGTGGTTT	Genotyping of Bonn <i>Mu</i> mutants via PCR
BonnMu NAC24_R	TGCGTGCGTGACCTTGTT	
MuTIR6	AGAGAAGCCAACGCCAWCGCCTCYATTCGTC	
NAC24qP CR_F	GGGTGGTTTAGGGTTCTCCG	Measurement of <i>NAC24</i> expression via RT-qPCR
NAC24qP CR_R	GATGAGCTCCTCGTCAGTCG	
NAC24qP CR_TG_F	TGGAGAGGTACTGGCCTGC	Measurement of <i>NAC24FLAG</i> transgene expression via RT-qPCR
NAC25qP CR_TG_R	GCGTCGAGGTCATGGTCCTT	
PIP2;5_F	TGTCGTCGTTGGTTGCCT	Measurement of <i>PIP2;5</i> expression via RT-qPCR
PIP2;5_R	CACAACAATCACACTAGCTTGGAA	
PIP1;1_F	CCCCTACTATGTTACGTGGAGTTC	Measurement of <i>PIP1;1</i> expression via RT-qPCR
PIP1;1_R	GCGGCATATTACACAATTGGTA	
EF1α_F	GATCTGAAGCGTGGGTATGTG	Measurement of <i>EF1α</i> expression via RT-qPCR
EF1α_R	GATGACCTGGGAGGTAAAGCTAG	
Actin1_F	GCCCTGCTGTATGAAATGGA	Measurement of <i>Actin1</i> expression via RT-qPCR
Actin1_R	AAAGGAACCAGCTAAAAGCAAAC	

GADPH_ F	TTGTTTCCCTTCCTGCTACC	Measurement of <i>GADPH</i> expression via RT-qPCR
GADPH_ R	AAACTGCAACCTCACCACAAG	
EcoRIHis TEV_NAC 24_F	GAATTCGGCGCGGGGCCATCATCATCATCATGGCGCG GGCGAAAACCTGTATTTTCAGGGCGGCGGGGCATGGA AGCATCAGCAGCAGC	Amplification of <i>NAC24</i> with TEV cleavage site and His-tag, for cloning in pGEX4T-1
NAC24_X hoI_R	CTCGAGGACTTGGTACCCGCCGGCAGG	
BamHI_ NAC24pQ E_F	TGTCTGGATCCGAAGCATCAGCAGCAGCAGG	Amplification of <i>NAC24</i> NAC domain for cloning in pQE60
NAC24pQ E_ MluI_R	AGACAACGCGTCTTGGCGAACACCCGGCACAC	
pPIP2;5Fr ag1_F(CY 3)	CGAGTGTAATAAGTGGCTAG	Amplification of CY-3 tagged pPIP2;5 fragments for EMSA
pPIP2;5_ _R	GCAGCCGAGCAATCGAATAAC	
pPIP2;5Fr ag2_F(CY 3)	GTTATTCGATTGCTCGGCTGC	
pPIP2;5Fr ag2_R	CGGTGTACAACCGGCCATGAG	
pPIP2;5Fr ag3_F(CY 3)	CTCATGGCCGGTTGTACACCG	
pPIP2;5Fr ag3_R	CTCCTCCGTTGCTTTTCACCA	
pPIP2;5Fr ag4_F(CY 3)	TGGTGAAAAGCAACGGAGGAG	
pPIP2;5Fr ag4_R	CAAGGACCACCTAATGCCT	
pPIP2;5Fr ag5_F(CY 3)	AGGCATTAGGTGGTCCTTG	
pPIP2;5Fr ag5_R	CTGCCTAATACGGGGGTTG	
pPIP2;5Fr ag6_F(CY 3)	CAACCCCCGTATTAGGCAG	
pPIP2;5Fr ag6	GCTAGTCTGGTGAGACTTGCTG	
pPDK1pe ak_F (CY3)	GGGACGGTGGTCTCTGGCGAGAAC	Amplification of <i>NAC24</i> binding

pPDK1peak_R	GTATTTAGGGGAACCGTTGCGG	region of <i>pPDK1</i> for EMSA
CASP6peak_F(CY3)	CCACCACACCCAAGCTGCATAAC	Amplification of NAC24 binding region of <i>CASP6</i> for EMSA
CASP6peak_R	CCACCATGGCCGCCACCTATG	
pLDSH5peak_F(CY3)	GGATGAACAGAACTCTGGGCGTGC	Amplification of NAC24 binding region of <i>pLDSH5</i> for EMSA
pLDSH5peak_R	CGCCTACAAGTCCCATTCACTTG	
pRHD3peak_F(CY3)	CTTTGTAATCTGATTACGTTAC	Amplification of NAC24 binding region of <i>pRHD3</i> for EMSA
pRHD3peak_R	ATAATGCCGAAGATGATGAAAGC	
pTBL32peak_F(CY3)	GAAGCACATACTAAAAGTTG	Amplification of NAC24 binding region of <i>pTBL32</i> for EMSA
pTBL32peak_R	GTTGAGATGTATTTCCCTCCAAAC	
pHSF1peak_F(CY3)	GCCTTTCTAAACTGTTATCTAGGG	Amplification of NAC24 binding region of <i>pHSF1</i> for EMSA
pHSF1peak_R	CTCATAGGTACAGAAATTGACC	
BK11peak_F(CY3)	CAGAGCGCCATCCAGGCCGCCATC	Amplification of NAC24 binding region of <i>BK11</i> for EMSA
BK11peak_R	CCGAATTTCCGATGAACACACGC	
pCNR8peak_F(CY3)	CAACGCACGGGCACTCATCTAG	Amplification of NAC24 binding region of <i>pCNR8</i> for EMSA
pCNR8peak_R	GAGAACCGGAGGGAGATGTG	
pAux14peak_F(CY3)	CCGGACAGTACTCTGCAGTAG	Amplification of NAC24 binding region of <i>pAUX14</i> for EMSA
pAux14peak_R	CAGACACGACGGGATCTCTTC	

pYG3peak_F (CY3)	GATCCGCGAAACCTAACGCGCTC	Amplification of NAC24 binding region of <i>pYG3</i> for EMSA
pYG3peak_R	CTCACAAAATGGGCAAAGCAGCCCG	
pMETP4peak_F (CY3)	CTCAGCCGTTTTCAATTTAACTG	Amplification of NAC24 binding region of <i>pMETP4</i> for EMSA
pMETP4peak_R	AAGCAGGCAGTCCACACATGATG	
pACOZ1peak_F (CY3)	TCCGACCTGATGGTGAAGTTGTGTG	Amplification of NAC24 binding region of <i>pACOZ1</i> for EMSA
pACOZ1peak_R	GCAAATACAAGCAGCAATCAAGGAG	
pPIN14peak_F (CY3)	CACCTGTTCAGTTCACTACAC	Amplification of NAC24 binding region of <i>pPILS6</i> for EMSA
pPIN14peak_R	GTCCACCTCGGGAAGTTCTTA	
MYB100peak_F (CY3)	TAGTTGCTCTAATGTCCTCTCCC	Amplification of NAC24 binding region of <i>MYB100</i> for EMSA
MYB100peak_R	CTTAGAAGGAGTTGCAACCTAC	
pARGOS1peak_F(CY3)	CTCTCTGAAAGAACAAGGAAGTAG	Amplification of NAC24 binding region of <i>pARGOS</i> for EMSA
pARGOS1peak_R	CTCGTCAGCACATGAATGAAGC	
p35S_EMSA_F (CY3)	CACGAGGAGCATCGTGG	Amplification of p35S fragment for EMSA
p35S_EMSA_R	GTCCTCTCCAAATGAAATG	
NACBS_F (CY3)	GCGCGCGTCCTTTGCGTGCGTGACACTTCACAGTGGCTGTAC	NAC24 binding site for EMSA
NACBS_R	GTACAGCCACTGTGAAGTGTACGCACGCAAAGGACGCGCGC	
NACBSmut6_F (CY3)	GCGCGCGTCCTTTGCGTGCGTAGACACTTCACAGTGGCTGTA	Mutated NAC24 binding sites for EMSA
NACBSmut6_R	TACAGCCACTGTGAAGTGTCTACGCACGCAAAGGACGCGCGC	
NACBSmut4_F (CY3)	GCGCGCGTCCTTTGCGTGCGGACACTTCACAGTGGCTGTAC	

NACBSm ut4_R	GTACAGCCACTGTGAAGTGTCCGCACGCAAAGGACGCG CGC	
NACBSm utTC_F (CY3)	GCGCGCGTCCTTTGCGCGCGTGACGCTTCACAGTGGCT GTAC	
NACBSm utTC_R	GTACAGCCACTGTGAAGCGTCACGCGCGCAAAGGACGCG GCGC	

Table S3: List of constructed plasmids with their corresponding names and applications.

Plasmid name	Purpose
DLR- <i>pPIP2;5FLuc::p35SRLuc::p35SNAC24</i>	DLR assay with <i>pPIP2;5</i> in maize mesophyll protoplasts
DLR- <i>pPIP2;5FLuc::p35SRLuc::p0NAC24</i>	
DLR- <i>PROMFLuc::p35SRLuc::p35SNAC24</i>	DLR assay with promoter of interest (<i>pPDK1</i> , <i>pLDSH5</i> , <i>pPRHD3</i> , <i>pTBL32</i> , <i>pAUX41</i> , <i>pMTPC3</i> , <i>pACOZ1</i> , <i>pARGOS1</i> , <i>pPIN14</i>) in <i>N. tabacum</i> leaf protoplasts
DLR- <i>PROMFLuc::p35SRLuc::p0NAC24</i>	
GB0110m- <i>pPIP2 ;5mVenus ::p35SmCherry ::p35SNAC24</i>	Transactivation assay in maize epidermal cells
GB0110m- <i>pPIP2 ;5mVenus ::p35SmCherry ::p0NAC24</i>	
USERpCAMBIA2300- <i>NAC24YFP</i>	Subcellular localization of NAC24 in maize mesophyll protoplasts
USERpCAMBIA2300- <i>YFPNAC24</i>	
BMS- <i>KanR::p35SmCherry::p35SNAC24FLAG</i>	Generation of stable BMS lines overexpressing <i>NAC24</i>
pIXHALO- <i>NAC24</i>	<i>In vitro</i> expression of HALONAC24 fusion protein
pGEX4T1- <i>NAC24</i>	Expression of GST-NAC24 protein in <i>E. coli</i>
pQE60- <i>NAC24</i> ₁₄₋₁₇₅	Expression of His-tagged NAC24 NAC domain in <i>E. coli</i>
pQE60- <i>NAC24R26A</i>	Expression of His-tagged mutants of NAC24 NAC domain in <i>E. coli</i>
pQE60- <i>NAC24R92A/R96A</i>	
pQE60- <i>NAC24K85A/R92A/R96A</i>	

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